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i. Acknowledgements

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ii. Abstract

Carbohydrate microarrays are a newly developed technology revolutionising the field of glycomics. Previously, it has been a difficult field to study due to the many challenges of glycan complexity. However, there is now the potential for it to become extremely accessible using carbohydrate microarrays to facilitate our understanding of carbohydrate-protein binding activities. Glycomic interactions are at the frontier of biological processes involving cell-cell interactions and have long been poorly understood. With the use of current immobilisation strategies adapted from protein array technologies, combined with a "gene chip" type microarray format, this study aims to produce a way of being able to create carbohydrate binding profiles of several plant lectins to simple sugars. In this report a cost effective method of creating reproducible carbohydrate microarrays in the lab is discussed as a new way to research into the field of glycomics.

The synthesis of 7 different 1-amino deoxy saccharide sugars is achieved that will act as a "library" of glycans for the microarrays. The functionalisation of glass slides to an NHS ester activated surface is performed to produce a method of covalent immobilisation. The above combination allow individual study of the binding profiles of different plant lectins, including: UEA I, DBA, SBA and RCA_{120}.

iii. Abbreviations

SAMS - Self assembled monolayers

NHS esters - N-Hydroxysuccinimde esters

APTES - (3-Aminopropyl)triethoxysilane

PNA - Peanut agglutinin

SBA – Soybean agglutinin

DBA - Dolichos biflorus agglutinin

UEA I - Ulex europaeus agglutinin

Con A - Concanavalin A

RCA_{120} - Ricinus communis agglutinin-120

PBS - Phosphate buffered Saline

PBST - Phosphate buffered Saline, Tween-20

NSB - Non-specific binding

GLU - Glucose

GAL - Galactose

LAC - Lactose

MAN - Mannose

RIB - Ribose

LYX - Lyxose

FUC - Fucose

1. Introduction

1.1 Glycomic interactions and their biological importance

Glycans are chains of single sugars (monosaccharides) which exist in a variety of chain lengths,[1] from several hundred to just one or two; they have been increasingly recognised as key participants in the biological processes in the body.[2] These kind of *glycomic interactions*[3] mostly play roles on cell surfaces acting as cell recognition and communication devices. Glycoproteins are an example of this, they comprise of a peptide backbone that is highly branched with oligosaccharide chains; it is these complex glycan structures that appear on a cells surface that are responsible for the human immunity system,[4,5] figure 1. Investigation of these interactions is crucial to understanding the fundamentals of many important biological processes. Thus, this study of "glycomics" is emerging as a front runner in the research field[6] after the genome research era. Due to the high complexity of glycan structures, and the wide range of protein binding possibilities there are in biological systems, their analysis has

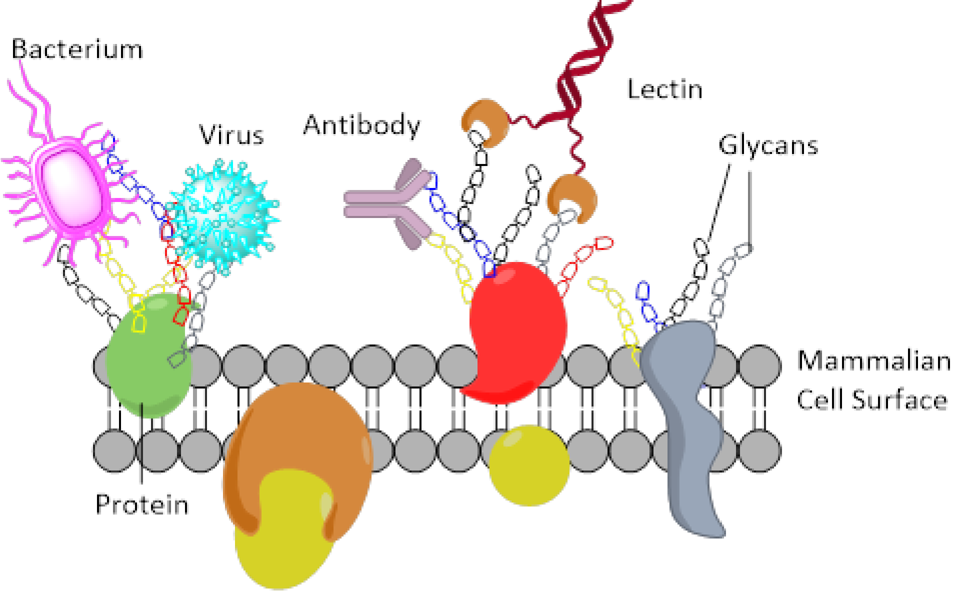


Figure 1, Diagram of a cells surface demonstrating glycan importance in cell interactions

proven to be extremely difficult. Secondly, unlike nucleic acids and proteins,[7] the biosynthesis of different oligosaccharides in the body is not template driven; it is not encoded in the genome.[2] This leads to a vastly diverse repertoire of structures; most of which are difficult to access via chemical synthesis. Furthermore, surface glycans are dynamic, so they cannot be directly studied using current technologies. This accumulation of complications has led to a lack of general methods for the synthesis and analysis of

these carbohydrate-protein interactions. Thus, so far, the majority of work previously done in this field has relied on expensive, time-consuming profiling and structural characterisation techniques. Some of these examples include high-resolution chromatography methods and exoglycosidase digestions combined with multiple analysis techniques.[8] However, understanding the variety of recognition systems that are involved in cell-cell communication systems still remains challenging. Lectins have historically been used to discriminate and detect glycan residues for bio sensor applications because of their commercial availability and high sensitivity. [9] The definition of a lectin is a carbohydrate binding protein other than an antibody or enzyme that interacts with carbohydrates non-covalently.[10] These interactions generally occur with the carbohydrate residues on a cells surface in a way that is usually specific and reversible.[11] They are found in the majority of living organisms from bacteria and viruses to plants and animals. Classic lectins usually contain more than two binding pockets for carbohydrates, thus, their binding profiles can be highly complex. The binding strength between lectins and glycans are relatively weak when there are a low number of ligands, but as soon as you begin to increase the number of binding sites there becomes an almost exponential increase in strength; this effect is known as the *cluster glycoside effect*.[12] Static arrays of monosaccharides and disaccharides have been evaluated by the specific binding profiles of plant lectins such as Concanavalin A (Con A); specific monosaccharide interactions were seen for each of the fluorescently labelled lectins used.[13] The use of this static array technique allows for a close approximation - a “snap-shot” image - of what might be occurring on a cell’s surface and offers great potential to the field of glycomics.

1.2 Microarrays and Contact Printing

Carbohydrate microarray technologies are a new generation of devices emerging in this field that are revolutionising the way in which glycomic interactions are being studied. The development of microarrays has already had a major impact in drug discovery programs as well as in biological research.[14,15] Their combined benefits of mass analysis and immobilised assay format has led to great success in areas such as genomics. Gene chips, which are essentially large oligonucleotide arrays, have been commercialised and are now widely used in gene expression profiling.[16] Due to the fact that biological functions of glycomic interactions have not been as extensively studied as those of nucleic acids and proteins; these current advances have led to interest in development of functional carbohydrate chips that can be used in the characterisation of cell activities.[13,17]

The main advantage to this type of analysis is the wide range of glycans (tens to hundreds) that can be placed on one static array. Generally speaking, microarray printing can be split into two categories: non-contact printing and contact printing. Non-contact printing involves a piezo-electronic printer that controls the sample delivery of solutions through a glass capillary via electrical signals.[18] This technique offers very precise printing results with very well distributed size and morphology of depositions; furthermore, this printing method recycles the sample solution to the source plate after printing which can be very important where only tiny amounts of rare samples are being printed. However, this technique is limited by the number of tips allowed on one instrument due to expense. Additionally, it requires special attention to its conditions such as the substrate moisture, stability and sample temperature and evaporation; this can result in printing taking several hours for just a few slides. Microarrays produced by a contact printing method involve a set of steel pins on an array robot. These pins have been delicately etched to have precise points to pick up tiny amounts of a sample. The pins are dipped into sample solutions from a multi-well source plate and transferred to the slides by directly blotting the sample on the surface. The total volume of solution delivered is dependent on the time the pin is in contact with the surface. Generally, the samples are first pre-blotted on a "pre-print" surface to reach a consistent morphology before printing the final array; this stage is tuned so that approximately 0.5nL of sample can be spotted reproducibly and rapidly. The final results are immobilised assays of 100-200 micron spots. This feature makes microarrays an appealing method for analysing carbohydrate-protein interactions as it allows the parallel investigation of a diverse range of interactions where only tiny sample quantities are required (on the microgram scale or less). This is primarily due to the spatial allowance of this high-throughput method where many substrates can be immobilised on a surface, meaning that hundreds or even thousands of investigations can take place in one small experiment. This technique provides an ideal method of mimicking the way carbohydrates are presented on a real cell-cell interface. Hence, why this has led to research being carried out using carbohydrate microarrays such as *Disney, Seeberger et al*. The binding specificities and profiles of bacteria were studied in order to be able to detect and harvest certain pathogens, then the microarray technologies were also used to screen for potential antibacterial susceptibility.[19,20]

1.3 Surface Immobilisation

To produce a suitable analysis strategy, the carbohydrates must be immobilised onto the surface so they are structurally and chemically stable, as they would be presented on a cells surface. There are two main approaches to surface modification; the first is the formation of self-assembled monolayers (SAMs) on a surface and the second is the deposition of polymeric or multi layers.[21] Both are very reliable techniques but they do offer different advantages: the formation of monolayers offers a better molecular control whereas the polymeric modification is usually less susceptible to change and more chemically robust.[22] The formation of SAMs can involve covalent and non-covalent interactions. Examples of non-covalent interactions involve the physical adsorption of proteins onto hydrophobic surfaces such as nitrocellulose, this works well due to the large surface area a protein offers and its ability to have multiple electrostatic interactions.[23] The issue with this technique for carbohydrate microarray studies is that the immobilised sugar substrates would have random orientations, so there is a probability that the proteins would demonstrate relatively weaker affinities to the target molecules. Covalent immobilisation can be site specific or site non-specific, the site specific immobilisation strategy provides the perfect opportunity to anchor a substrate down in a preferred orientation. In previous work, this technique has demonstrated the site specific covalent

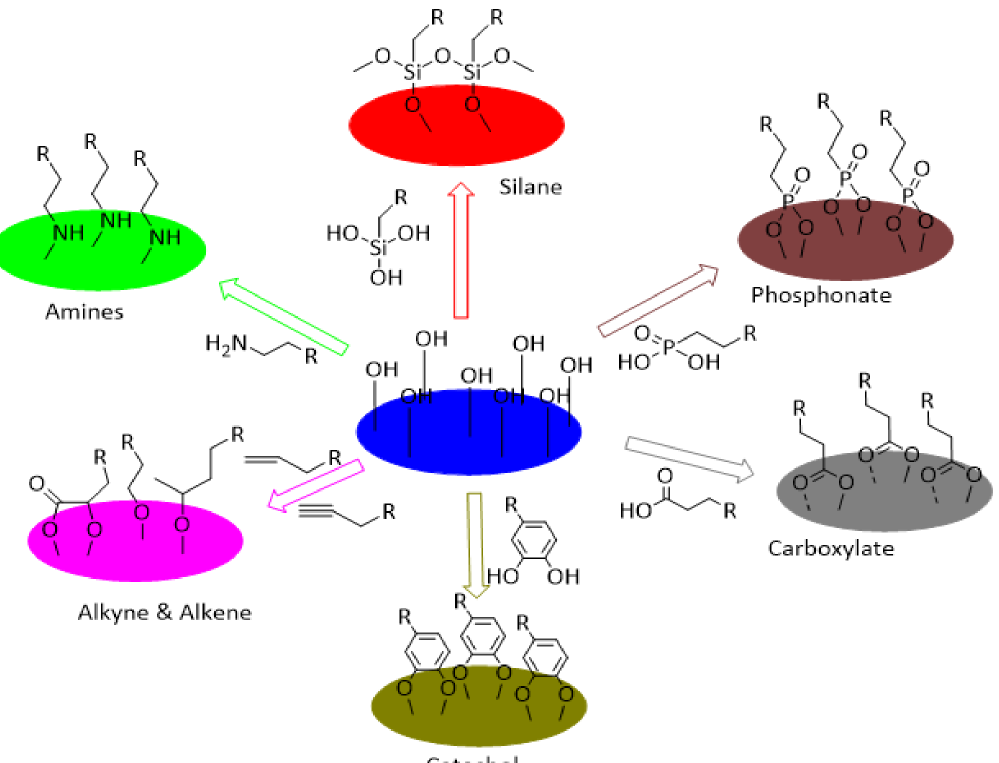


Figure 2: Examples of currently used technologies in the modification of an oxide surface

immobilisation of small molecules, which produced ligands that were presented uniformly across a surface.[24,25] The best studied SAMs involve the use of a thiol monolayer on a gold surface, due to the favourable formation of the Au-S bond, it allows easy synthesis of these kinds of surfaces.[26] However, if the modification uses monodentate ligands, the surface is generally not very stable due to the weak individual gold-sulphur bond and can easily be broken if not handled properly. The most stable SAM functionalised surfaces involve strongly covalently bonded atoms such as C-C bonds or Si-C, but these are more difficult to achieve as they require higher activation energies to produce.[27] Oxides are a favourable intermediate as they are easily accessible and form strong bonds to metals and semiconductors such as silicon; the formation of hydroxylated surfaces allow easy modification, see figure 2. The modification of particular interest in this project is the silane surfaces; these are extremely strong starting points for further modification; they are chemically stable and easily accessible.[28] Not only this, but, there is a wide range of functional groups that will readily react with them. Alkylsilanes in the form SiR_3X, SiR_2X_2, $SiRX_3$ (where X is a leaving group, typically a halide or hydride) have been a hugely popular go-to for covalent modification, primarily due to the rapid covalent linkage to the OH anchoring group. These substrates can form multivalent links to the surface providing extra stability to the monolayer; furthermore, branched alkyl chains then provide an easy pathway to further modification. Usually, the covalent attachment of free glycans can be difficult as they lack a selectively reactive functional group and thus direct immobilisation on commercially available slides is challenging. By chemically modifying the glycans it becomes much simpler to achieve immobilisation. Efficient reactions have been studied for this application including amino and thiol modification.[29,30] The use of *N*-hydroxysuccinimide (NHS) esters and epoxy modified slides as a covalent immobilisation technique is now being commonly used.[31,20] This is due to its high reactivity towards primary amine groups, which provides an ideal platform for biomolecular substrates containing free amine groups such as proteins. The NHS ester activated slides are selective to only primary amines whereas epoxy slides are more reactive and can be used on secondary and aromatic amines. In this project the use of NHS ester functionalised surfaces as well as amine substituted glycans to create microarrays will be demonstrated; this combination leads to the formation of a chemically stable covalent amide product, see figure 3.

NHS ester Surface

Primary amine
on synthesised glycan

Stable amide bond formed
between surface and sugar

Figure 3: Reaction between primary amine and NHS ester surface to give stable amide

1.4 Current Areas of application and Interest

One of the main attractions of this type of research is the potential for development of anti-adhesion therapy: the prevention of adhesion of pathogenic organisms in host tissues.[32] In many systems, the adhesion between infectious organisms and the host are mediated by lectins present on the bacteria's surface. These bacterial lectins bind to complementary carbohydrates that are found on cell surfaces. Through the use of microarray technology, if these lectins binding profiles could be determined and understood could it could lead to the developments of synthetic mimics, such as glycopolymers.[33] This type of synthetic derivative could interfere with interactions between an invading pathogen and host to prevent the spread of, or even initial infection of the species. Not only can these interactions be inhibited by glycopolymers, but, small molecule inhibitors are also a viable solution as they have very favourable interactions within the sugar-binding pocket.[34] This type of research has shown great promise: in one example aromatic α-mannosides were found to be extremely potent inhibitors of a type 1 fimbrated E.*coli* having affinities in the nanomolar range.[19] Another key attraction for studying these interactions is the application as a tool in diagnostics. The idea is to create a carbohydrate array platform that can be used as a biosensor, due to the way that many different cell types can bind to carbohydrates, the cells' "carbohydrate fingerprint" could say what type of cell/pathogen is present.[35] Some previous work has shown that using monosaccharide coated surfaces has compared the relative binding of different lectins to generate a training algorithm that enables the correct identification of the bound lectin.[36] This is primarily done by utilising each lectins unique binding "barcode" which is determined by profiling them against a range of monosaccharides.

2. Project Aims

The aims of this particular project develop a technique to mimic the interactions occurring on a cells surface, via the analysis of glycan microarrays and fluorescently labelled lectins in an attempt to improve the field of research into glycomics. The project first involves the synthesis of a range of amino substituted glycans that will act as the "library" for the basis of a high-through put technique. Secondly, the chemical modification of plain glass slides to allow the immobilisation of the carbohydrates and proteins onto the surface and provide a suitable platform for the experiments to be conducted on. Finally it will aim to produce a method of fabricating stable and reproducible microarrays to be used to analyse and profile fluorescently labelled lectins.

3. Results and Discussion

3.1 Synthesis of 1-amino de-oxy sugars

For the first stage of this project, the synthesis of 7 1-amino deoxy sugars was conducted. The reaction proceeded by the equilibrium shown in figure 4, by adding large amounts of ammonium carbonate ($(NH_4)_2CO_3$) strongly favours the formation of the amino substituted saccharide. Reaction details and yields shown in table 1. These glycans were chemically modified so they could then be easily immobilised to a surface, thus acting as a glycan "library".

Figure 4: Mechanism of reaction for 1-amino substitution of D-form reducing sugar

1- Amino deoxy Sugar	Molecular Mass (g)	ESI-Quad Peaks	
		[M+H⁺]	[M+Na⁺]
Glucose	179	*180*	202
Galactose	179	*180*	202
Lactose	341	*342*	364
Mannose	179	*180*	202
Ribose	149	-	172
Lyxose	149	-	172
Fucose	163	-	186

Table 1: Product peaks observed from ESI-Quad MS

The standard procedure of synthesis as described by Dirk Vetter, Mark A. Gallop, 1995 involved making up a solution of the D-sugar starting material in D2O at 5% w/w. Ammonium carbonate is then added at an approximate ratio of 1:10 sugar: salt, crude yield is removed after by direct cryodesiccation. However, not all products were collected

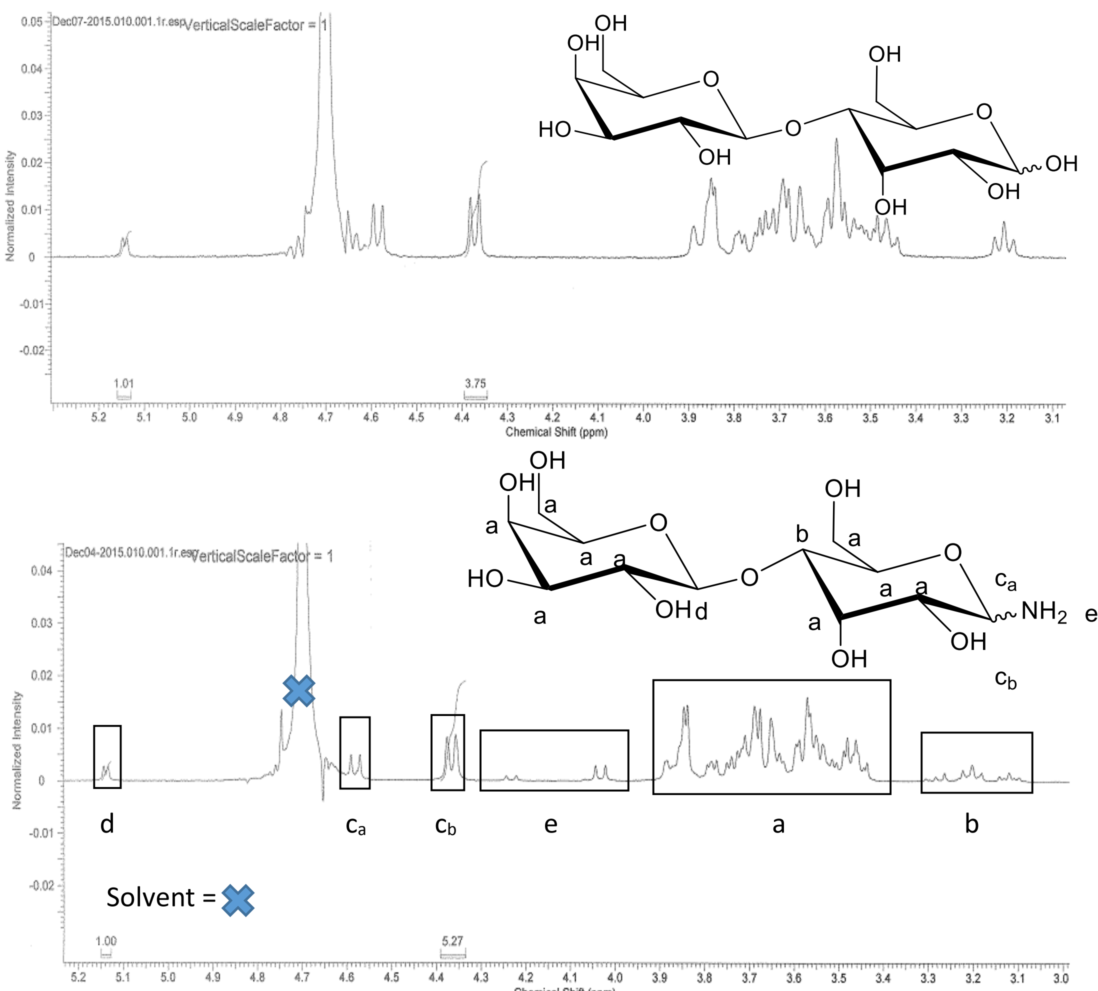


Figure 5: (TOP) Proton NMR of D-Lactose starting material, (BOTTOM) Proton NMR of 1-amino deoxy substituted product

via this method. Due to the high salt content required in the small solutions, the samples were unable to undergo immediate freeze drying. After attempted and failed dilutions were made, the ammonium carbonate had to be removed by heating, 55°C, over an extended period of 5 hours to promote decomposition of the salt and evolution of CO_2. To prevent destruction of heat sensitive carbohydrate products, 55°C was the maximum temperature used. The solutions were then rotary evaporated instead in a water/methanol, the crude product was then freeze dried to produce the final product. The mechanism for the reaction can be found in figure 4. Characterisation was performed by NMR and ESI-Quad MS (for full assignment see experimental). Characterisation by mass spectrometry found that for each of the products one of either the $[M+H^+]$ or $[M+Na^+]$ was visible on the spectrum, the corresponding expected and observed are shown in table 2. ^{1}H NMR characterisation (figure 5) by comparing starting material and product showed that the amine peaks were now observed in the product spectra and not in the starting material. Furthermore the anomeric peaks are clearly observed at 4.37ppm and 4.59ppm,

corresponding to the β or α position hydrogen, using integral data these were observed at a 5:2 ratio, respectively. This data means that the amine substituents can be found in both the β or α position. The large area of indistinct peaks between 3.5 – 3.9ppm correspond to the large number of protons on the sugar rings in different conformations, which leads to complex multiplets and hence is very difficult to characterise.

3.2 Surface Modification of Glass Slides

A suitable surface for the immobilisation of the microarrays was needed to be prepared. Previous work in this field has used gold coated glass slides,[13] glass slides modified with thiol groups as linkers,[30,37]and also nitrocellulose surfaces have been a popular choice. The NHS ester surface was chosen for its successful background in protein, enzyme and peptide immobilisation[38,31]and it's formation of a chemically inert immobilised product, an amide bond upon reaction with a primary amine (see figure 9). In order to produce the NHS ester slides, plain glass slides were modified in a three step conversion to produce the new surface (see figures 6 and 7). The first step involved the chemical cleaning with piranha solution which is a very strong oxidant solution (it should be treated with extreme caution as it reacts violently with organic material). This stage would cause hydroxylation of the surface to produce free oxide groups. The second stage involved producing the tether groups, an aminosilane, (3-aminopropyl)triethoxysilane (APTES), was used in order to leave free primary amine groups to allow NHS ester addition. This was followed

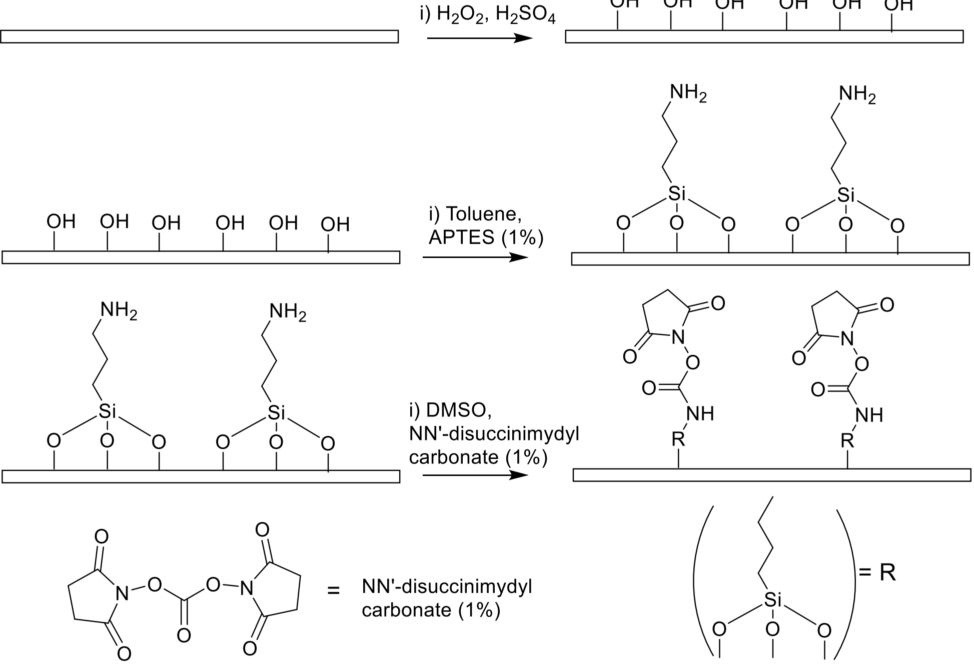


Figure 6: Reaction scheme for synthesis of NHS surface activated slides

by the addition of NN’-disuccinimydyl carbonate, to produce the NHS ester activated surface. In order to determine if the surface modification at was successful, one slide was removed at each stage and was taken for water contact angle measurements.. As demonstrated in figure 8, after silanisation there is a clear change in hydrophobicity of

Figure 7: Reaction Mechanism for formation of NHS ester surface from APTES functionalised surface

the surface whereby it changes from a relatively hydrophilic surface (polar OH surface groups) to a much more hydrophobic one (aminosilane). This observation is expected for surface modification due to the hydrophobic alkyl chain which has been added to the surface. Further modification of the surface (addition of NHS ester) led to another change in surface hydrophobicity, although not as drastic as previously observed. Now that a probable change in the chemical composition of the surface had been observed, several tests were conducted to confirm the surface was the intended NHS activated one.

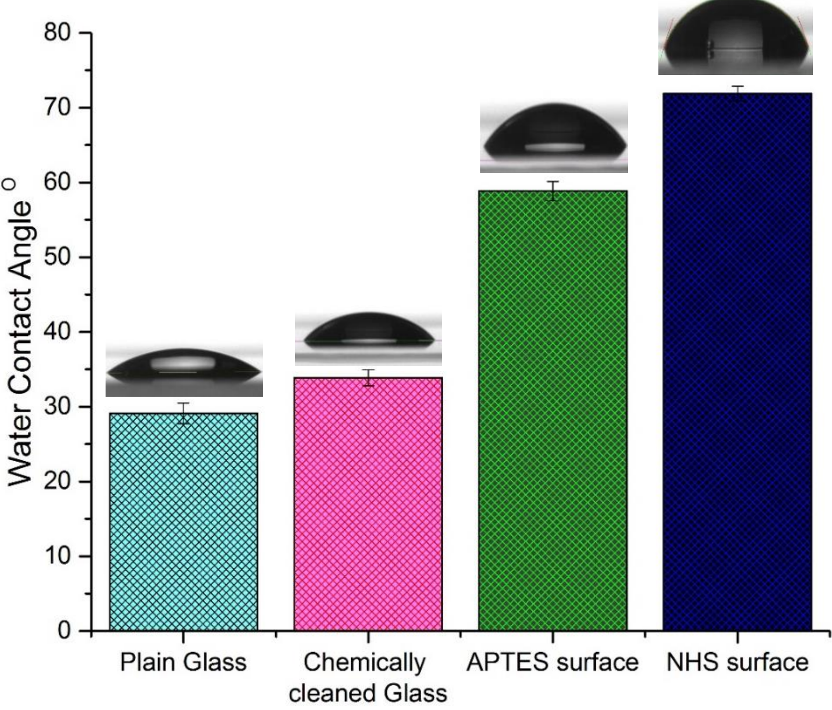


Figure 8: Graph showing water contact angles taken at each stage of surface modification, each measurement had a minimum of 5 water droplets analysed

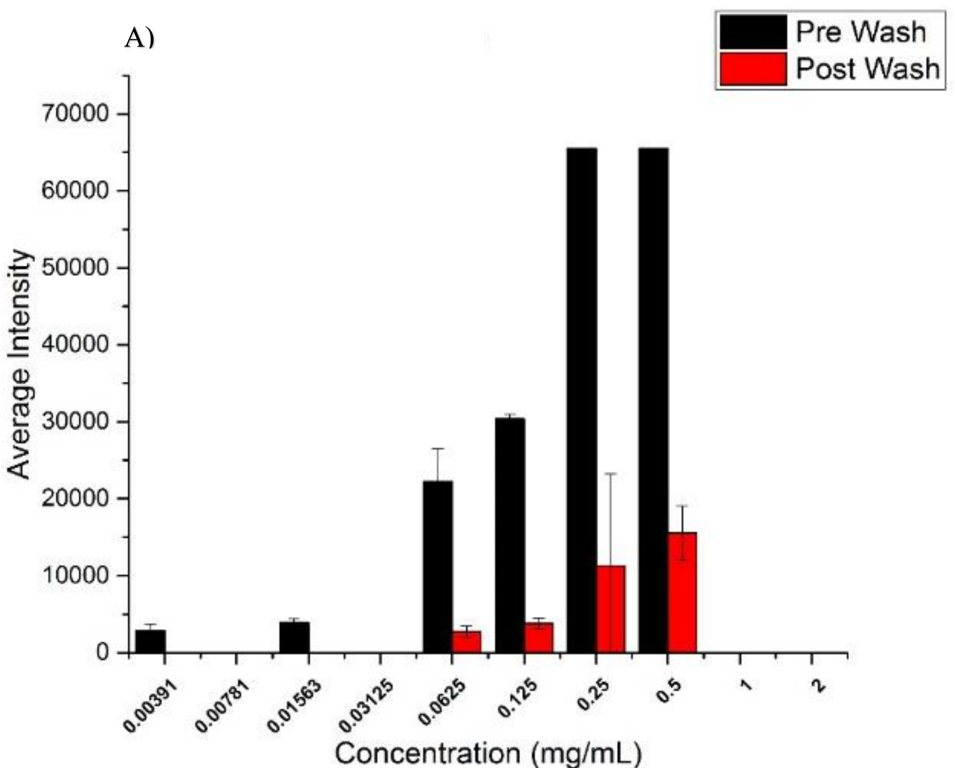
D-form free glycan

Amino substituted glycan

= Silane linker

Figure 9: Reaction scheme showing immobilisation strategy of carbohydrates to form stable amide covalent bond to surface

As it is known NHS esters are highly selective towards primary amines, 1-amino deoxy sugars and fluorescently labelled lectin proteins were printed onto the surfaces of the slides. The 1-amnio deoxy sugars which were synthesised to act as the "library" of sugars for the project were used as a qualitative analysis, figure 10, this is due to them not being



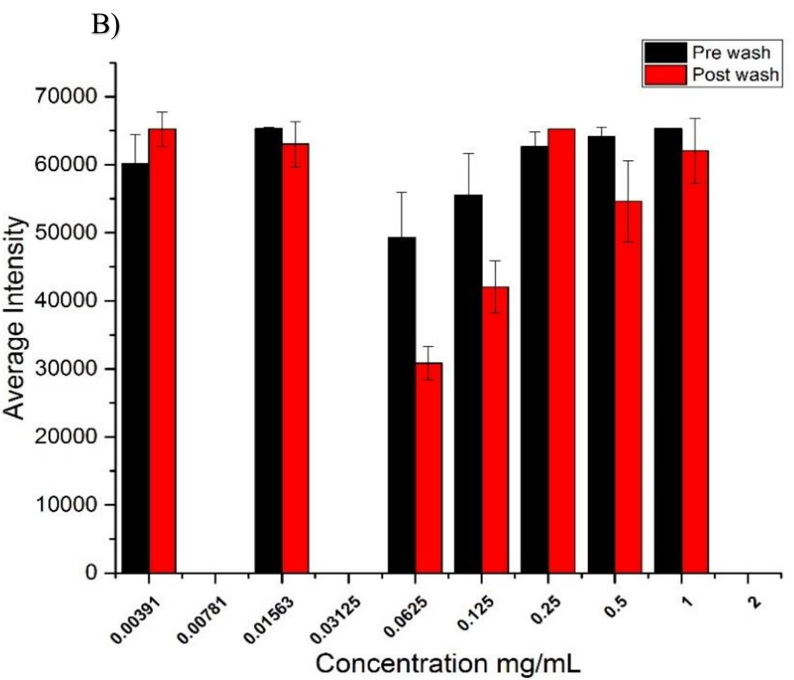


Figure 10: Average intensities for fluorescently tagged Peanut agglutinin (PNA) lectin on (A) plain glass slides and (B) NHS activated slides, before and after thorough washing with dH$_2$O

fluorescently tagged so thus intensity values cannot be measured as the fluorescence observed is not quantifiable. The fluorescently tagged lectins used were to act as a quantitative test, figure 10, so the binding intensities could be shown numerically as well as visually. Figure 10, demonstrates that when the lectins were printed on to a plain glass surface there was a minimal amount of binding observed. Direct washing of the substrates caused the majority of deposited lectin to be removed; this is strong evidence to suggest no covalent linkage formed. The low values seen from the post-washing of the plain glass slides are probably representative of small amount of protein physisorbed to the surface through non-covalent interactions. By comparison the NHS ester surface demonstrates

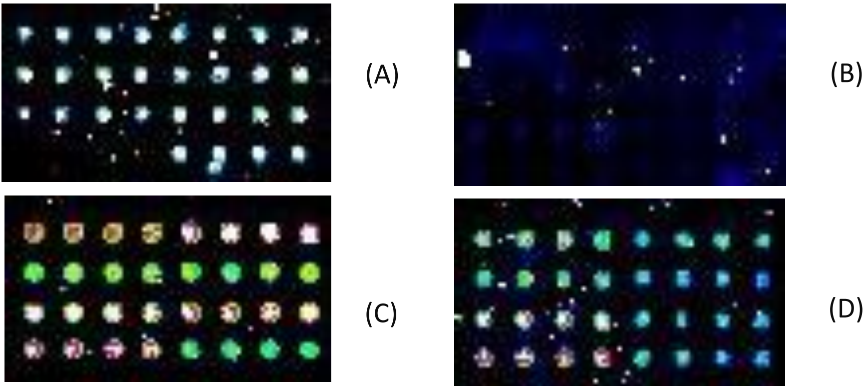


Figure 11: Images of carbohydrate prints on plain glass and NHS ester surface before and after washing with Phosphate buffered saline (PBS) and dH2O
A) Plain glass carbohydrate print – pre wash, B) Plain glass carbohydrate print – post wash, C) NHS modified surface – pre prwash, C) NHS modified surface – post wash.

that after thorough washing the deposited samples are almost unaffected, thus providing strong evidence for formation of a substrate-surface covalent linkage. The carbohydrate microarrays, figure 11, strongly show further evidence of covalent tethering to the surface; direct visual comparison between the microarrays shows a much greater carbohydrate presence on the NHS ester surface post wash. Although it can be said that it is highly likely NHS surface modification has occurred; the actual composition of the surface has not been concluded. For immobilisation purposes it is assumed the ideal situation would be a self-assembled monolayer of tether molecules to allow uniform presentation of the substrates. However, this could be seen as potentially uncharacteristic of a real cell - as there is no guarantee glycans would be presented in such a format, hence why, it is not a necessity to obtain a SAM in this case.

3.3 Printing Controls

After suitable immobilisation was achieved for the carbohydrate microarrays; the printing conditions and strategies needed to be determined in order to produce an efficient, reproducible strategy. Firstly the mechanics of printing needed to be defined, so that the observed microarray can be understood; a diagram to explain this is shown in figure 12. Preparations for printing strategy were extremely important to ensure s useful microarray was constructed. Once the schematics of the contact printer had been fully understood, it was then possible to produce a range of microarray substrates which could have tailored

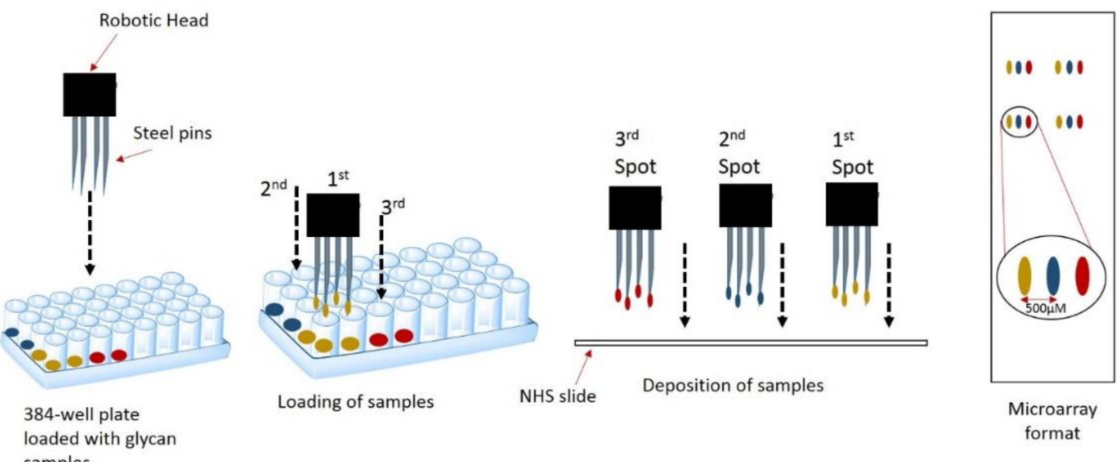


Figure 12: Diagram demonstrating the printing schematics of the robotic array instrument

features depending on the necessity of the print. Shown in figure 13 is an explanation of the main strategy used for the control prints; this print contained blocks of the same glycan in decreasing concentrations. As can be seen by the demonstrated image, with the correct

planning it was possible to apply this print fairly easily and reproducibly. An alternative

GLU GLU
GAL GAL
LAC LAC
MAN MAN
LYX LYX
RIB RIB
FUC FUC
2.00E00 mg/mL
7.81E-03 mg/mL
Decreasing Concentration
Diameter of 100-200µM
NHS slide

Figure 13: Printing strategy for the control prints plus example of microarray

print strategy was also developed to produce a microarray containing all glycans in one area at one particular concentration. The methodology behind this technique was to be able to conduct multiple incubations on one slide by having hydrophobic barriers between the congregations of sugars; thus maximising potential for mass screening. This strategy also required a lot less preparation of samples which improved efficiency in printing. Details of this print are shown in figure 13, an extra saccharide raffinose (RAF), which did not have an amine group, was included as a control substrate in these prints. After initial printing was conducted it was observed that spot intensities were relatively weak and this was originally thought to because the carbohydrates were not labelled and were unlikely to be seen clearly through fluorescence imaging. However, after reading

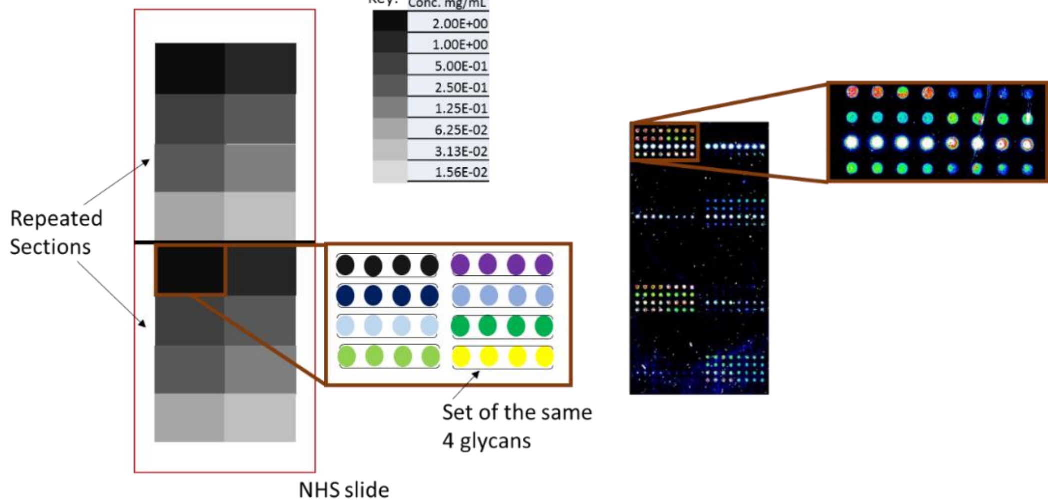
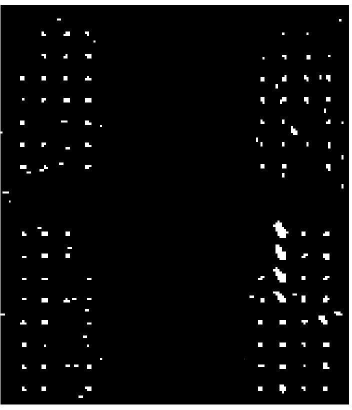


Figure 14: Diagram detailing microarray print involving blocks of the glycans at the same concentration, example microarray (right)

previous work from groups such as Fais , Karamanska et al, [17,39] it was understood that

the binding of substrate to surface could be increased by adding an incubation period of high humidity (>75%) of 1 hour after printing of the microarrays. This incubation period has been highly insisted in previous work such as protein microarrays,[23,40] it is something that has often been overlooked; now the same methodology is now being applied to carbohydrate microarrays.[41] The importance of the incubation period is shown in figure



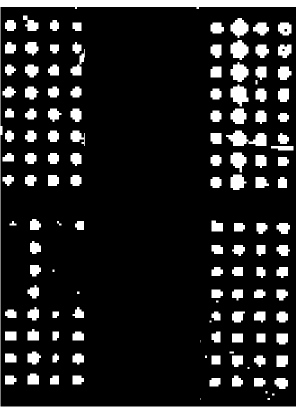
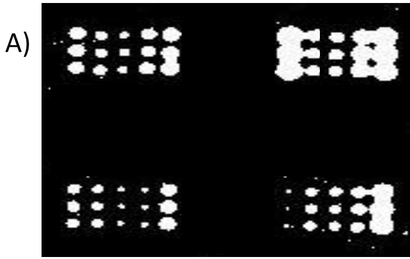


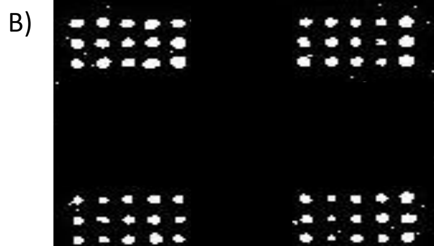
Figure 15: Demonstrating importance of incubation in >75% humidity after printing. Two microarrays were printed, one was incubated after printing.
(LEFT) Carbohydrate microarray printed on NHS surface with no incubation
(RIGHT) Carbohydrate microarray printed on NHS surface with 1 hour incubation at >75% humidity

14. To test the quality of microarrays that were being produced, commercialised NHS ester slides were purchased from Nexterion® for direct comparison against the synthesised slides. Prints were conducted in parallel using the strategy explained in figure 13, both sets of slides were incubated for 1 hour after printing; the final results are shown in figure 16. Results from both the purchased and synthesised slides gave good spot

A)



B)



C)

D)

Figure 16: Comparison of bought NHS ester slides vs. Synthesised NHS ester slides after washing with PBS and dH_2O. All images are segments of printed microarray showing two Glucose (right) and two Galactose (left) spot sequences.A) Purchased NHS ester slide 1, B) Purchased NHS ester slide 2
D) Synthesised NHS ester slide 1, D) Synthesised NHS ester slide 2

morphology and resolution, however, the purchased slides were found to have better consistency between microarrays as compared to the synthesised ones, qualitatively. This was probably due to the topography of the surfaces; the purchased slides are defined monolayers that incorporate a polymer tether with an NHS linker at the end.[42] The resulting surface is an extremely smooth surface that will allow the substrates to be consistently spotted to a certain morphology. Whereas for the synthesised slides it is assumed the topography is not as smooth; thus, due to the roughness of the surface the deposition of carbohydrates does may not always correspond with the end of a NHS tether leading to less substrate-surface binding. Furthermore, the density of tethers is also an unknown factor meaning there can be no certainty to equal distribution of the sugars as it would be dependent on the amount of covalent linkages that could be formed. It is for this reason that the following experiments were to be carried out with the remaining purchased Nexterion® slides.

3.4 Determination of a Suitable Blocking Agent

Once the carbohydrate microarray has been created, there is still the potential for non-specific binding (NSB) to occur; due to the amount of available tether sites that have not been occupied by amino-substituted glycans. If the microarrays were to be directly

Pre Wash *Post Wash*

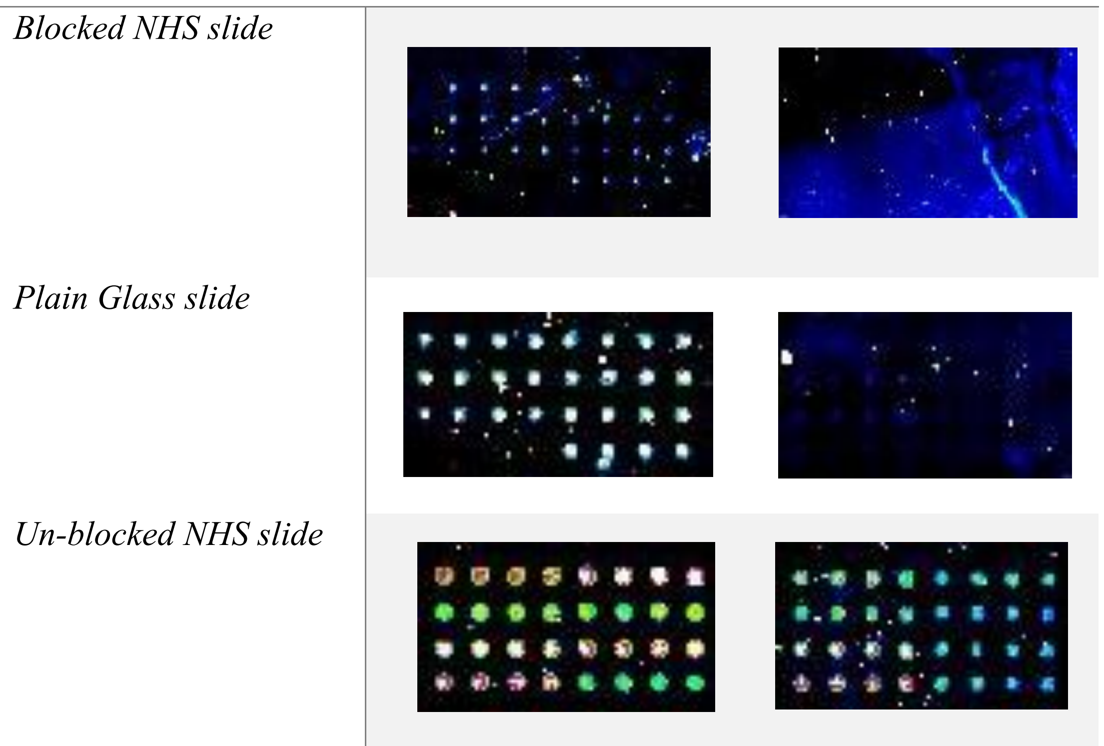


Table 2: Details of control experiemnt for printing of carbohydrate on blocked NHS surface, plain glass and un-blocked NHS surface

incubated with lectins, free primary amine groups on the proteins would covalently bind to the surface causing a poor signal to noise ratio. This NSB needs to be minimised by the saturating of these unoccupied tethers that remain free on the surface of the slide. A collective term for the species that achieve this is "blocking agents";[43] blocking agents are substances that reduce NSB without taking active part in the specific assay reaction; there will be minimal interference between lectin – glycan interactions. Blocking reagents[44] and methods are generally vary quite widely as no standardised procedure has been determined for all carbohydrate microarray applications. The two main classes of blocking agents are proteins and detergents (commonly non-ionic). Protein blockers have two main purposes; they block unoccupied sites on a surface and they stabilise biomolecules bound to the surface. Detergent blockers mainly block ionic and hydrophobic biomolecule – surface bonding. Utilising this information an extremely commonly used protein, blocker bovine serum albumin (BSA), was tested, as was a very successful non-ionic detergent blocker, Tween-20.[45] Each blocking agent was tested separately and in combination using advised concentrations from ELISA technical guide

Blocking Solution	Components	Wt.%
1	PBS	-
	Tween-20	0.05
2	PBS	-
	BSA	2
3	PBS	-
	BSA	2
	Tween-20	0.05

Table 3: Details of blocking solutions tested

to surface blocking.[43] Solutions were incubated for 30 minutes each and then washed using phosphate buffered saline, images were taken before and after blocking. Each slide was then incubated with fluorescently labelled PNA lectin for 1 hour. A control microarray that received no blocking was run alongside; see table 1. The individual Tween-20 solution seemed to offer little in the way of surface blocking as there was very large areas of background fluorescence that strongly suggested protein-surface covalent binding, however, there was a slight visibility improvement as compared to the control – thus it cannot be said that the Tween-20 offered no background minimisation at all. The

individual BSA blocking solution showed promising signs of minimisation of non-specific binding, the microarray spots were still visible. Despite this, there is still apparent

	Pre- block	***Post-block***	***Post-PNA incubation***
Control		(no block)	
Tween-20 0.05%			
BSA 2%			
BSA 2% Tween-20 0.05%			

Table 4: Details of blocking trial solutions and the results of each microarray, pre-block, post-block and after 1 hour incubation with a fluorescently labelled PNA lectin
Note: Only a section of the microarray has been shown here, colour differences are for visual aid only.

background fluorescence detected in the image. The combined use of the two types of blocking agents managed to produce not only an unchanged microarray after blocking but it eliminates the majority of background non-specific binding, there is still small amounts as a zero background is almost impossible to achieve due to electrostatic and hydrophobic interactions. However, by maximising the contrast in the fluorescence it was possible to produce suitable images that not only allowed visualisation of the spots but extraction of intensities from the fluorescently tagged PNA binding to the sugar substrates. A further control experiment was run, this involved the printing of a carbohydrate microarray onto a surface blocked slide, in comparison to a non-blocked NHS ester surface to provide further evidence that the reagent was successfully preventing covalent or non-covalent surface binding. The slides were printed in parallel and incubated for 1 hour at >75% humidity, followed by washes with dH_2O and phosphate buffered saline Tween-20 (0.05%) (PBST); the results of this control are shown in table 4. The results above show extremely strong evidence that once a surface is blocked using the BSA, Tween-20 combination the NHS tethers have been deactiviated, thus making

the surface inert. This provides the ideal situation to get the best signal-to-noise ratio possible and allow more accurate measurements.

3.5 Testing of Microarray Strategy Using PNA

From the previous results and data the next experiment was then conducted to attempt data extraction. As before, carbohydrate microarrays were printed on Nexterion® NHS ester slides and blocked following the procedure mentioned earlier utilising a BSA, Tween-20 combination. The slides were then incubated for 2 hours in a dark humidity chamber, after which they were washed with PBST and dH2O, then dried using nitrogen air. Images of slides were taken before and after incubation; figure 17. It must first be noted, lectins are inherently promiscuous, and will bind many sugars, they are only different in their relative affinities; these results aim to demonstrate a lectins binding profile; thus binding to most glycans is expected. In previous literature it is stated PNA has a higher affinity towards galactose moieties than other glycans.[46,47] The results shown below seem to correspond with previous findings, as a slightly higher

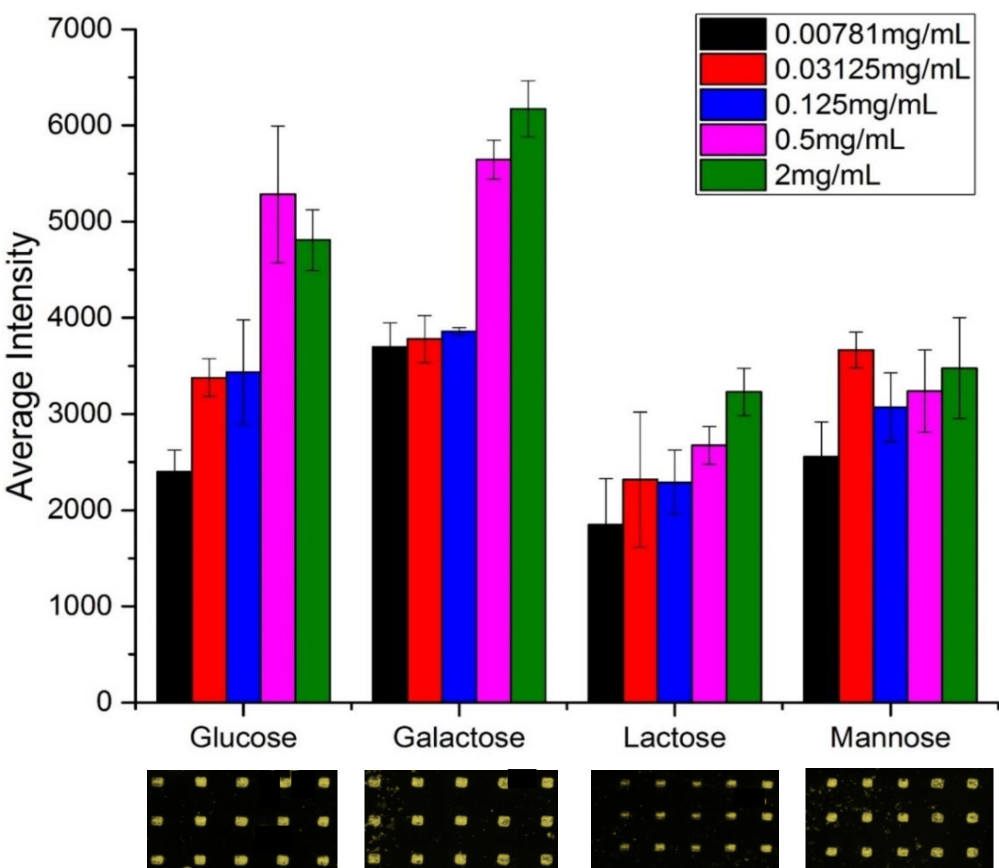


Figure 17: PNA lectin incubation results for four arrayed glycans, corresponding segments shown below

affinity is seen for the galactose than both mannose and lactose. The binding between glucose and galactose is very similar, this is probably in relation to their very similar

structures, but as expected there is a range of binding seen. The important feature is a good correlation between a larger intensity and a higher concentration; thus pointing towards successful binding of the lectin to each substrate.

3.6 Further Lectin Incubation: UEA I, SBA, DBA, RCA120

Further tests were then performed on more fluorescently labelled lectins; these were performed on the alternative microarray strategy (see figure 14) to try and maximise results. Initially it was planned to run two incubations simultaneously on a single slide to reduce cost, see figure 18. An issue arose from this methodology concerning the fluorescence images; the hydrophobic barrier that was being used was emitting vast amounts of fluorescence causing great difficulties detecting the microarray spots. Due to the highly sensitive nature of the microarray surfaces; removal of the barrier after incubation would only cause disruption to the system and create large amounts of error in the data therefore this strategy was abandoned and single lectin incubations were run as before. Figure 19 shows the results for the Soybean agglutinin (SBA) incubation.

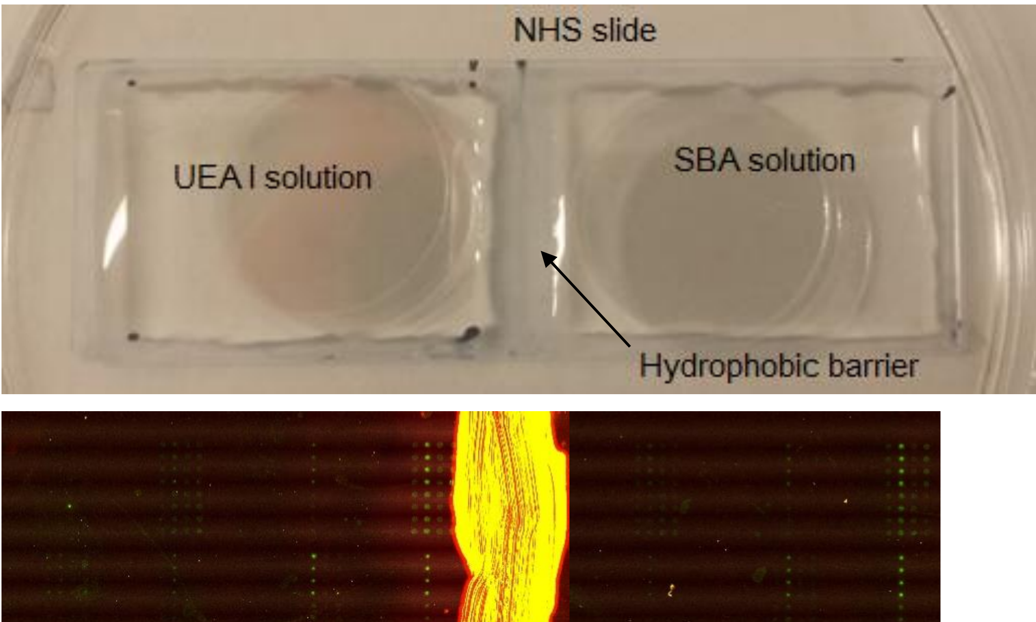


Figure 18: (TOP) Image demonstrating technique for multiple incubations on single slide, solution concentrations at 0.1mg/mL
(BOTTOM) Image showing hydrophobic barrier – fluorescnce disruption

Previous literature has shown SBA to have a binding preference of α- and β- Galactose residues;[48,49] from the data collected, it was observed that SBA showed a much stronger galactose binding intensity in comparison to other glycans. Alongside this there seems to be weak binding to other glycan residues with a slightly greater affinity towards the

glucose and mannose substrates. Interestingly the highest concentration of lactose (1mg/mL) has shown an intense peak suggest large binding affinity – this could be placed down to the structure of lactose (a disaccharide of glucose and galactose). At the higher concentration it is likely that the galactose residues are more accessible and thus a greater affinity is observed for this particular concentration. Figure 20 shows the results for the

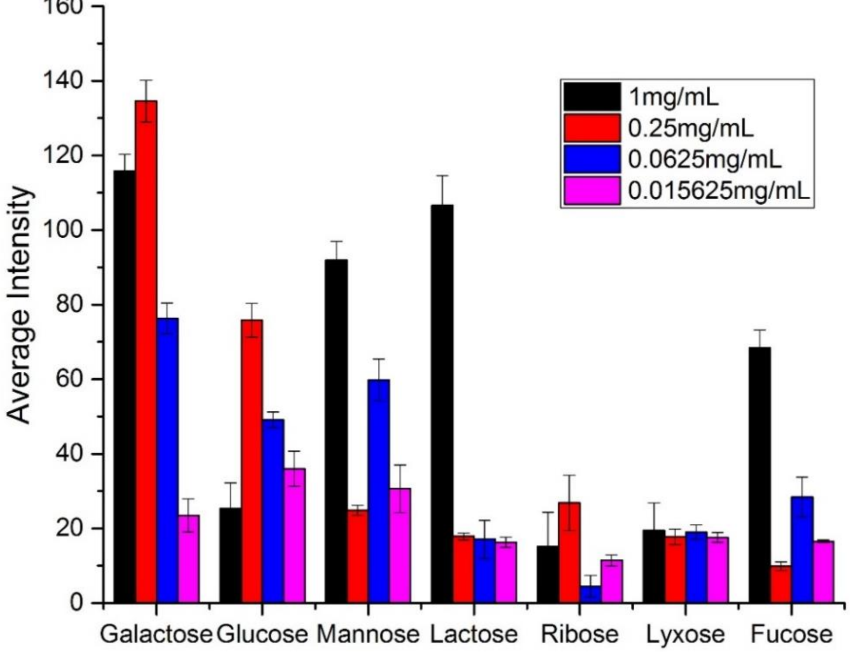


Figure 19: SBA lectin incubation results

Ulex europaeus agglutinin (UEA I) lectin incubation. Previous literature has shown that the UEA I lectin has a preference for fucose binding[50], but also has been known to bind strongly to none fucose[46,51] containing glycans. The results observed see a very broad

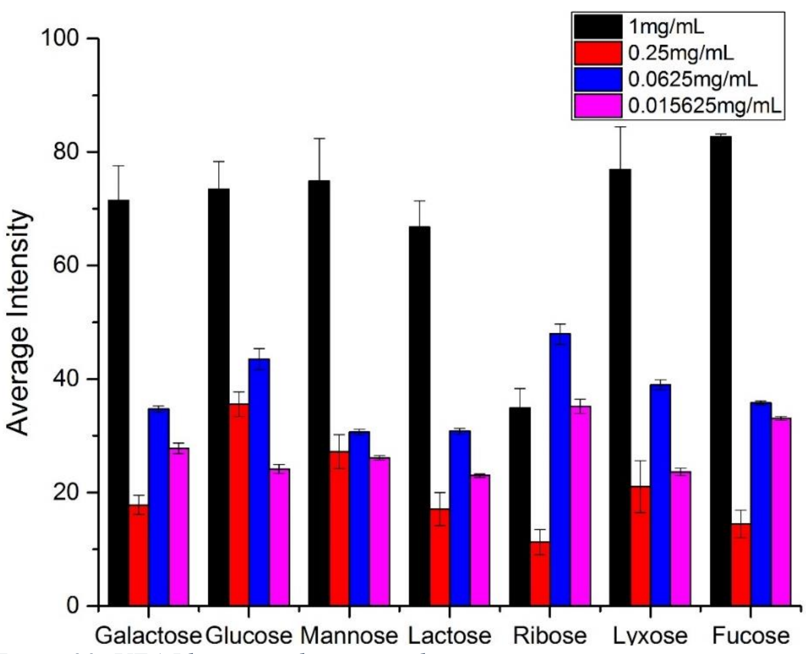


Figure 20: UEA I lectin incubation results

spectrum of affinities which corresponds to previous literature, although it could be noted that the fucose intensities are relatively large – it is not sufficient to say there is any specific preference for this binding interaction.

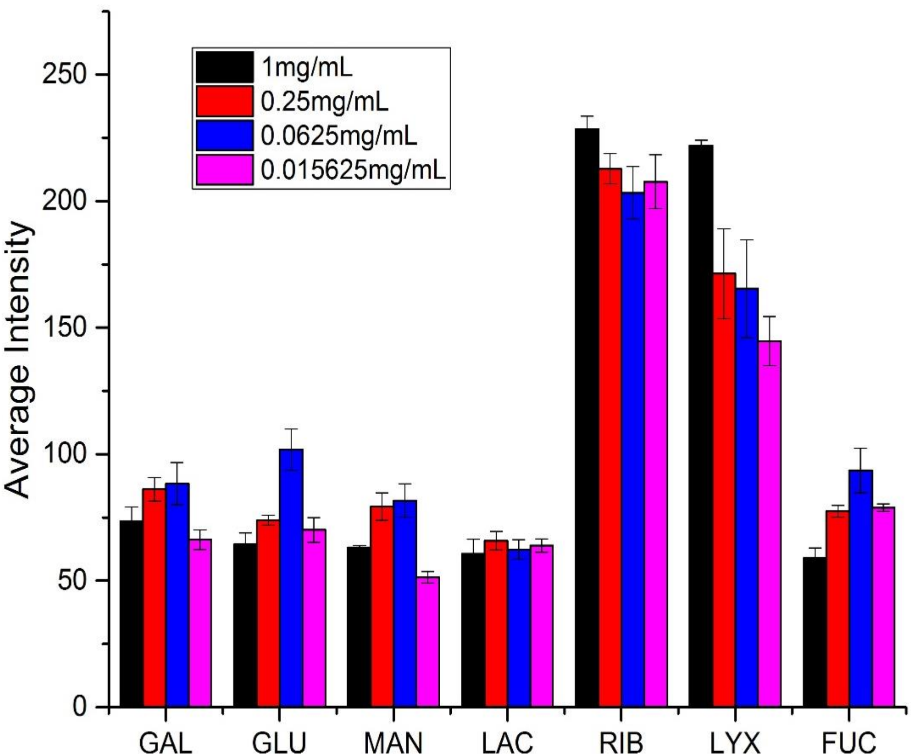


Figure 21: DBA lectin incubation results

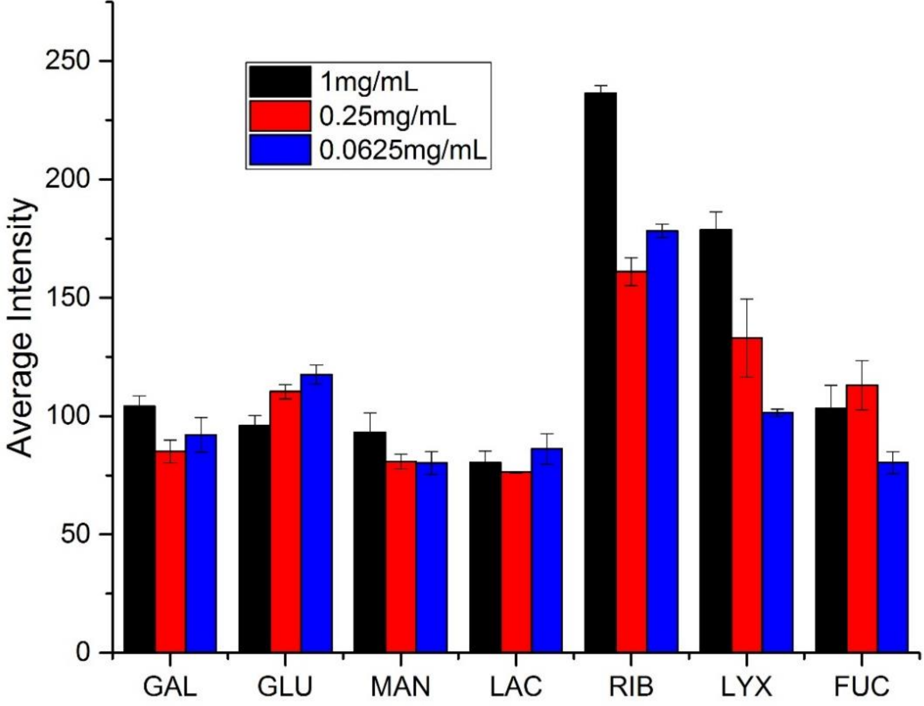


Figure 22: RCA_{120} lectin incubation results

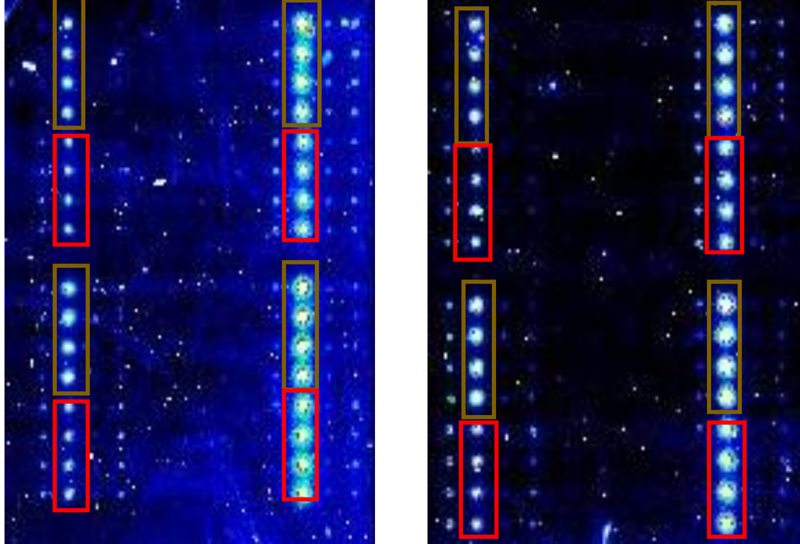


Figure 23: (LEFT) carbohydrate microarray BEFORE incubation with DBA (RIGHT) carbohydrate microarray BEFORE incubation with RCA_{120}
Highlighted boxes: Ribose, Lyxose

Figure 22 and 24 show the data extracted from the Dolichos biflorus agglutinin (DBA) and Ricinus communis agglutinin 120 (RCA_{120}) lectin incubations. The glycans ribose and lyxose intensities were found to be very large, this is contrary to previous literature, which states that DBA[49] and RCA_{120}[52] have a binding preference for galactose residues. It is noted that the microarray prints for these experiments were printed separately from the previous two lectin incubations due to space constraints in the microarray printing chamber. Figure 21 shows the images of the carbohydrate microarrays post-block but pre-incubation; it can be seen that certain spots have extremely high background fluorescence (Highlighted in figure 23).

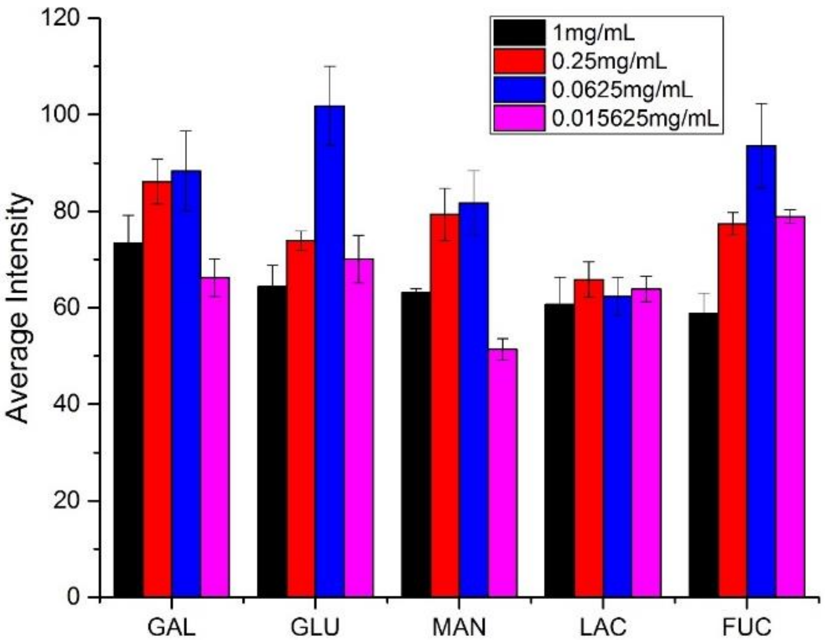


Figure 24: DBA lectin incubation results with lyxose and ribose removed

These highlighted spots all correspond to the intensities shown are not real representation of the lectins binding to the sugar substrate; figure 24 and 25 show the binding profile with these two glycans removed for DBA and RCA_{120}, respectively. For DBA, what can be observed is that there appears to be binding across the range of different saccharides with no real evidence for selectivity only a weaker affinity to lactose in comparison to the other glycans. It could be noted that this lectin has no dependence to concentration on binding; i.e. there is a maximum amount of glycan pockets on the structure of DBA which it cannot exceed, hence the binding affinity is capped. However, more data would need to be collected to support this. For the RCA_{120} lectin results, figures 22 and 25, only values for 3 concentrations were extracted due to poor visibility on parts of the microarray. A similar situation as the DBA is observed with not enough evidence to suggest a favoured glycan residue, although the mannose and lactose residues are noted to have slightly lower values than the rest. It is thought that alternative blocking procedure might be more effective to improve the sensitivity of this technique. Due to the small size of the sugars present on these microarrays and the use of a large protein blocker BSA, although BSA does not interfere with the lectin-glycan interactions, it is thought that they may hinder binding to the substrates simply via steric hindrance. A small molecule blocker may be more appropriate for these kind of applications, such as ethanolamine.[53]

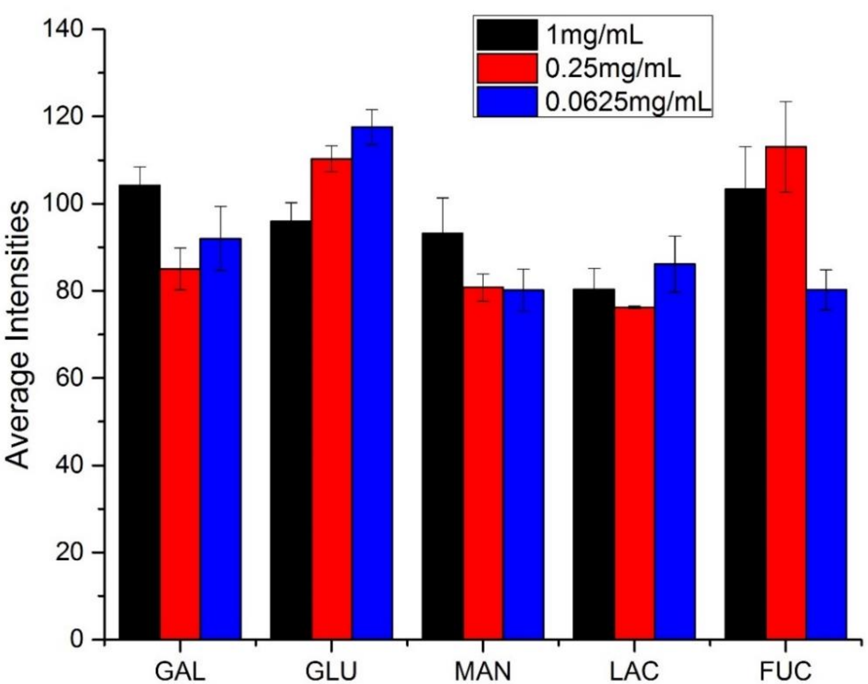


Figure 25: RCA120 lectin incubation results with lyxose and ribose removed

4. Conclusions and Future Work

The synthesis of 7 1-amino deoxy sugars was successfully completed in order to create a library like base of glycans to use in carbohydrate microarray experiments. Full characterisation by NMR and ESI-Quad mass spectroscopy was performed for their analysis.

The work done in this project has used known methods of surface modification using silane modification techniques in order to fabricate an immobilisation surface that is suitable for carbohydrate microarray applications. This work used developments from previous research into protein microarray fabrication and applied them to this newly submerging field. The microarrays that were created using the synthesised NHS ester activated surfaces were close in standard to the purchased Nexterion® slides, and thus, one day soon, this strategy could be seen as a cost effective manner of creating these carbohydrate microarrays.

It has been observed that a combined use of protein and detergent blockers produces a lower signal-to-noise ratio when compared to the use of these reagents on their own. Furthermore the use of this blocking technique minimised the non-specific binding interactions of the fluorescently labelled lectins to the surface such that it was possible to measure binding intensities of the lectins to the arrayed glycans. Using the techniques described the binding profiles of plant lectins to 7 different glycan residues at a range of concentrations. SBA was found to have a higher binding preference for galactose residues, while UEA I , DBA and RCA_{120} were observed to bind all glycan residues to a similar level. The technique still requires further development to increase sensitivity and be able to determine more accurate and detailed binding profiles. However, it can be said that this work is able to give a general profiling image of a proteins binding specificities and it was possible to see some distinction between each binding profile.

With use of a larger library of glycans and an improved technique of spot analysis this method new technology of carbohydrate microarrays to determine protein binding profiles could revolutionise the study of glycomic interactions. Some improvements that could be made to the study in reference to future work would be to trial some alternative blocking reagents to hopefully improve the sensitivity of the measurements to determine more accurate binding affinities; as mentioned previously a small molecule reagent may be better suited to microarrays of this nature. Furthermore,

more research into this technique would involve the incorporation of a much larger sample library, containing not just small sugars but larger oligosaccharide structures too which could compare to those found on a cells surface. In addition to this it would mean the testing of a wider range of lectin proteins, including pathogenic bacteria lectins, such as the cholera toxin lectin CTx, to have a positive impact into anti-adhesion therapy research.

The aim of this project was to achieve a reproducible carbohydrate microarray analysis technique to mimic a cells surface. The final results have shown strong promise for further developments into this area and it has been shown that it is possible to produce a simplified solution to understanding these complex interactions.

5. Experimental and Methods

5.1 Instrumentation

5.1.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

All NMR spectra were obtained using a Bruker DPX-300 (300 MHz) or DPX-400 (400 MHz) spectrometer, all submitted samples were done in D_2O obtained from Aldrich.

5.1.2. Mass Spectrometry

All MS analysis was obtained using a Bruker Esquire 2000 electrospray instrument. The $[M+H^+]$ peaks and sodiated $[M+Na^+]$ peaks were quoted.

5.1.3. Microarray Fluorescence Scanner

The primary method of analysis throughout the project was fluorescence imaging. This was done by having the lectins directly labelled using FITC (fluorescein Isothiocyanate) labelling.[54] This type of analysis allows a sensitive and real-time observation of many lectin-carbohydrate images even able to monitor weak carbohydrate-lectin interactions (Kuno et al., 2005). Images were taken using an Agilent G2565CA scanner (2µM resolution) which uses a standard two colour scanning technique of SHG-YAG laser (532 nm) and a helium-neon laser (633 nm). Each slide had a marked top left corner, away from the microarray spots, slides were then placed into the holders so the image would correspond to the original slide orientation.

5.1.4. Microarray Contact Printer

To construct the carbohydrate microarrays, a 4-pin Array-IT SpotBot® 3 microarray contact printer was used. Loading deck that accommodates 1 microplate (384-well) for samples and a slide substrate deck that accommodates 14 substrates of standard size (25 mm x 76 mm). The printing chamber offered humidity control between 10-80% RH.

5.2 Synthesis of Amino-substituted sugars

From 6 D-form monosaccharides: Glucose (1.00 g, 5.5 mmol), Galactose (1.00 g, 5.5 mmol), Mannose (1.00 g, 5.5 mmol), Ribose (0.50 g, 3.3 mmol), Lyxose (0.50 g, 3.3 mmol), Fucose (0.25 g, 1.5 mmol) and 1 D-form disaccharide, Lactose (1.00 g, 2.9 mmol), the synthesis of 7 1-amino deoxy sugars was performed (*as Dirk Vetter, Mark A. Gallop, 1995*).[55] Each of the saccharides were weighed out to the stated amounts, they were then made into 5% w/w solutions with D_2O (see table 5). Each solution was placed in a vial then saturated with ammonium carbonate at approximately a 1:10 ratio of sugar: salt. Each of the solutions were allowed to stir at room temperature for 6 days, if a solution

became no longer saturated, more ammonium carbonate was added. After 6 days had elapsed the solutions underwent gravity filtration to remove the undissolved ammonium carbonate. Once complete each of the vials were frozen to below -18°C, then freeze dried for 12 hours to remove excess D_2O and ammonium carbonate. Unsuccessful freeze dried samples were diluted using 80cm^3 of distilled water. Instead of immediate cryodesiccation the remaining sugars were heated at 55ºC for 4 hours to try and promote the decomposition of the remaining ammonium salts. The temperature was not raised higher than 55ºC so to avoid caramelisation of the sugars. To the resulting solutions 20 cm^3 of methanol was added to make an 80:20 water: methanol mix, the solutions were then rotary evaporated. Once all the solvent had been removed there remained a small amount of viscous liquid, this was then diluted in 10 cm^3 of dH_2O, frozen to below -18°C, then freeze dried for 12 hours, leaving the dry amino-substituted sugar product. Once all the products had been isolated, NMR and ESI-Quad samples were prepared in D_2O and methanol, respectively, and run for characterisation.

Reducing Sugar	Amount used (g)	D_2O used (mL)	$(NH_4)_2CO_3$ added initially (g)	Additionally $(NH_4)_2CO_3$ (g)	Yield (g)
Glucose	1.000	20	9.020	-	0.3418
Galactose	1.000	20	9.080	-	0.4732
Lactose	1.000	20	9.120	-	0..3806
Mannose	1.000	20	9.100	-	0.2467
Ribose	0.500	10	4.140	3.170	0.1932
Lyxose	0.500	10	4.070	3.180	0.1104
Fucose	0.250	5	2.520	1.410	0.1811

Table 5: Details of amino sugar synthesis

5.2.1. ^{1}H NMR assignments:

1-amino-deoxy lactose

^{1}H NMR (D_2O) 300MHz, δppm: 3.22 (1H, tt, H^4), 3.43-3.87 (12H, m, H^3,H^4,H^6,H^7,H^9), 4.05 (1H, d, H^1), 4.24 (1H, d, H^1), 4.37 (1H, d, $H^{2β}$), 4.59 (1H, d, $H^{2α}$), 5.14 (1H, d, H^8)

1-amino-deoxy glucose

^{1}H NMR (D_2O) 300MHz, δppm: 2.51-3.34 (6H, m, H^3, H^4, H^5 ,H^6 ,H^7), 3.67 (2H, d, H^1), 5.03 (1H, d, $H^{2\beta}$), 5.39 (1H, d, $H^{2\alpha}$)

1-amino-deoxy mannose

^{1}H NMR (D_2O) 300MHz, δppm: 2.74-3.35 (6H, m, H^3, H^4,H^5 ,H^6 ,H^7), 3.83 (2H, s, H^1), 5.31 (1H, d, $H^{2\beta}$), 5.86 (1H, d, $H^{2\alpha}$)

1-amino-deoxy galactose

^{1}H NMR (D_2O) 300MHz, δppm: 2.84 (2H, t, H^3,H^4), 2.96-3.26 (4H, m, H^5,H^6,H^7), 3.41(2H, d, H^1), 4.08 (1H, d, $H^{1\beta}$), 5.74 (1H, d, $H^{1\alpha}$)

1-amino-deoxy fucose

^{1}H NMR (D_2O) 300MHz, δppm: 1.13 (3H, d, H^7), 3.32-3.88 (4H, m, H^3,H^4,H^5,H^6), 4.02 (2H, d, H^1) 4.73 (1H, d, $H^{2\beta}$), 5.12 (1H, d, $H^{2\alpha}$)

1-amino deoxy ribose

^{1}H NMR (D_2O) 300MHz, δppm: 3.23-4.02 (5H, m, H^3, H^4, H^5, H^6), 5.13 (1H, d, $H^{2\beta}$), 5.27 (1H, d, $H^{2\alpha}$)

1-amino deoxy lyxose

^{1}H NMR (D_2O) 300MHz, δppm: 3.15-3.95 (5H, m, H^3, H^4, H^5, H^6), 4.25 (2H, s, H^1), 4. 45 (1H, d, $H^{2\beta}$), 5.15 (1H, d, $H^{2\alpha}$)

5.3 Functionalisation of NHS ester slides

The functionalisation of glass surfaces was done to produce N-hydroxysuccidimide (NHS) ester functionalised surfaces in a three step process. One slide was removed at each stage to be used for contact angle measurements. Plain glass solid surfaces were chemically cleaned using piranha solution (**caution – reacts violently with organic material**). All glassware was first cleaned with distilled water, then acetone followed by thorough drying. 9 mL of H_2SO_4 (98%) was placed in the reaction vessel and placed on ice allowing to cool for 5 minutes, 3 mL of H_2O_2 (30%) was then added dropwise – this reaction is extremely exothermic – to produce the piranha solution. The glass slides were then immersed in the solution and left to clean for twenty minutes. Once complete, the slides were removed by the edge of the slide using tweezers and washed with dH_2O, ethanol and finally dH_2O again, the slides were dried with a stream of nitrogen air. After this point the slides were kept in the same orientation (modified surface upwards) so to not disturb the surface. All glassware was cleaned and dried with dH_2O and nitrogen air (important no water in the next step to prevent autopolymerisation of (3-aminopropyl)triethoxysilane (APTES). A solution of APTES (0.5 mL, 1% v/v in toluene) was made up, the hydroxylated slides were then submerged in the solution and left to react for 2 hours. The reaction was kept dry at all times. Once the time had elapsed, the slides were removed carefully and cleaned with Toluene (5 x 2 mL) and dH_2O (5 x 2 mL)

and dried between each step using nitrogen air. A solution was prepared of the NHS ester *NN*'-disuccinimydyl carbonate (0.5 g, w/w% in dimethylsulphoxide (DMSO)), the aminosilane functionalised slides were submerged in the solution and left to react for 1 hour. The slides were then carefully removed, washed with DMSO (5 x 2 mL) followed by dH_2O (5 x 2 mL) and then dried with nitrogen air. After the surface modification was complete, contact angle measurements were then taken for each slide (plain glass, hydroxylated glass, amino-silane (APTES), NHS ester).

5.4 Control Print Experiments

Serial dilutions of 5 fluorescently labelled lectins in phosphate buffered saline solution (PBS) (10 mM), Peanut agglutinin (PNA); Soybean agglutinin (SBA); Ulex europaeus agglutinin (UEA I); Dolichos biflorus agglutinin; Concanavalin A (Con A), were made up in a 384 well plate. The first concentration was 2 mg/mL, then ten dilutions were conducted by a factor of 2. NHS ester slides were removed from -20ºC storage, rinsed gently with dH_2O and dried using nitrogen air, slides were left to reach room temperature for 10-15 minutes. Plain glass slides and NHS ester modified slides were placed in the contact printer, the printing chamber was allowed to humidify up to >75%. The 384 well plate with lectin dilutions was placed inside once the humidity had been reached. After printing, slides were left incubated for 1 hour at >75% humidity. The slides were carefully removed and fluorescence images were taken. All slides were dip washed twice in dH_2O (25 mL) for 10 seconds and then left to air dry for 20 minutes, after which, images were then taken of the slides one more time. Serial dilutions were then made for the 1-amino de-oxy sugars, dilutions were once again done in PBS (10 mM) in a 384-well plate with a starting concentration of 2mg/mL, followed by dilutions of two-fold for 8 repetitions. The same conditions as above were used for printing. Dip washing was then done with PBS (10 mM) once and dH_2O once for 10 seconds each, after which they were left to air dry for 15-20 minutes and finally stored in a dry sealed container.

5.5 Blocking Tests

Three solutions were prepared, BSA (0.4g, 2% w/w in PBS 10mM), Tween-20 (10µL 0.05% v/v in PBS 10mM), BSA(0.4g, 2% w/w) /Tween-20 (10µL, 0.05% w/w) (in PBS 10 mM). Carbohydrate microarray slides were submerged in each solution in a sealed incubation chamber at room temperature for 30 minutes. After incubation the slides were removed and dip washed in PBS solution (10mM) and then allowed to air dry for 20

minutes, after which fluorescence images were taken. The two Tween-20 containing blocks were then submerged in a solution of PNA lectin (0.1 mg/mL in PBST (Phosphate buffered saline, Tween-20 (0.05% v/v)). The BSA (2%) block was submerged in a solution of PNA (0.1 mg/mL in PBS (10mM)). The solutions were stored in a dark incubation chamber at 75% humidity for 1 hour. After removal from the solution, Tween-20 blocked slides were dip washed twice with PBST followed by dH_2O for 10 seconds and then left to air dry for 15-20 minutes, while BSA (2%) was washed with PBS and dH_2O.

5.6 Carbohydrate Microarray Lectin Incubations

5 different lectins; PNA, SBA, DBA, UEA I, Ricinus communis agglutinin- 120 (RCA_{120}) were diluted in PBST to produce concentrations of 0.1 mg/mL. Blocked carbohydrate microarrays were removed from storage and placed inside a dark chamber at >75% humidity for 10 minutes. A layer of lectin solution was placed onto the microarrays surface using a glass pipette. The chamber was then re-sealed and the microarray was left incubated for 2 hours. After incubation the substrates were dip washed twice with PBST followed by dH_2O for 10 seconds and then left to air dry for around 15-20 minutes.

5.7 Analysis of Images: ImageJ and Agilent extraction software

Two pieces of software were used for the analysis of the images, initially the Agilent feature extraction software was used to view the corresponding Tagged Image File (TIF) output files, however, it was discovered that using this software the only method of calculating intensity values was through manually picking peaks from a generated intensity graph, this resulted in large human error and inaccuracy. In an attempt to eliminate this issue the images were transferred to ImageJ. As the Agilent fluorescence software is able to extract only the green colour fluorescence; which is used in FITC labelling. ImageJ is also capable of doing this task by splitting an image into its' discrete colour channels; thus it was possible to only show the intensities from the green colour channel. Furthermore, the use of this software allowed determination of an average intensity over a specific area as shown in figures 26 and 27; this eliminated error in manually collecting intensity values.

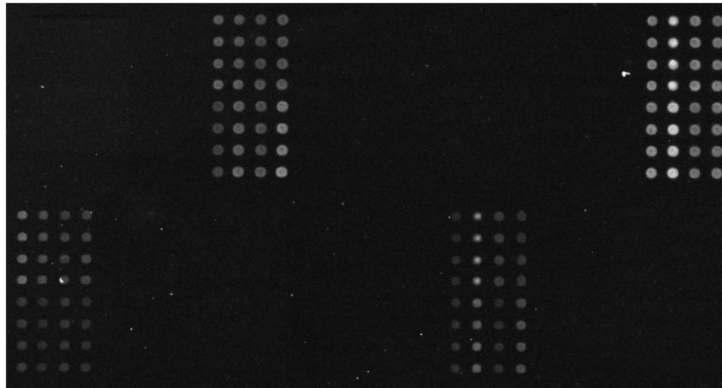


Figure 26: Green channel extraction from ImageJ software

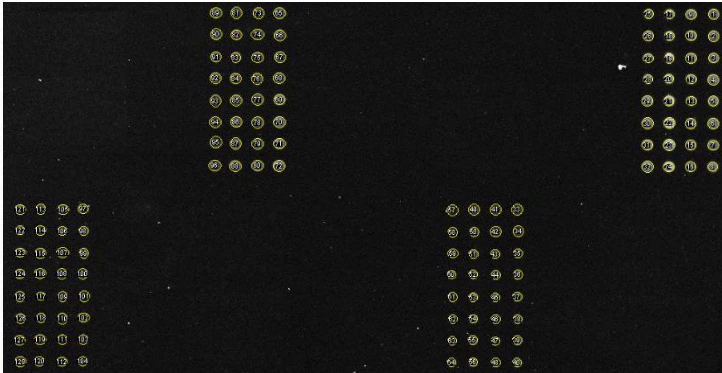


Figure 27: Example of intensity calculation over spot area

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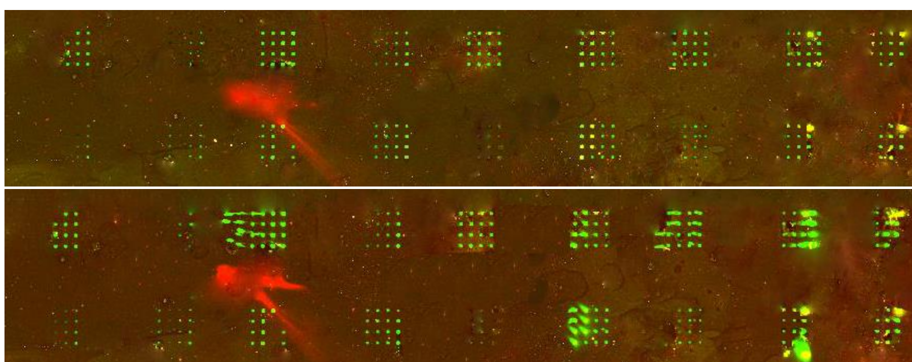
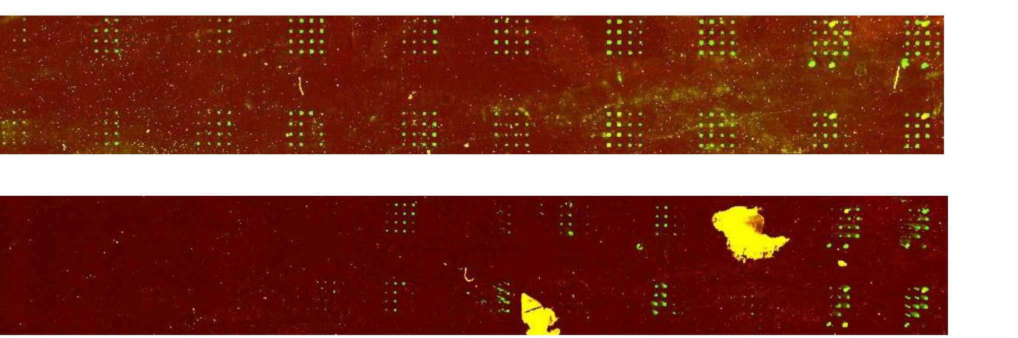
7. Appendices

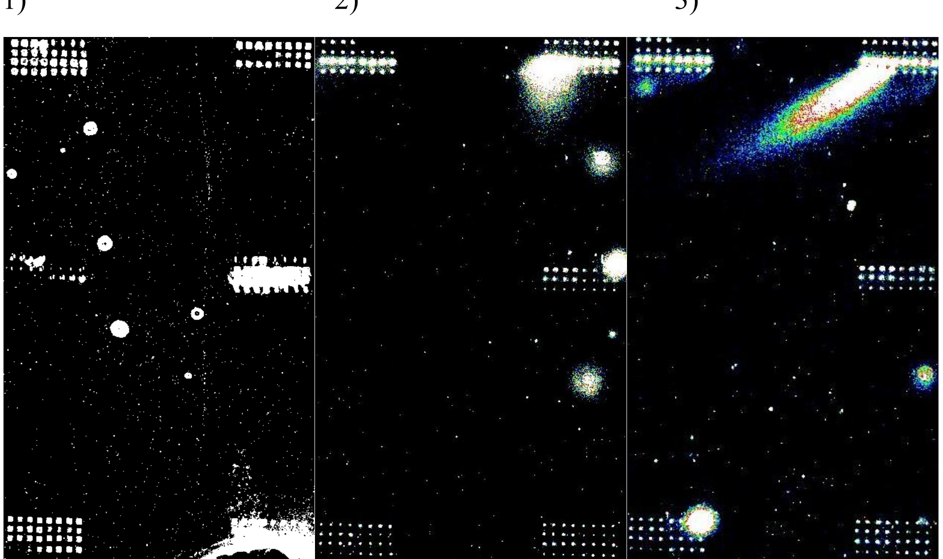


Images of PNA printed onto synthesised NHS ester surfaces pre-wash (top), post-wash (bottom)



Images of PNA printed on plain glass slide, pre-wash (top), post-wash (bottom)

1) 2) 3)



3 carbohydrate microarrays printed with 200 µM spot distance, spots were too close together and excessive smearing would occur meaning loss of spot resolution. Thus, spot distance of 500 µM was used in the report to improve analysis of microarrays. (1) Synthesised NHS surface, (2)(3) Purchased NHS surface.