**Investigation of whole cell meningococcal glycan interactions using high throughput
glycobiology techniques: glycan array and surface plasmon resonance**

Tsitsi D. Mubaiwa, Lauren E. Hartley-Tassell, Evgeny A. Semchenko, Christopher J.
Day, Michael P. Jennings, Kate L. Seib*

Institute for Glycomics, Griffith University, Gold Coast, Queensland, Australia

* Address correspondence to k.seib@griffith.edu.au

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Abstract

A growing body of evidence suggests that glycans are important for meningococcal host-pathogen interactions and virulence. The development of glycobiology techniques such as glycan array analysis and surface plasmon resonance (SPR) has increased awareness of the importance of glycans in biological processes and has increased the interest of their study. While these techniques are more routinely used with purified proteins, there is growing interest in their applicability to cell-based studies, to better emulate host-pathogen interactions *in vivo*. Here we describe the use of glycan array analysis and SPR for the investigation of glycan binding by *Neisseria meningitidis* cells. Used together, these methods can help identify and characterise *N. meningitidis* glycointeractions.

1. Introduction

The ability of bacteria to bind host glycans has long been linked to pathogenesis (1). In some cases, differential glycan binding results in different levels of virulence between strains (1). The development of glycobiology techniques, such as glycan arrays and surface plasmon resonance (SPR), has facilitated a greater understanding of the role of glycans in various biological processes. Glycan array technology has rapidly become a standard for the detection of glycan interactions (2). It is a high throughput technique that can be used to identify glycan binding by bacterial, viral, mammalian cells or purified biomolecules (3). Much like DNA microarrays, glycan arrays contain a large number of glycans that are immobilised onto a solid support (2). Fluorescently labelled ligands are incubated onto the slides to allow binding, which is then visually detected using a laser scanner (4) (Fig. 1). Surface plasmon resonance is a quantitative bioanalytical technique which provides association and dissociation kinetics of a reaction (4). It is a highly sensitive technique that requires small amounts of glycans and can be used to detect dissociation constants (affinities) in the picomolar range (4). In this method, a ligand is captured or immobilised onto a sensor chip, and the

analyte in solution is flown over the chip to allow binding (4). Reactions are detected and quantified based on a change in mass (4).

In prior studies, we used glycan array analysis as a screening tool to characterise the glycointeractome of the *Neisseria meningitidis* serogroup B strain MC58 (5). Glycan array analysis of the wild type MC58 strain allowed us to determine the glycan binding properties of the organism, and by comparison the binding properties of mutant strains lacking major outer membrane structures, we determined the meningococcal surface structures potentially responsible for mediating the interactions (5, 6). This approach can be applied to similar investigations of other meningococcal serogroups and mutant strains.

2. Materials

Prepare all solutions using deionised water and analytical grade reagents. Filter all buffers using 22 µm filters, prior to their use.

2.1 Glycan array analysis

1. Glycan arrays (e.g. Dextra GAK001) (*see Note 1*).
2. Phosphate buffered saline (PBS) pH 7.4; weigh 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄ and 0.24 g KH₂PO₄ and transfer to a 1 L graduated cylinder. Add MilliQ water to a final volume of 1 L and adjust pH to 7.4, using HCl.
3. Array PBS: PBS pH 7.4 with 1.8 mM MgCl₂ and 1.8 mM CaCl₂ (*see Note 2*).
4. *Neisseria meningitidis* grown on BHI agar plate.
5. 20µM Bodipy-SE 558 (Invitrogen D2219) (*see Note 3*). Prepare dye stock to 40 mM in anhydrous DMSO (*see Note 4*). Transfer 40 µL of the dissolved dye to a clean 50 mL conical polypropylene centrifuge tube. Add 960 µL of 100% ethanol and mix. Add 19 mL of PBS and mix. Aliquot dye into 250 µL lots and store at -20 °C, in the dark until required.
6. 2.5% formaldehyde in PBS.

7. Bovine serum albumin (BSA) made up to 0.5% in array PBS.
8. 50 mL conical polypropylene centrifuge tubes.
9. Forceps for slide handling.
10. 10 mL tubes.
11. Shallow plastic container for washing (e.g. pipette box lid).
12. Slide scanner capable of scanning within excitation/emission of selected dye (595/625 nm for Bodipy-SE 558) and at $<10\ \mu\text{M}$ resolution (*see Note 5*).
13. 37°C incubator.

2.2 SPR using amine coupling (C1) sensor chips (BIAcore T200)

1. BIAcore T100/T200/S200 (GE Healthcare).
2. Series S C1 sensor chip (GE Healthcare).
3. 10 mM sodium acetate solutions, pH ranging from 3.0-5.5 in 0.5 pH increments (adjusted with HCl).
4. Running buffer: PBS pH 7.4.
5. 50 mM NaOH.
6. Amine coupling kit (e.g. GE Healthcare) containing 0.39 M 1-ethyl-3-(3-dimethylaminopropyl) - carbodiimide (EDC), 10 mM N-hydroxysuccinimide (NHS) and 1M ethanolamine.
7. Formaldehyde (2.5%) fixed bacterial cells at $\text{OD}_{600} = 0.1$.
8. 4 mL, 1.5 mL, 7000 μL vials, 96 well plates and plate seals.
9. BioNormalizing solution (70% glycerol).

3. Methods

3.1 Glycan array analysis of whole cells

3.1.1 Preparation of bacterial cells

1. Grow bacteria on solid media (e.g. 1x BHI agar plate) overnight at 37°C at 5% CO₂. Harvest cells into 5 mL of PBS and gently resuspend. Remove excess media by centrifuging cells at $771 \times g$ for 5 minutes, and discarding supernatant. Repeat wash in 5 mL PBS.
2. Resuspend cell pellet in 250 μ L PBS.
3. Label cells by adding equivalent volume of 20 μ M Bodipy-SE 558 to cell suspension. Incubate at 37°C for 30 minutes.
4. Centrifuge cells at $771 \times g$ for 5 minutes and remove excess dye. Wash cells three times in 5 mL of PBS. Resuspend cells in array PBS and adjust cell density to OD₆₀₀ = 0.1 (*see Note 6*).

Preparation of glycan array slide:

5. Pre-block slide by immersing in 50 mL tube with 0.5% BSA.
6. As per instruction manual for glycan slides, carefully remove the plastic protector from the gene frame which has the window cut out. Carefully apply each gene frames to the slide starting with the first gene frame on the top left-hand corner. Place the second gene frame directly underneath the top gene frame on the very left-hand side of the slide. The third gene frame aligns directly below the second gene frame (**Fig. 2**).

3.1.2 Hybridisation of sample to array slide

1. Remove the top plastic protector from the gene frame and pipette 65 μ L of labelled cell sample into the left-hand edge of the gene frame area, without touching the slide (*see Note 7 and Fig. 3*). Work quickly to seal the gene frame using the coverslip, avoiding air bubbles.
2. Place slide in a humidified chamber to and incubate at 37 °C for 15-30 min in the dark (*see Note 8*).

3.1.3 Washing and fixing of array slide

1. After incubation, place array PBS into a clean plastic container (e.g. the lid from a pipette tip box). Submerge slide under the solution. Carefully remove each coverslip and gene frames using forceps. Be careful not to touch the glass slide.
2. Fix cells by immersing slide into 50 mL tube with 2.5% formaldehyde and incubating at room temperature for 20 minutes.

3. Prepare three 50 mL tubes containing 45 mL array PBS.
4. Transfer array to first tube of array PBS and wash by gentle inversion for 2 minutes. Repeat washes with remaining tubes of array PBS.
5. Transfer array slide to a clean 50 mL tube and dry by centrifuging at $200 \times g$ for 4 minutes. Slides must be completely dry before scanning or storage. To avoid photo-bleaching, store slides in the dark (*see Note 9*).

3.1.4 Scanning and analysis of array slides

1. Scan slide at 558/568nm, and at 10 μm resolution (*see Note 10*).
2. Analyse using microarray scanner software and a “.gal” file (*see Note 11*).
3. A positive interaction is identified as a visible group of spots that have a fluorescence intensity which is statistically significantly greater than the slide background and empty (blank control) spots (**Fig. 1 (3)**).

3.2 SPR using bacteria cells immobilised onto a C1 sensor chip

3.2.1 Preparation of bacterial cells

1. Grow bacteria on solid media (e.g. 1x BHI agar) overnight at 37°C at 5% CO₂. Harvest cells into 5 mL of PBS and gently resuspend. Remove excess media by centrifuging cells at $1000 \times g$ for 5 minutes, and discarding supernatant. Repeat wash three times in 5 mL PBS.
2. Fix cells by adding equal volume of 5% formaldehyde (final concentration = 2.5%) and incubate at room temp for 5 minutes. Pellet cells at $1000 \times g$ for 5 minutes and discard supernatant. Resuspend cells in 5 mL of PBS and centrifuge at $1000 \times g$ for 5 minutes. Discard supernatant and repeat wash twice in PBS. Resuspend cells in PBS and adjust cell number to OD₆₀₀ = 1 ($\sim 10^8$ colony forming units (CFU)).

3.2.2 Set up of BIAcore C1 chip

1. pH scout of bacteria:
 - a. Follow the BIAcore method template (Wizard) for surface preparation, pH scout.

- b. Dilute cells 1:10 ($\sim 10^7$ CFU) (*see Note 12*) in 10 mM sodium acetate solutions of varying pH (ranging from pH 3 to 5.5). Run cell samples over flow path 1 at a flow rate of 5 μ L/min with a contact time of 120 seconds. Clean chip with 0.05 M NaOH.
- c. Analyse results. For immobilization, select the pH which shows a curve with the highest response units (RU).

2. Immobilisation of bacteria to C1 chip

- a. Prepare cells in 10mM sodium acetate of optimal pH (as determined in pH scout above).
- b. Select 'immobilisation' in surface preparation methods.
- c. Follow the Wizard set up.
- d. Select chip type as C1. Select amine as the coupling method. Select 'Flow Cell 1', and select 'blank immobilisation'. For test samples (bacteria) check the flow cells to be used and select time and flow based immobilisation. Immobilise for 900 seconds at a flow rate of 5 μ L/min. Aim to capture approximately 200 RU of bacterial cells.

3. BIAcore assays to determine affinity of glycan interactions

- a. Select 'new method' for BIAcore T100/T200, and 'LMW single cycle' for BIAcore S200.
- b. Set the temperature to 25°C, and the concentration of analytes to μ M. Set for 1 Hz recording. Use dual channel detection if analysing one immobilised ligand or multi-channel detection if testing more than one ligand at the same time.
- c. Set up one cycle with the purpose to be sample and link it to cycle_1. Cycle_1 contains the following steps (*see Note 13*): Sample: single cycle kinetics, flow rate of 30 μ L/min. Contact time of 90 seconds and dissociation time of 600 seconds. Regeneration 1: use a regeneration buffer that will cause minimum damage to the immobilised cells such as the sodium acetate buffer used for immobilisation. Contact time 120 seconds, flow rate 30 μ L/min.

4. Set up each analyte with two rows in the table. Row 1: 5x zero concentration, as non-binding control, using the running buffer. Row 2: Dilution series. On first run set up a 1:5 dilution series (0.16 μ M, 0.8 μ M, 4 μ M, 20 μ M and 100 μ M). Once a concentration range is known then

subsequent runs can use a 1:2 dilution series around the saturation point. Ensure that the same running buffer (same bottle) that was used for making dilutions is used as the running buffer for the experimental run.

5. Prime system before commencing run. For S200 software, delete control sample and solvent correction. Insert a regeneration step in the sample overview, and remove the carry-over control.
6. Analyse using the BIAcore evaluation software.

3.2.3 Evaluation of affinity interactions

1. Select surface bound kinetics/affinity or affinity. Select the flow cell for analysis and the specific sample to analyse.
2. Fit curve at 4 seconds before injection stop, with a window of 5 seconds.
3. Use the Steady state affinity fit model. Due to fast association/dissociation, kinetic models cannot be used.

4. Notes

1. Glycan arrays may be sourced from other suppliers including RayBiotech (GA-Glycan-100-1).
2. 1X stock of array PBS must be prepared daily as crystals will form over time. 20 X stock solutions can be made and stored without issues.
3. Alternative labelling for cells include CFDA-SE (Invitrogen C1157), and intrinsic fluorescent markers such as green fluorescent protein (GFP). Check laser scanner specifications prior to selecting dye/label.
4. Aliquot 40 mM dye stocks into 40 µL batches and store at -20 °C in the dark until required.
5. Check laser scanner specifications prior to selecting a fluorescent dye or label.
6. If required, cells can be fixed in 2.5% formaldehyde for 20 minutes. Prior to incubation on the array, excess formaldehyde should be removed by washing 3X in array PBS.

7. Gloves must be worn at all times when using the Dextra glycan array slides. The glycans are printed on the surface of the slide, so extreme care is needed when handling the slide. The barcode area is safe to touch.
8. For a humidified chamber, a plastic box such as a pipette tip box with 1 cm water in the bottom may suffice. Ensure the slide is flat and elevated above the water, not submerged.
9. In general, scan slides using 100% laser power with a PMT of 50-70% gain. In the case of low signal, increase PMT gain so that control spots can be visualised.
10. Scan slide at a wave length appropriate for the fluorophore used.
11. Image analysis can be performed using most microarray analysis software including Innopsys (Mapix), ScanArray (Perkin Elmer) and Spotfinder (TIGR).
12. The number of bacterial cells used (CFU/mL determined from optical density at 600 nm (OD_{600})) is dependent on capacity of BIAcore flow cells (200 nL for T200) and size of bacteria cells. A lower CFU/mL may be required for larger cells. Aim for 50 CFU/ μ L for larger cells.
13. In the BIAcore S200 software, delete control sample, solvent correction and start up.

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References

1. Higgins MA, Whitworth GE, El Warry N et al (2009) Differential recognition and hydrolysis of host carbohydrate antigens by *Streptococcus pneumoniae* family 98 glycoside hydrolases. *J Biol Chem* 284(38):26161-26173
2. Oyelaran O, Gildersleeve JC (2009) Glycan arrays: recent advances and future challenges. *Curr Opin Chem Biol* 13(4):406-413
3. Tiralongo J, Moran AP, (2010) Bacterial lectin-like interactions in cell recognition and adhesion. In: Moran AP, Otto H, Brennan PJ et al (ed) *Microbial glycobiology: structures, relevance and applications*. San Diego
4. Varki A, Etzler ME, Cummings RD, et al. (2009) Principles of glycan recognition. In: Varki A, Cummings RD, Esko JD et al (eds) *Essentials of glycobiology*, 2nd edn. La Jolla, California
5. Mubaiwa TD, Hartley-Tassell LE, Semchenko EA et al (2017) The glycointeractome of serogroup B *Neisseria meningitidis* strain MC58. *Sci Rep* 7(1):5693
6. Mubaiwa TD, Hartley-Tassell LE, Semchenko EA et al (2018) The Bexsero *Neisseria meningitidis* serogroup B vaccine antigen NHBA is a high-affinity chondroitin sulfate binding protein. *Sci Rep* 8(1):6512

Figure 1. Overview of glycan array analysis. 1). Functionalised glycans are printed on to a glass slide using robotic technology. 2). Fluorescently labelled proteins or cells are incubated onto the slide to allow binding. 3). The slide is scanned to determine binding to the glycans printed on the array. Binding is detected as four adjacent fluorescent spots that have a fluorescence intensity which is significantly greater than the slide background and empty spots.

1 **2**

Figure 2. Positioning of Gene Frames onto array slides. 1). The gene frame is aligned with top and left-hand edges of the glass. 2). The second gene frame is placed directly under the top gene frame, and the third under the second gene frame.

1 **2**

Figure 3. Application of samples onto array slides. 1). Remove plastic protector from gene frame and add sample. 2). Seal gene frame with coverslip. Gently roll the coverslip on. Then repeat for second and third samples.