Synthesis and applications of cell wall glycopolymer fragments from Staphilococci and Enterococci

Berni, F.

Chapter 3

Development of a teichoic acid microarray tool for antibodies profiling

D. van der Es*, F. Berni*, W. F. J. Hogendorf, N. Meeuwenoord, D. Laverde, A. van Diepen, H. S. Overkleeft, D. V. Filippov, C. H. Hokke, J. Huebner, G. A. van der Marel, J. D. C. Codée; *Chem. Eur. J.*, **2018**, *24*, 4014–4018.

INTRODUCTION

Our immune system protects us against possible dangerous infections.[1] The first line of defence is constituted by the innate immune response which is characterized by a non-specific but fast reaction, to recognize and eradicate pathogens.[2] The second arm of the immune system, the adaptive immune system, develops during the lifetime of an individual as the adaptation to infections by pathogens. This immune response develops slower, but lasts longer and serves to protect form reinfection with the establishment of immunological memory.[3]

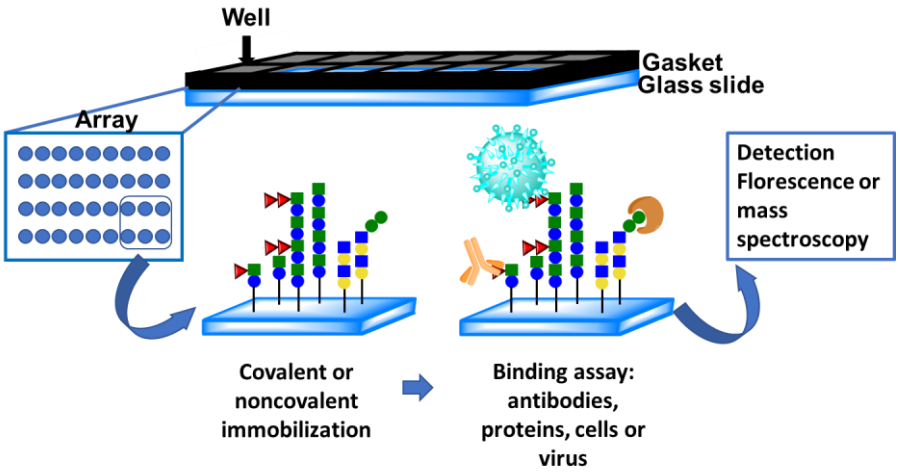
The development of vaccines for the prevention of infectious diseases relies on the activation of the adaptive (humoral and cellular) immune response. The ultimate goal is to confer long-term protection and the nature of the vaccine determines the type of antigen-specific immune effectors and the production of immune memory cells.[4] One of the parameters for the evaluation of vaccine efficacy is the detection of high affinity antibodies. These are released by B-lymphocytes and upon maturation into memory cells the isotype of the immunoglobulins (Ig) switches from low-affinity IgM to high affinity IgG.[1,5] The evaluation of the (specific) recognition of antigens of the pathogen constitutes a critical parameter for vaccine and diagnostic purposes but also for basic and clinical research.[6] In the last decades anti-carbohydrate antibody profiling has significantly advanced thanks to the advent of glycan-microarray technology, which allows the analysis of carbohydrate-mediated interactions in a high throughput manner using minute amounts of material.[7] For example, Gildersleeve and co-workers used this tool to probe the anti-carbohydrate antibody profile of sera of 48 healthy volunteers against 122 different glycans (98 well-defined synthetic fragments and 24 natural glycoproteins). High levels of IgG antibodies were detected against α-L-rhamnose (α-Rha), β-L-rhamnose (β-Rha), the Forssman disaccharide as well as the well-known α-Gal epitope.[8]

Generally, a glycan microarray is created by positioning different carbohydrates (from natural sources or chemically and/or enzymatically synthesized) in a dot matrix array fashion on a glass microscope slide via an automated arraying robot (Figure 1).[9] On a single slide multiple arrays can be printed, as the applied gasket can contain up to 64 wells for the simultaneous screening of different samples. The immobilization of the fragments can occur either via noncovalent interaction or covalent coupling and once printed the slides can be stored for several months in the dark and inert atmosphere. Carbohydrate-microarrays have been employed for the analysis of glycan binding specificity of not only antibodies but also proteins (such as lectins from the immune system), viruses and cells, through fluorescent measurement or mass spectroscopy detection.[10]

As described in Chapter 1, teichoic acids (TA) are immunogenic cell-wall components present in many Gram-positive bacteria species. Since the isolation from native sources leads to heterogenous mixtures, synthetic strategies have been developed both in solution and using automated solid phase techniques, to deliver well-defined TA-fragments.[11] Libraries have been generated comprising both glycerol phosphate (GroP) and ribitol phosphate (RboP)-based TAs. [12,13,14] In order to evaluate these compounds in a high throughput fashion, herein the development of a TA-microarray is described as a qualitative tool to access the binding specificity of monoclonal antibodies as well as

polyclonal sera. The availability of this technique enables the rapid screening of binding interactions to establish structure-immunogenicity relationship studies, aid in monoclonal antibody development and profile sera for both basic and clinical research.

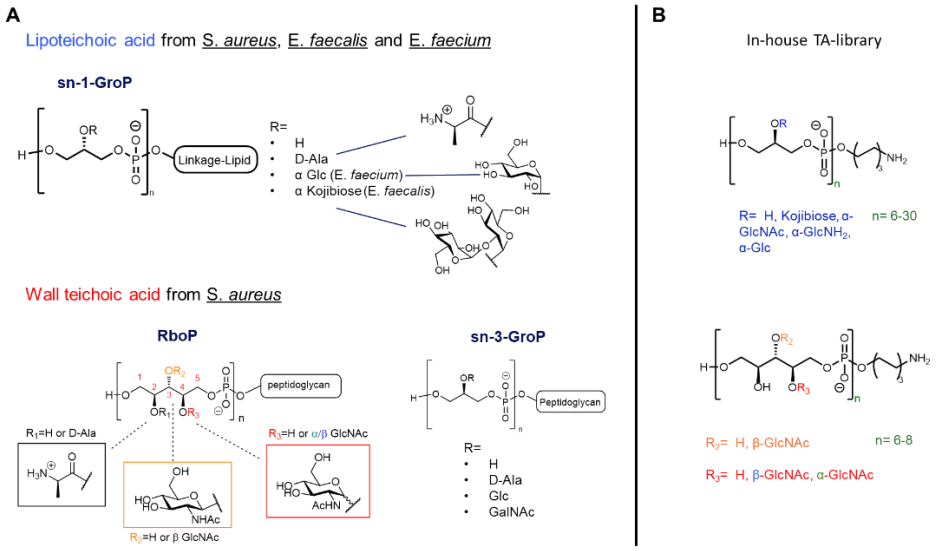
Figure 1: Schematic representation of a glycan-microarray assay.



RESULTS AND DISCUSSION

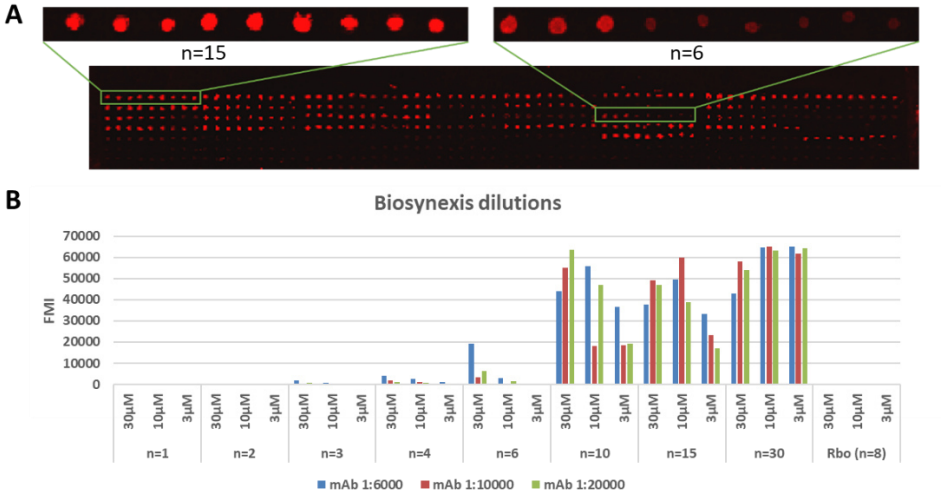
In Figure 2, the structures of the TAs found in *Staphylococcus aureus* and *Enterococcus faecalis* and *faecium* are shown (A), with a schematic overview of the TA-library that has been previously been generated. The well-defined GroP and RboP based fragments differ in the number of repeating units, the type of glycosyl substituent, the position of the carbohydrates along the chain and the degree of substitution. All of the fragments are equipped with the same aminohexanol linker for ligation purposes. The primary amine was used for the immobilization on reactive surfaces and for this purpose epoxide functionalized glass slides were chosen for the development of the TA-microarray.[15, 9]

***Figure 2**: A) TA-structures found in Enterococcos faecalis and faecium and Staphylococcus aureus. B) General overview of the in-house generated TA-library.*



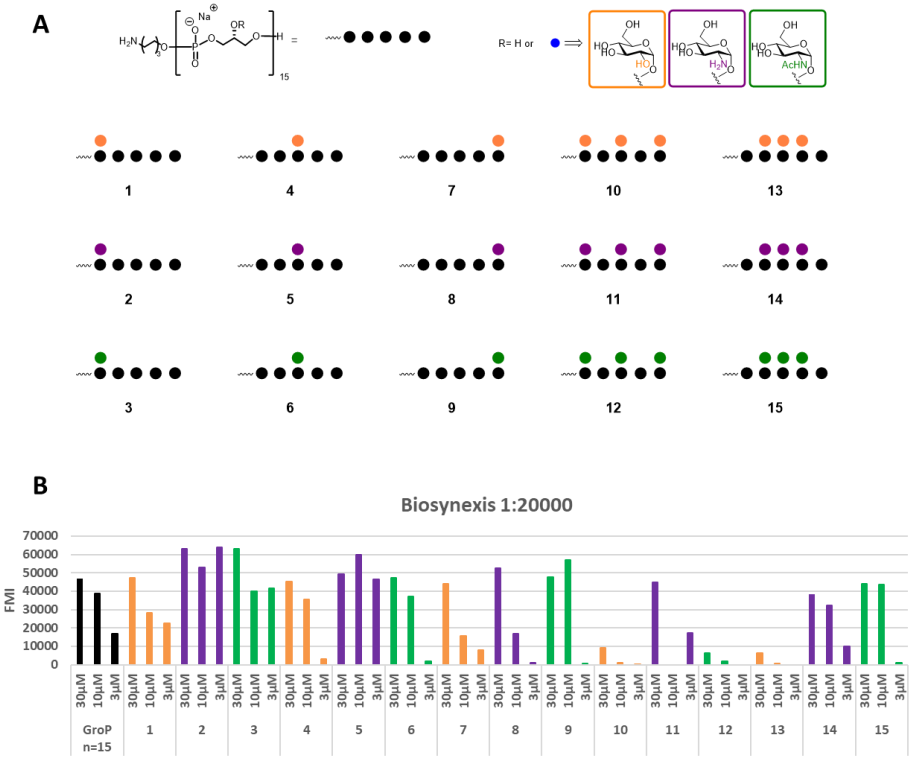
To assess the TA microarrays, first the binding specificity of a commercially available monoclonal antibody (mAb, Biosynexis)[16] was evaluated. This mAb is a mouse monoclonal antibody that has been raised against native S.*epidermidis* LTA, the structure of which is characterized by a GroP repeating unit having D-alanine moieties as the major substituent at the C-2 position.[17] Different dilutions of the antibody were employed (1:6000, 1:10000, 1:20000) and binding was detected by fluorescent scanning of binding of a secondary antibody for which a rabbit anti-mouse IgG labelled with DyLight 650 was used. In Figure 3A an example of an illustrative scan is provided, with a focus on two unsubstituted GroP fragments, a pentadecamer and a hexamer, showing good morphology and homogenicity among the different spots.[18] The binding of the mAb to non-substituted fragments differing in the number (n) of repeating units are shown in Figure 3B. In this experiment a non-substituted RboP octamer was used as negative control. A clear length-dependence was observed for the binding of the mAb to the oligo-GroP chains. The array also revealed better binding at higher concentration of the TA fragments. Little binding was detected for fragments with three, four or six repeating unit, while the interaction significantly increased for the 10-, 15- and 30-mer. Of note, the measured fluorescence is not always linearly proportional to the different dilutions of the mAb or the concentration of the printed TAs. The intensity of the detected signal may be affected by minor differences in density, orientation and conformation of each compound on the array, and the constructed tool is therefore only used as a qualitative assay.[19]

***Figure 3**: Example of scan image and microarray results of Biosynexis mAb on unsubstituted GroP fragments. A) Scan image of Biosynexis at 1:10000 dilution and focus on spots related to pentadecamer GroP (left top) and hexamer GroP (right top). B) Array results of Biosynexis at different dilutions: 1:6000 (blue), 1:10000 (red) and 1:20000 (light green). Values refer to the average of the fluorescent median intensity of three spots for each compound printed at 30 µM, 10 µM and 3 µM concentrations. n= number of repeating unit (GroP); Rbo (n=8), ribitol phosphate octamer from WTA of S. aureus.*



As shown above, TAs from the opportunistic pathogens *E. faecalis*, *E. faecium* and *S. aureus* contain α-kojibiose (D-glucose-(α-1,2)-D-glucose), α-glucose or *N*-acetyl α-glucosamine residues, respectively.[20] The array used above was expanded with a set of fragments based on a pentadecamer glycerolphosphate backbone carrying one or three carbohydrate (glucose, glucosamine or *N*-acetyl-glucosamine) appendages (Figure 4A). The compounds also differ in the position of the substituents along the chain. In the case of the monosubstituted fragments the glycosyl moiety has been introduced at the beginning (**1**, **2**, **3**) or middle (**4**, **5**, **6**) or at the end of the chain (**7**, **8**, **9**), relative to the position of the linker. The three glycosyl residues were introduced at the end, middle and beginning of the chain (**10**, **11**, **12**) or on neighbouring residues in the middle of the chains (**13**, **14**, **15**). Binding to the array was evaluated using Biosynexis at a dilution of 1:20000. Figure 4B shows diminished binding to the fragments carrying a terminal substituent (**7**-**12**). This reveals the preference of the mAb to bind to non-substituted GroP-chains. It also indicates that binding of the epitopes is best when the epitopes are most exposed (not hindered by a terminal carbohydrate appendage).

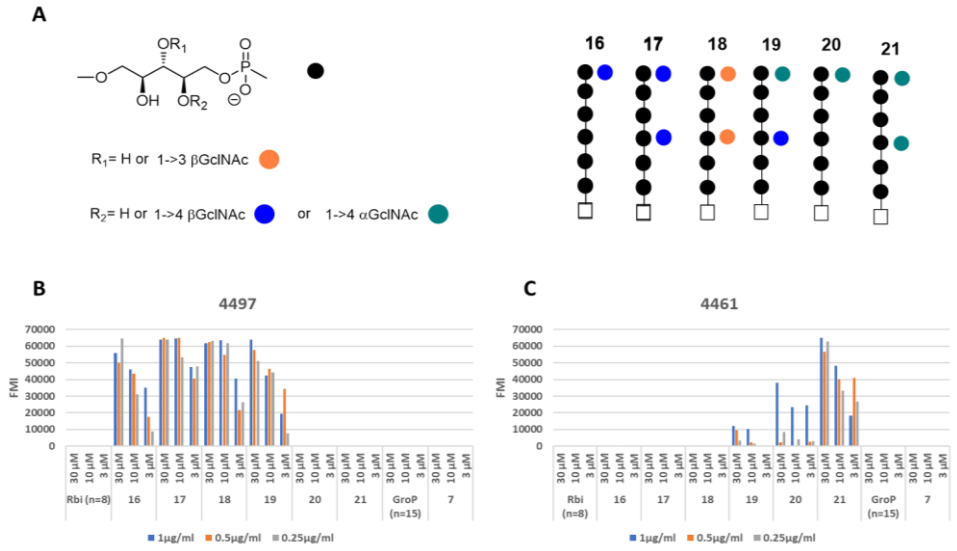
***Figure 4**: Glycosylated GroP pentadecamers. A) Schematic overview of the expanded library of pentadecamers (**1**-**15**). B) Array results of Biosynexis at 1:20000 dilution. Values refer to the average of the fluorescent median intensity of three spots for each compound printed at 30 µM, 10 µM and 3 µM concentrations. n= number of repeating unit (GroP); different colors depict the type of glycosyl substituent (Glc=orange, GlcNH$_2$=purple, GlcNAc=green).*



Lapardus and co-workers recently generated monoclonal antibodies from B-cells of patients infected by *S. aureus* and they have mapped the binding specificity of the antibodies using biosynthesis knock-out strains.[21] The antibodies were found to be directed towards WTA from *S. aureus* specifically recognizing either the β-GlcNAc or the α-GlcNAc glycosylated RboP. More recently, binding studies were performed at the molecular level using fully synthetic RboP fragments or enzymatically[22] glycosylated RboP-oligomers. To establish binding preferences of the monoclonal antibodies 4497 and 4461, raised against β-GlcNAc or α-GlcNAc substituted WTA, a TA-microarray was generated presenting a set of well-defined WTA RboP fragments. The library used is shown in Figure 5A and it includes, alongside the unsubstituted RboP octamer,[22] several hexamers differing in the anomeric configuration of the carbohydrate appendages, as well as the position and number of *N*-acetyl-glucosamine substituents.[21] Both antibodies were used in three different concentrations (1 µg/ml, 0.5 µg/ml and 0.25 µg/ml) and detection was performed using a goat anti-human IgG secondary antibody with an Alexa Fluor® 488 fluorophore. The non-substituted GroP pentadecamer and compound **7** were

used as negative controls. As previously reported, the monoclonal antibody 4497 specifically recognizes both the 1,3- and 1,4-β-GlcNAc glycosylated RboP hexamers (**17**, **18**) and the presence of a single glycosyl substituent is sufficient for recognition (**16**, **19**). The monoclonal antibody 4461 shows clear specificity for the RboP hexamers decorated with α-1,4-GlcNAc substituents (**19**-**21**) with a seemingly lower affinity than antibody 4497. Interestingly, GroP based TA **7** is not recognized, suggesting that the RboP backbone (or unit) plays a crucial role in antibody binding.

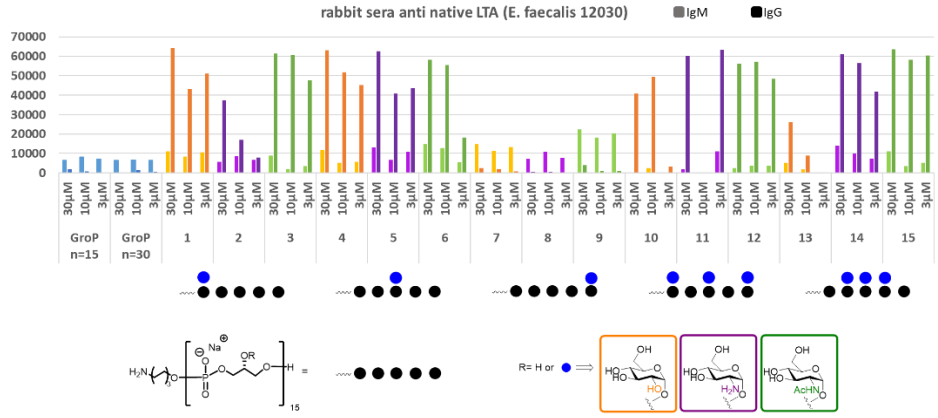
***Figure 5**: A) Schematic overview of the RibP-based library (**16**-**21**). Array results of 4497 (B) and 4461 (C) at different dilutions: 1 µg/ml=blue, 0.5 µg/ml=orange and 0.25 µg/ml=grey. Values refer to the average of the fluorescent median intensity of three spots for each compound printed at 30 µM, 10 µM and 3 µM concentrations. n= number of repeating unit.*



After the assessment of the arrays using monoclonal antibodies for both GroP and RboP fragments, the newly generated TA-microarrays were employed for the analysis of more complex biological samples. At first the binding of IgM and IgG antibodies in rabbit sera, obtained after immunization with native LTA of *E. faecalis* strain 12303, was evaluated using the GroP TA microarray.[19] Binding of IgM and IgG antibodies was detected using anti-rabbit IgM- and IgG secondary antibodies, labelled with DyLight 650 and DyLight 550 reporter groups, respectively. In Figure 6 the results are shown, where the IgM signal is depicted in a lighter shade of colour than the IgG signal. Binding to the non-substituted fragments is depicted in blue, while the colour pattern of Figure 4B is used to show binding of the substituted TAs, to differentiate the different glycosyl substituent. As it can be seen in Figure 6, relatively low binding is detected for the IgM antibodies as a result of a low titer or affinity in comparison to the IgG antibodies. The recognition seems to be non-specific. In contrast, the IgG antibodies are directed towards fragments with

glycosyl substituents (**1**-**6**, **10**-**15**), but interestingly no binding was detected for pentadecamers bearing the glycosyl appendage at the end of the chain (**7**-**9**).

***Figure 6**: TA-microarray analysis on rabbit sera after immunization with native LTA from E. faecalis 12303 (1:1000 dilution). Values refer to the average of the fluorescent median intensity of three spots for each compound printed at 30 µM, 10 µM and 3 µM concentrations. n= number of repeating unit (GroP); different colors depict the type of glycosyl substituent (Glc=orange, GlcNH$_2$=purple, GlcNAc=green).*



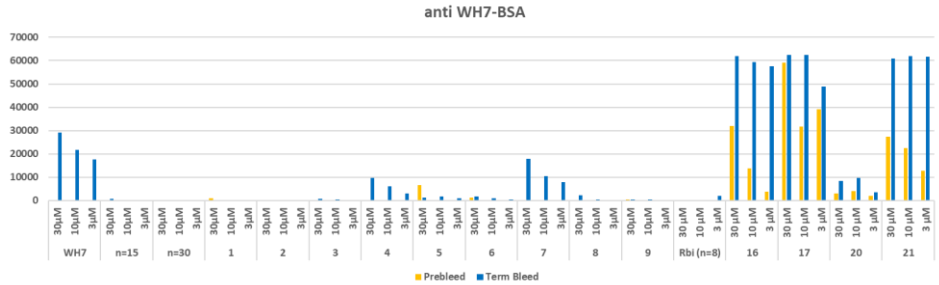
Previously a fully synthetic GroP hexamer bearing a glucosyl substituent at the terminal residue (**WH7**) was used to generate a model vaccine against *E. faecalis*.[23] It was conjugated to BSA as carrier protein, and the conjugate vaccine was able to induce opsonic and protective antibodies against the targeted Gram-positive bacterium. Although the rabbit sera generated after immunization was evaluated for bacterial killing ability, no structural analysis of the antibody repertoire was reported. Therefore, the generated TA-microarray was employed to unravel the preferential binding of the IgG antibodies using the protocol as described above. In Figure 7A the results are reported for the anti-WH7-BSA serum at 1:500 dilution, revealing high specificity of the IgG antibodies towards fragments bearing a glucosyl substituent at the terminal part of the chain or in the middle. The other glycosyl substituted TA fragments and the non-substituted GroP chains are not recognized.

Subsequently, two other **WH7** conjugates were developed, where the synthetic GroP TA was conjugated to either detoxified tetanus toxoid (TT)[24] or the zinc ABC transporter substrate-binding lipoprotein (AdcA) from *E. faecium*.[25] Binding of antibodies in the sera, raised using these conjugates is reported in Figure 7B and 7C, from which it becomes apparent that in these two conjugates elicited a higher antibody titer and the antibodies showed a broader recognition profile, with some recognition of other types of glycan appendages as well as binding to the non-substituted 30-mer.

***Figure 7**: Microarray analysis of rabbit sera raised against different WH7 conjugates. (A) anti WH7-BSA, (B) anti WH7-TT, (C) WH7-AdcA. Values refer to the average of the fluorescent median intensity of three spots for each compound printed at 30 µM, 10 µM and 3 µM concentrations. n= number of repeating unit (GroP); different colors depict the type of glycosyl substituent (Glc=orange, $GlcNH_2$=purple, GlcNAc=green). All three sera were used at 1:500 dilution.*

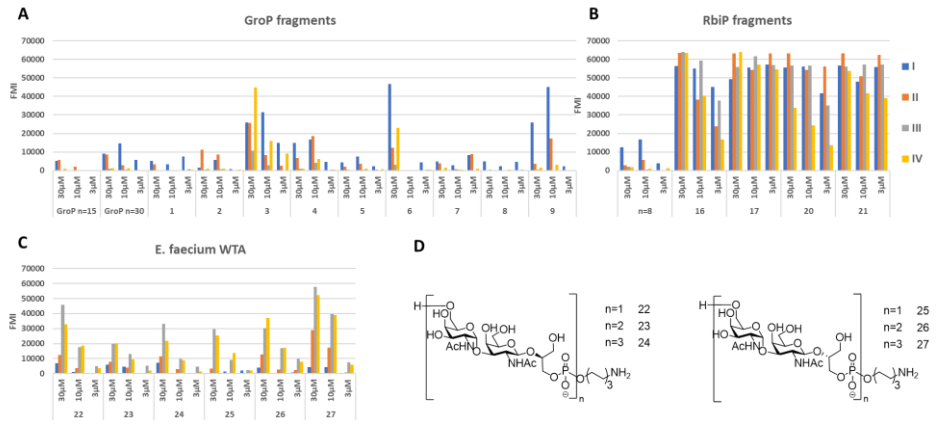
The opsonic activity of the anti-**WH7**-BSA rabbit sera has previously also been evaluated against an *E. faecium* clinical isolate and a community acquired *S. aureus* strain, to explore the potential of model vaccine to combat other multidrug resistance Gram-positive bacteria.[23b] It was observed that the serum generated against **WH7**-BSA was cross-reactive towards these tested strains. When the serum was analysed on the TA-microarray, presenting *S. aureus* WTA fragments, IgG binding to RboP based TA was observed (Figure 8, blue). To exclude the possibility that these antibodies were generated upon immunization with the synthetic GroP conjugate, the pre-bleed serum (taken prior to the immunization) was also tested. Figure 8 reveals the presence of antibodies recognizing the GlcNAc-functionalized RboP fragments in the pre-bleed serum (yellow). This may indicate that the observed opsonic activity towards S. *aureus* could have come from these pre-existing antibodies. Antibodies against the GroP based fragments are present only in the final bleed after immunization with **WH7**-BSA, corroborating the previous results.

Figure 8: *comparison of sera before and after immunization. Prebleed (yellow) and terminal bleed (blue) upon immunization with WH7-BSA conjugate were analyzed on the TA-microarray, both at 1:500 dilution. Values refer to the average of the fluorescent median intensity of three spots for each compound printed at 30 µM, 10 µM and 3 µM concentrations.*



The detection of pre-existing anti-TA antibodies indicates that the TA-microarray could be used as part of the evaluation of an immunization protocol using a TA-based vaccine. Indeed, enterococci and staphylococci are commensal bacteria and IgG antibodies can be present also in the sera of healthy donors.[26] The IgG repertoire against TA-based structures may vary depending on pathogen exposure during the lifetime of an individual. In Figure 9, the anti-TA antibody repertoire of 4 healthy donors was evaluated using the TA-microarray. In panel A binding to the GroP based fragments is shown, where binding to RboP and E. *faecium* WTA are shown in panel B and C respectively. The structures of E. *faecium* WTA fragments are depicted in Figure 9D.[27] As it can be seen, there is a substantial difference among the four subjects in panel A and C. Serum **I** (blue) has a broader content of IgG antibodies against the GroP fragments, while sera **III** (grey) and **IV** (yellow) have relatively higher titer of IgGs directing towards compounds **22**-**27**. In panel B, high signals were instead detected towards **16**, **17**, **20** and **21** across all 4 subjects. It has been previously observed that high level of IgG antibodies can be present in serum from both healthy individuals and infected patients.[28] In particular, GlcNAc substituted RboP oligomers have been identified as one of the immunodominant antigen in *S. aureus* species.[29,20b]

***Figure 9**: Microarray analysis on human sera from healthy volunteers. Four human sera from healthy volunteers were used at 1:200 dilution: **I** (blue), **II** (orange), **III** (grey) and **IV** (yellow). IgG antibodies were detected using goat anti-human IgG secondary antibody, Alexa Fluor® 488 conjugate. Values refer to the average of the fluorescent median intensity of three spots for each compound printed at 30 µM, 10 µM and 3 µM concentrations.*



CONCLUSION

Glycan microarrays have become important tools for the evaluation of glycan binding protein at the molecular level. The high throughput nature of this technology allows the rapid screening of large libraries of glycans, the binding of which can then be further evaluated using other techniques, such as surface plasmon resonance, ITC, X-ray crystallography or STD NMR.[10] In the context of TA-vaccine development, a large library of GroP and RboP based fragments has been generated. This chapter has described the construction of a TA-microarray to probe the binding of monoclonal and polyclonal antibodies from different sources. Epoxide functionalized micro array glass slides were used to immobilize the compounds, that had been equipped with an aminohexanol for this purpose. The feasibility of the technology was at first assessed using monoclonal antibodies generated against either GroP or RboP fragments. Specific binding could be detected although it also became apparent only qualitative binding data could be generated. Next, the arrays were used to probe more complex biological samples such as rabbit sera, obtained in immunization experiments using different TA sources and model vaccine candidates. The arrays have revealed that a very specific immune response can be generated when synthetic TA-conjugate vaccines are used. Preliminary screening of serum from healthy individuals indicates that *S. aureus* WTA featuring GlcNAc-RboP elements is a commonly recognized antigen. The arrays developed here can be expanded when new structures become available. The inclusion of TA fragments carrying D-Ala substituents will be of particular importance as this modification is known to play an important role in TA-biology. The arrays can be used to probe binding to many other biomolecules such as biosynthesis enzymes and lectins.

EXPERIMENTAL SECTION

TA-microarray construction

Synthetic well-defined GroP and RobP based teichoic acids were dissolved in MiliQ in order to obtain three different concentrations (75 µM, 25 µM and 7.5 µM) and from each solution 8 µL were added in a 384-wells V-bottom shape (Genetix, New Milton, UK). Subsequently 12 µL of a solution containing 16% of DMSO in spotting buffer (Nexterion Spot, Schott Nexterio) were added in each well, obtaining a final concentration of respectively 30 µM, 15 µM and 3 µM. Alongside the well-defined TA fragments, wells containing only MilliQ, DMSO and spotting buffer in the same proportion were also included as negative and background control. The plate can be stored at -20°C and used multiple times depending of course on the size of the preceding printing (how many arrays and how many slides performed).[9] Each compound was printed in triplicate on epoxysilane-coated glass slides (Slide E, Schott, Nexterion) by contact printing using the Omnigrid 100 microarrayer (Genomic Solutions, Ann Arbor, MI) equipped with SMP3 pins with uptake channels that deposit 0.7 nl at each contact. Each array was printed five or eight times on each glass slide. Printed slides were incubated overnight at room temperature at sufficient humidity to prevent drying of the spots and to allow covalent binding to the epoxysilane via reaction with primary amines. The slides were stored in the dark until used, while the plate at -20°C.

General binding assay

The slides were washed with PBS (3x) and subsequently all unreacted sites on the arrays were blocked by shaking the slides for 1 hour with ethanolamine (0.25 ml, 0.05M in PBS containing 20 mg/ml of BSA). The slides were flushed with PBS and PBS containing 5% of Tween® 20 subsequently and finally each array was rinsed with PBS containing 1% of Tween® 20. After removal of the PBS containing 1% of Tween® 20, the arrays were shaken with 0.25 ml of the appropriate sample (monoclonals or sera) diluted with PBS containing 1% of Tween® 20 and 10 mg/ml of BSA for 60 minutes. The slides were flushed with PBS and PBS containing 5% of Tween® 20 subsequently and finally rinsed with PBS containing 1% of Tween® 20 subsequently. After removal of the PBS containing 1% of Tween® 20, the arrays were shaken with 0.25 ml of fluorescent secondary antibody conjugates, differing upon the type of sample used, diluted with PBS containing 1% of Tween® 20 and 10 mg/ml of BSA for 30 minutes in the dark. The slides were flushed with PBS, PBS containing 5% of Tween® 20 and MilliQ subsequently. The slides were dried by centrifugation and fluorescent measurements were performed using Agilent G2565BA microarray scanner system (Agilent technologies) with 10 µm resolution, using two lasers (532 nm and 635 nm). Data and image analyses were performed with GenePix Pro 7.0 software (Molecular Devices, Sunnyvale, CA, USA) as described previously.[8] Fluorescence intensities were quantified and corrected for background/non-specific antibody adhesion by subtracting the fluorescence at blank spots, where only spotting buffer was printed without GTA fragment. The average of the triplicate spots was calculated and visualized in bar graphs using Microsoft Excel.

Binding assay using Biosynexis monoclonal antibody

Commercially available (IBT Bioservices) mouse anti-S. epidermidis monoclonal antibody Biosynexis (0.92 mg/ml) was diluted at 1:6000, 1:10000 and 1:20000 and used as described in the general binding assay. Goat anti-mouse IgM heavy chain secondary antibody Alexa Fluor® 488 conjugate (Invitrogen, A21426) and goat anti-mouse IgG (H+L) secondary antibody Alexa Fluor® 555 conjugate (Invitrogen, A21422) were used for detection at 0.5 μg/ml concentration as described in the general binding assay.

Binding assay using 4497 and 4461 monoclonal antibodies

4497 and 4461 monoclonal antibodies were obtained as previously described[22] and used in three different concentrations (1 μg/ml, 0.5 μg/ml and 0.25 μg/ml). as described in the general binding assay. Goat anti-human IgG secondary antibody Alexa Fluor® 488 conjugate (Invitrogen, A-11013) was used at 0.5 μg/ml concentration as described in the general binding assay.

Binding assay using rabbit sera

Rabbit sera raised against the putative vaccine candidate were obtained as previously described (native LTA from *E. faecalis*[23], **WH7**-BSA[23], **WH7**-TT[24] and **WH7**-AdcA[25]). The sera raised against native LTA from *E. faecalis* was diluted at 1:1000, while **WH7** conjugates and the pre-bleed of **WH7**-BSA immunization at 1:500 as described in general binding assay. Detection of IgM and IgG antibodies was performed using specific goat anti rabbit secondary antibodies, labelled with DyLight® 650 (AB_96982) and DyLight® 550 (AB_10942173) reporter groups respectively at 0.5 μg/ml as described in general binding assay.

Binding assay using human sera

Blood was drawn from four healthy volunteers and allowed to clot for 15 minutes at room temperature. After centrifugation for 10 min at 3,220 xg at 4°C, serum was collected and stored at -80°C. Each serum was used at 1:200 dilution as described in general binding assay and IgG detection was performed using goat anti-human IgG secondary antibody Alexa Fluor® 488 conjugate (Invitrogen, A-11013) at 0.5 μg/ml concentration as described in the general binding assay.

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