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Serum antibody screening by surface plasmon resonance using a natural glycan microarray

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Abstract A surface plasmon resonance (SPR) based natural glycan microarray was developed for screening of interactions between glycans and carbohydrate-binding proteins (CBPs). The microarray contained 144 glycan samples and allowed the real-time and simultaneous screening for recognition by CBPs without the need of fluorescent labeling. Glycans were released from their natural source and coupled by reductive amination with the fluorescent labels 2-aminobenzamide (2AB) or anthranilic acid (AA) followed by high-performance liquid chromatography (HPLC) fractionation making use of the fluorescent tag. The released and labeled glycans, in addition to fluorescently labeled synthetic glycans and (neo)glycoproteins, were printed on an epoxide-activated chip at fmol amounts. This resulted in covalent immobilization, with the epoxide groups forming covalent bonds to the secondary amine groups present on the fluorescent glycoconjugates. The generated SPR glycan array presented a subset of the glycan repertoire of the human parasite *Schistosoma mansoni*. In order to demonstrate the usefulness of the array in the simultaneous detection of glycan-specific serum antibodies, the anti-glycan antibody profiles from sera of *S. mansoni*-infected individuals as well as from non-endemic uninfected controls were recorded. The SPR screening was sensitive for differences between infection sera and control sera, and revealed antibody titers and antibody classes (IgG or IgM). All SPR analyses were performed with a single SPR array chip, which required regeneration and blocking of the chip before the application of a serum sample. Our results indicate that SPR-based arrays constructed from glycans of natural or synthetic origin, pure or as mixture, can be used for determining serum antibody profiles as possible markers for the infection status of an individual.

Keywords Carbohydrate-binding protein detection · Glycan microarray · Natural glycans · Serum screening · Surface plasmon resonance

Abbreviations

2AB	2-aminobenzamide
AA	anthranilic acid
CAA	circulating anodic antigen, -6)[GlcA(β1–3)]GalNAc(β1-
CBP	carbohydrate-binding protein
FGn	Fuc(α1–3)GlcNAc
FFGn	Fuc(α1–2)Fuc(α1–3)GlcNAc
FFFGn	Fuc(α1–2)Fuc(α1–2)Fuc(α1–3)GlcNAc
FLDN	Fuc(α1–3)GalNAc(β1–4)GlcNAc
FLDNF	Fuc(α1–3)GalNAc(β1–4)[Fuc(α1–3)]GlcNAc
HILIC	hydrophilic interaction liquid chromatography
HPLC	high-performance liquid chromatography
KLH	keyhole limpet hemocyanin
LDN	GalNAc(β1–4)GlcNAc
LDNF	GalNAc(β1–4)[Fuc(α1–3)]GlcNAc
Le^X	Lewis X, Gal(β1–4)[Fuc(α1–3)]GlcNAc
MALDI-TOF-MS	matrix-assisted laser desorption/ionization-time of flight-mass spectrometry
PLSDA	partial least square for discriminant analysis
RP	reversed phase
SPR	surface plasmon resonance
Sm	*Schistosoma mansoni*

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Introduction

Interactions of carbohydrate-binding proteins (CBPs) with their glycan ligands are important for mediating many biological processes. In the past decade, several glycan microarrays have been developed to investigate the binding between CBPs and glycans [1–9]. These arrays make use of printed glycan slides in combination with fluorescence detection, and differ mainly in the way the glycans are immobilized, as reviewed by Culf *et al*. [10]. Another tool for analyzing protein–carbohydrate interactions is surface plasmon resonance (SPR). The advantage of SPR is its ability to measure label-free and real-time binding of CBPs to glycans. In the past, several studies of protein–carbohydrate interactions have been performed by SPR, but only recently Karamanska *et al*. described the analysis of a glycan array by SPR using a commercially available SPR instrument, the Biacore™ Flexchip instrument [11]. Forty biotinylated oligosaccharides were spotted onto neutravidin-coated gold slides to study their interactions with plant lectins and a recombinant Fc fusion protein of human Siglec-7 [11].

We here present an SPR-based glycan microarray using the Biacore Flexchip apparatus for screening of interactions between glycans and proteins, which methodology is comparable with our recently developed glass slide glycan microarray [8]. Glycans are labeled by reductive amination with conventional fluorescent tags, *e.g*. 2-aminobenzamide (2AB) or anthranilic acid (AA), and subsequently purified and quantified by high-performance liquid chromatography (HPLC) making use of the fluorescent tag. Thereafter, covalent immobilization of the labeled glycans is achieved on epoxide-activated Biacore Flexchip affinity chips via the secondary aromatic amine group which represents the bridge between fluorescent tag and glycan moiety [8]. The method is broadly applicable because it facilitates the purification and immobilization of glycans from both natural and synthetic sources. Due to the covalent linkage, the glycan array can be reused after regeneration and blocking, making it suitable for analyzing multiple protein samples with a single glycan microarray. By printing a set of schistosome-related glycan structures, we could measure anti-carbohydrate immunoglobulin G and immunoglobulin M profiles in sera of humans infected with the parasite *Schistosoma mansoni*, establishing the usefulness of the method for the simultaneous detection of serum proteins to a multitude of glycans.

Materials and methods

Monoclonal antibodies were produced as described previously [12] and sera from *S. mansoni* infected individuals (n=13) were similar to a previous study [13]. The detection of *S. mansoni* eggs in the feces of patients was used as an indicator of infection with *S. mansoni*. These sera were collected in areas in Kenya, where schistosomiasis is endemic. Negative control sera (n=8) were from Dutch blood donors with no history of schistosomiasis.

Various commercially available oligosaccharides were from Dextra Laboratories (Reading, UK) and Sigma-Aldrich (Zwijndrecht, The Netherlands). Moreover, natural glycans were isolated from ribonuclease B (RNase B) and keyhole limpet hemocyanin (KLH), both obtained from Sigma-Aldrich; from *S. mansoni* adult worms and *S. mansoni* eggs, both isolated from infected hamsters; and from human serum (healthy donor). RNase A, BSA, HSA, fetuin, and asialofetuin were from Sigma-Aldrich. Circulating anodic antigen (CAA) was purified previously [14]. BSA-Lewis X, Gal(β1–4)[Fuc(α1–3)]GlcNAc (Le^X) (8 glycans/BSA), BSA-GalNAc(β1–4)GlcNAc (LDN) (8 glycans/BSA), BSA-Fuc(α1–3)GalNAc(β1–4)GlcNAc (FLDN) (13 glycans/BSA), BSA-GalNAc(β1–4)[Fuc(α1–3)]GlcNAc (LDNF) (5 glycans/BSA), BSA-di-CAA (14 glycans/BSA), BSA-tri-CAA (7 glycans/BSA), BSA-tetra-CAA (5 glycans/BSA), and BSA-penta-CAA (4 glycans/BSA) oligosaccharide conjugates were prepared as described previously [15]. HSA-di-Le^X and HSA-tri-Le^X (average 22 glycans/HSA) were a kind gift of Dr. B. Appelmelk (Vrije Universiteit Medical Center, Amsterdam, The Netherlands). BSA-Fuc(α1–3)GlcNAc (FGn) (11 glycans/BSA), BSA-Fuc(α1–2)Fuc(α1–3)GlcNAc (FFGn) (10 glycans/BSA), and BSA-Fuc(α1–2)Fuc(α1–2)Fuc(α1–3)GlcNAc (FFFGn) (9 glycans/BSA) were prepared previously [16]. A selection of the glycoconjugates and (neo)glycoproteins used for the glycan SPR chip is shown in Fig. 1. The anti-class antibodies rabbit anti-human IgG (γ-chains) and rabbit anti-human IgM (μ-chains) were from Dako (Glostrup, Denmark) and used at 10 and 25 μg/ml, respectively. Glycan structures were drawn using GlycoWorkbench software (http://www.eurocarbdb.org).

Glycan release

N-Glycans from the biological sources RNase B, human serum, and KLH were released by PNGase F (peptide N-glycosidase F, Roche Diagnostics, Mannheim, Germany) after protein reduction and denaturation. Released glycans were purified using sequentially reversed phase (RP)- and graphitized carbon cartridges, as described previously [17]. The purification of N-glycans from *S. mansoni* adult worm glycoproteins has been described before [17]. *S. mansoni* egg glycolipids were prepared by organic solvent extraction of glycolipids [18], Folch partitioning [19], and desalting using an RP C18 cartridge [18]. Glycan moieties were released from the glycolipids by recombinant ceramide glycanase (endoglycoceramidase II from *Rhodococcus* sp.; Takara, Otsu, Japan) and obtained in the aqueous flow-

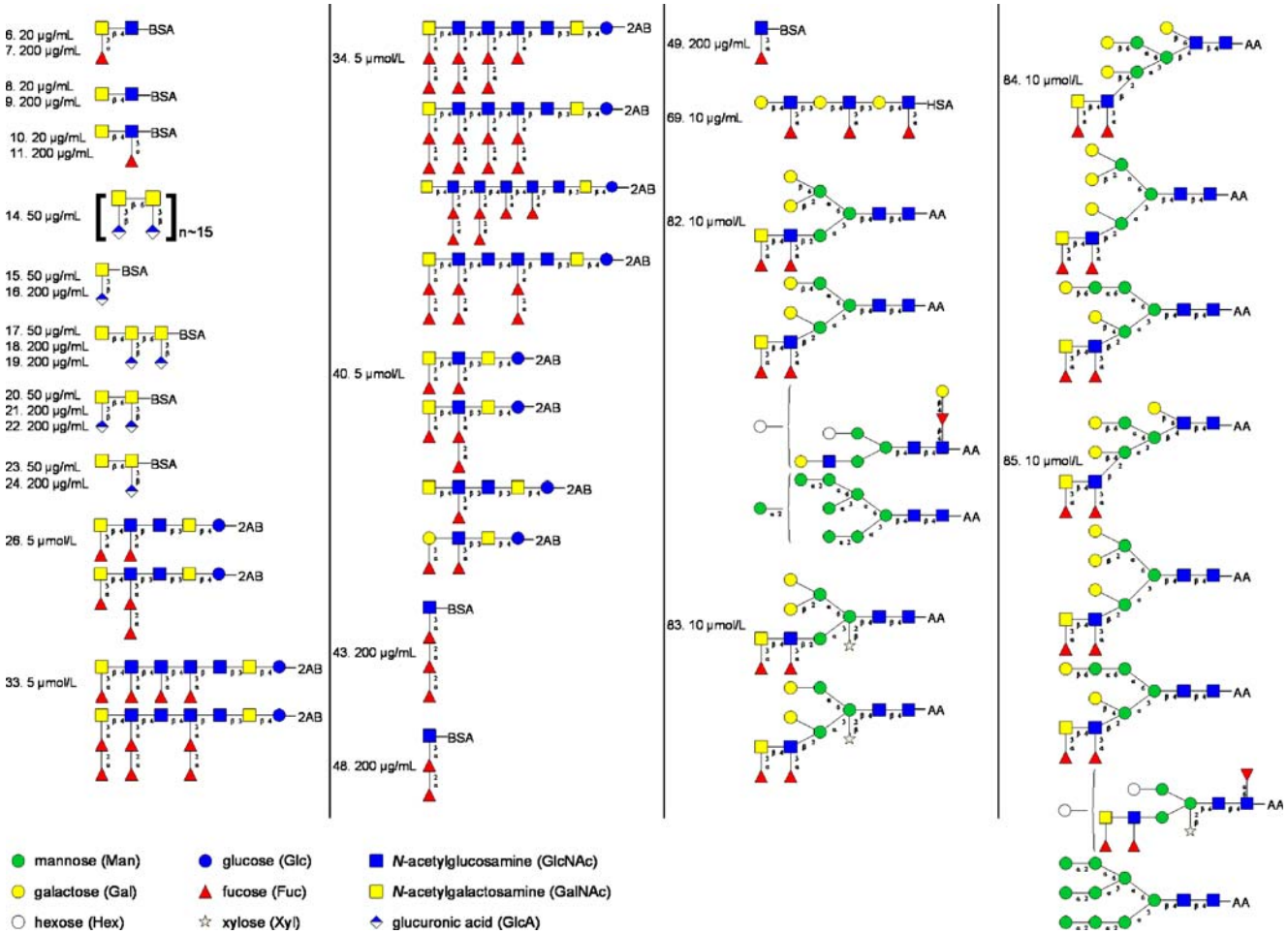

Fig. 1 Structural schemes of part of the glycoconjugates and (neo) glycoproteins that were spotted on the epoxide affinity chip. Nature of the depicted glycan structures: 6–11, 15–24, 43, 48 and 49, synthetic BSA-oligosaccharide conjugates; 14, glycoprotein CAA; 26, 33, 34 and 40, isolated from *S. mansoni* egg glycolipids, second dimension HPLC fractions 10.1, 15.1, 15.2 and 8.2, respectively; 69, synthetic HSA-oligosaccharide conjugate; 82–85, isolated from the glycoprotein KLH, first dimension HPLC fractions 20–23

through of an RP C18 cartridge [18]. All glycan fractions were dried before labeling.

Glycan labeling and purification

Natural and synthetic glycans were labeled with the fluorescent compounds 2-aminobenzamide (2AB) or anthranilic acid (AA; both from Sigma-Aldrich) by reductive amination using the mild reducing agent sodium cyanoborohydride [20] (Fig. 2a). The labeled glycans were purified by hydrophilic interaction liquid chromatography (HILIC) (Amide-80 column, 4.6 mm × 25 cm, particle size 5 µm, Tosoh Bioscience, Stuttgart, Germany) (Fig. 2b). Eluent A consisted of 50 mM formic acid (pH 4.4); eluent B consisted of eluent A/acetonitrile 20/80 (v/v). A linear gradient from 100% to 40% eluent B was applied at a flow rate of 1 ml/min. In general, fluorescence detection was performed at λ_{ex}–λ_{em} 360–425 nm, but for large amounts of glycan λ_{ex}–λ_{em} 280–500 nm was used. Fractions were collected and analyzed by matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS). Three HILIC fractions of *S. mansoni* egg glycolipid-derived glycans [*Schistosoma mansoni* (Sm) egg fr. 8, 10 and 15] were fractionated in a second dimension (named Sm egg fr. x.y), using RP-HPLC on a Hypersil ODS column (2 mm × 25 cm, particle size 3 µm; Thermo, Waltham, MA) at 200 µl/min. Eluent A consisted of H_2O with 0.1% formic acid/acetonitrile (99.6/0.4, v/v), eluent B consisted of H_2O with 0.1% formic acid/acetonitrile (5/95, v/v). Gradient conditions: 0−6.5 min at 5% eluent B, followed by a linear gradient to 50% eluent B in 25 min. Fractions were collected and analyzed by MALDI-TOF-MS(/MS) (Fig. 2c).

MALDI-TOF-MS(/MS)

MALDI-TOF-MS(/MS) was performed on an Ultraflex II mass spectrometer (Bruker Daltonics, Bremen, Germany). Fragment analysis was achieved by laser-induced decom-

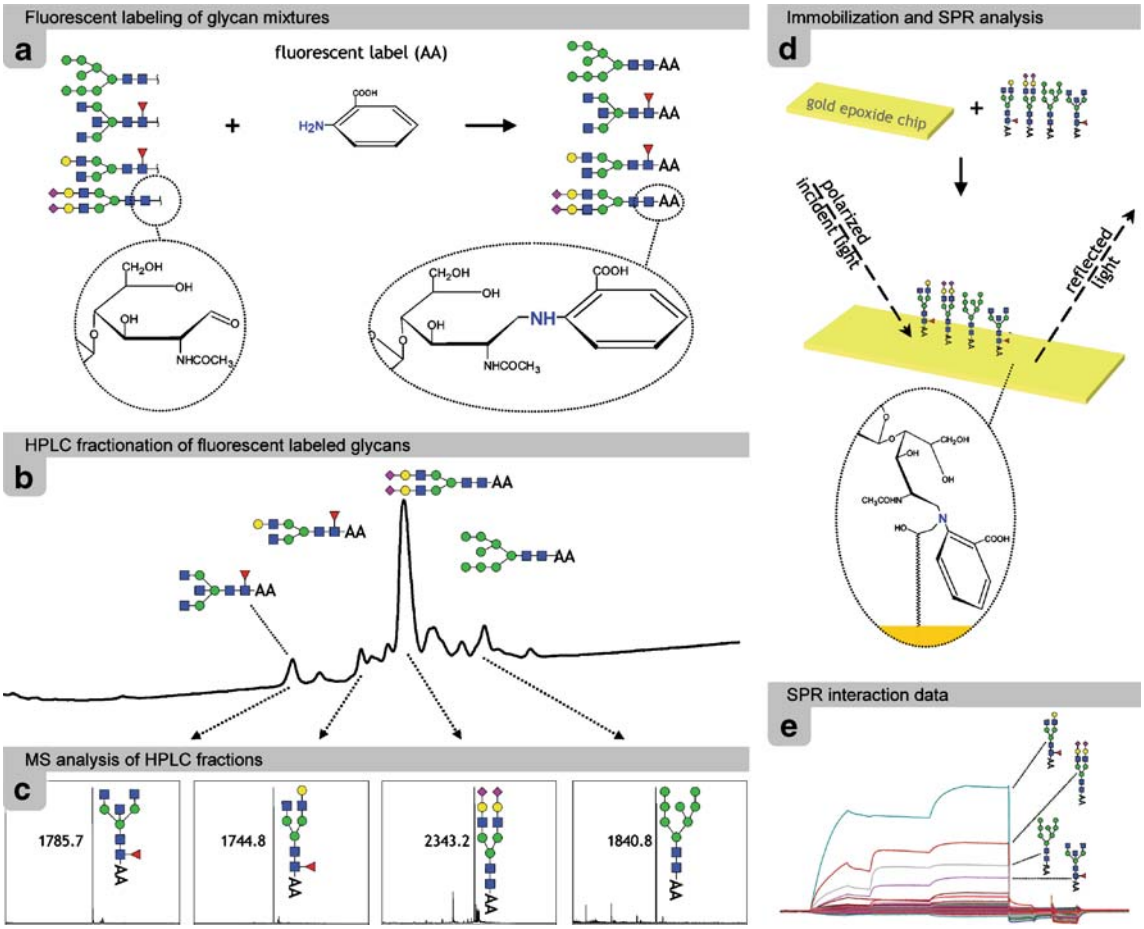

Fig. 2 Scheme of the SPR approach as presented in this paper. **a** Glycans (in mixtures) are derivatized with fluorescent labels; **b** Fractionated by HPLC; **c** Analyzed by MALDI-TOF-MS; **d** Immobilized on homemade epoxide affinity chips and analyzed by SPR; **e** SPR interaction data

position using the LIFT TOF/TOF-MS/MS facility. Samples (0.5 μl) were spotted with 1 μl of 6-aza-2-thiothymine (ATT, Sigma-Aldrich) or 2,5-dihydroxybenzoic acid (DHB, Bruker Daltonics) matrix, and analyzed in the positive or negative (reflectron) mode.

Epoxide chip preparation

An epoxide-activated SPR chip was created using a methodology similar to the one introduced by Löfås and Johnsson [21, 22] and Ro *et al.* [23] (Fig. 3). Gold affinity chips (GE Healthcare, Uppsala, Sweden) were treated with 10 mM 11-mercapto-1-undecanol (Sigma-Aldrich) in ethanol for 12 h. To activate hydroxyl groups, the chips were immersed in 0.4 M NaOH/2-methoxyethyl ether (Sigma-Aldrich) (50/50, v/v) containing 0.6 M epichlorohydrin (Sigma-Aldrich) for 4 h. After activation, a dextran solution (0.3 mg/ml) in 0.1 M NaOH was added for 20 h. Prior to printing, the chips were again immersed in 0.4 M NaOH/2-methoxyethyl ether (50/50, v/v) containing 0.6 M epichlorohydrin for 4 h, and washed with water, ethanol and water again. All reactions were performed at room temperature and a rubber mold (GE Healthcare) was used in order to keep the reagents on the active surface of the chip.

Printing

Analytes were dissolved in 20 μl of 50/50 (v/v) H_2O/spotting buffer (Nexterion Spot, Schott Nexterion, Jena, Germany) in 384-well V-bottom plates at concentration ranging from 1 to 100 μM for AA and 2AB labeled glycans, and 10 to 500 μg/ml for BSA/HSA glycoconjugates. For (heterogeneous) HPLC fractions, the concentration is given for the sum of the glycans that are present in that fraction. In total, 144 samples were printed (12×12 array; volumes ~13 nl) on an epoxide-modified sensor surface (11×11 mm) by non-contact piezo dispensing using a multichannel nanopipettor (STARplus; Hamilton, Bonaduz, Switzerland). Printed slides were incubated overnight at room temperature at sufficient humidity to prevent drying of the spots. The most relevant structures present in the array can be found in Fig. 1.

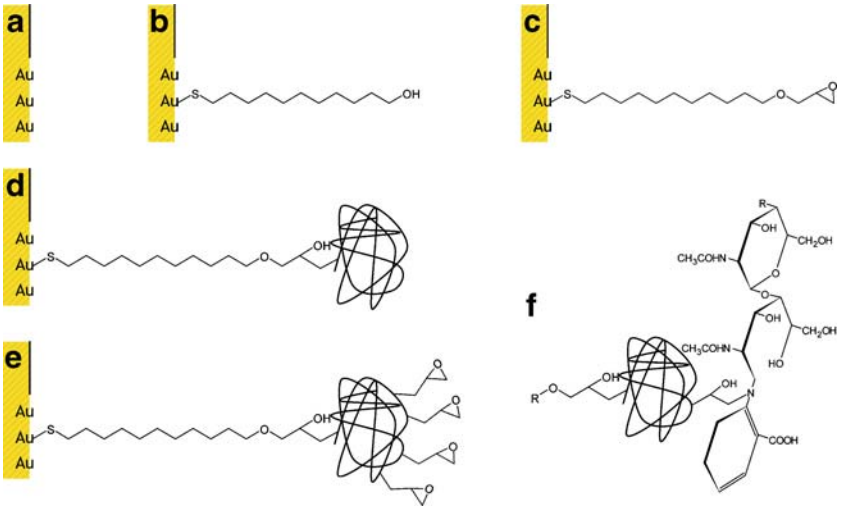

Fig. 3 Preparation of the epoxide affinity chips. **a** Bare gold affinity chip; **b** SAM of 11-mercapto-1-undecanol; **c** SAM of epoxide; **d** SAM of dextran; **e** Second SAM of epoxide; **f** Glycan immobilization via the epoxide group

Biacore flexchip SPR

The printed affinity chips were loaded into a Biacore Flexchip (GE Healthcare). Regions of interest (ROI) on the chip were defined while the printed chip was dry. The spot diameter was 200−400 μm, but the diameters of the ROIs and reference ROIs were set at 225 and 200 μm, respectively. Running buffer consisted of PBS-T (0.3% Tween20 in PBS, v/v), deactivation buffer consisted of PBS-T/Biacore Flexchip Blocking buffer (GE Healthcare) (9/1, v/v) with 2.5 mM ethanolamine, blocking buffer consisted of 3% BSA in H_2O (w/v), and regeneration buffer consisted of 100 mM HCl. Sera were diluted 50× in running buffer. Experiments were performed at 750 μl/min for sera and 1,000 μl/min for other solvents, at 25°C. Each SPR experiment consisted of a cycle of running buffer, regeneration buffer, water, blocking buffer, running buffer, sample, running buffer, anti-IgG solution, running buffer, anti-IgM solution and running buffer. Sera and anti-class antibody solutions were pumped for 12.5 min, followed by 3 min running buffer. One printed affinity chip was used for all experiments described in this manuscript.

Data analysis

Reference corrected SPR data was analyzed with Biacore Flexchip software by subtracting the 'baseline' value from the 'binding_late' value. The processed data of the anti-IgG and anti-IgM response were summed and used for data analysis and interpretation. Only secondary antibody signals were used for sample evaluation. GraphPad Prism 4 was used to plot the data and to calculate statistical significances. Statistical analysis [partial least square for discriminant analysis (PLSDA)] was performed with Simca-P+ software (V11.5; Umetrics, Umeå, Sweden). For PLSDA, data was normalized by subtraction of the blanks.

Results and discussion

The aim of this study was the development of an SPR glycan array method based on immobilization of fluorescently labeled natural glycans as well as other glycoconjugates for label-free, online, in-depth analysis of multiple protein–carbohydrate interactions. For this purpose, the sensor surface of the affinity chip was modified with an epoxide coating for immobilization of glycans that were labeled by reductive amination and for the (neo)glycoproteins (Fig. 3). The surface modification comprised several self-assembled monolayers, as described previously by Löfås [22]. In summary, a hydroxyl-surface was obtained by reacting the thiol group of 11-mercapto-1-undecanol with the gold surface (Fig. 3a,b). The exposed hydroxyl groups then reacted with epichlorohydrin to form an epoxide monolayer (Fig. 3c), which was allowed to react with dextran (Fig. 3d). The randomly linked highly flexible hydrophilic dextran layer was supposed to reduce nonspecific protein adsorption and acted as a spacer for further modification. Following the protocol introduced by Ro *et al.* [23], a second epoxide monolayer was created on the dextran layer (Fig. 3e), which was used for covalent glycan immobilization (Fig. 3f). Epoxide reacts with amine-modified glycans, which makes it possible to covalently immobilize glycoconjugates that have been reductively aminated with established labels, like AA, 2AB and diaminopyridine [24, 25]. During reductive amination, the primary aromatic amine on these labels reacts with the aldehyde of the

oligosaccharide forming an imine (Schiff base), which is subsequently reduced to a secondary aromatic amine by hydride reagents. The formed secondary aromatic amine group subsequently reacts with the epoxide groups on the affinity chips (Fig. 2a,d). In total 144 samples, from either natural or synthetic origin, were spotted on the epoxide-affinity chip (see Fig. 1 for a selection of the spotted glycoconjugates) by non-contact printing. Most of these 144 samples represented glycans labeled with AA or 2AB (Fig. 2a), which were obtained after HPLC fractionation (Fig. 2b) and analyzed by MALDI-TOF-MS(/MS) for purity and structural features (Fig. 2c). The prepared glycan affinity chip was used for SPR experiments (Fig. 2e).

Optimization of SPR screening

Before the first SPR run of the printed glycan chip was performed, unbound glycan samples were removed by washing with running buffer, followed by deactivation of remaining epoxide groups. In order to be able to screen multiple samples in an efficient manner using a single glycan chip, an SPR program was developed with regeneration and blocking steps between the applications of protein samples. Several buffers and solvents were tested for regeneration and blocking in combination with monoclonal antibodies, anti-class antibodies, and diluted human sera in order to achieve SPR base-line stability, reduce nonspecific binding and allow the recovery of the chip (data not shown). These tests resulted in the following protocol: the chip was flushed with running buffer, regeneration buffer, H_2O, blocking buffer, and again running buffer. Following these preparation steps, human serum was pumped across the glycan chip to observe antibody binding. A further 3-min flush of running buffer was used to observe the dissociation of antibodies. The amounts of IgG and IgM that were bound to individual spots on the chip were monitored by applying the secondary antibody anti-human IgG followed by anti-human IgM. Each of these steps was followed by a flush of running buffer to obtain dissociation data. With this method, one printed SPR glycan array was used for screening the 21 serum samples included in this study, demonstrating the robustness of the method. One serum sample was analyzed for four times over a period of 4 months and provided comparable intensities and binding profiles (average relative standard deviation (RSD) of 13% for the 20 highest responses (anti-IgG or anti-IgM, after normalization on the most intense peak). This proved the stability of the chip and no deglycosylation of the immobilized glycans by glycosidases.

Screening of human sera

To exemplify the potential of the printed glycan microarray for the simultaneous detection of anti-glycan proteins in sera from *S. mansoni* infected individuals (positive sera, PS) and negative control sera from Dutch blood donors with no history of schistosomiasis (negative sera, NS) were analyzed. As an example, Fig. 4 shows the resulting SPR sensorgrams (a, c and e) of two of the positive sera (#2022 and #2052) and one negative serum (#NS21) with the corresponding bar charts (b, d and f). Each line in a, c and e corresponds with one glycan spot on the array, whereas each bar in b, d and f corresponds with anti-human IgG or anti-human IgM responses of the spots. The sensorgrams and bar charts provide information on serum levels of anti-glycan antibodies and their class. The two infection sera (Fig. 4a,b,c and d) mainly contained antibodies against BSA-FGn (see structure 49 in Fig. 1), BSA-FFGn (48), BSA-FFFGn (43) and BSA-FLDN (7), which were of the IgG and IgM class for PS #2022 (a and b) and mainly of the IgG class for PS #2052 (c and d). Besides, Fig. 4e and f show that also the control serum #NS21 contained anti-glycan antibodies, with the highest response obtained for HSA-tri-Le^X (69 in Fig. 1). In addition, association and dissociation information was obtained for antibodies during the running buffer step after the application of the sera (data not shown). Overall, these results show the suitability of the SPR glycan microarray for analyzing anti-carbohydrate antibodies from serum samples.

Data analysis

Combining all interaction data in one chart (Fig. 5a) reveals differences between the negative sera (NS; $n=8$) and positive sera of *S. mansoni* infected individuals (PS; $n=13$). Rabbit anti-human IgG and anti-human IgM responses were summed and plotted versus the glycoconjugates on the affinity chip. Several glycoconjugates, including BSA-FLDN, BSA-LDNF (11), BSA-FFFGn, BSA-FFGn, BSA-FGn, KLH glycans in fr. 19–24 (82–85 for fr. 20–23), and Sm egg glycans in fr. 10–16, 8.2 (40), 10.1 (26) and 15.2 (34), showed higher antibody binding for most of the PS than for the NS. These observations are in agreement with previous findings [13]. The recognized glycan samples were analyzed by MALDI-TOF-MS, *e.g.*, the spectrum of KLH fr. 22 (Fig. 6a) indicated a major peak with *m/z* 2214.9 ($[M–H]^-$), which is consistent with the glycan composition Hex6HexNAc4Fuc2-AA. MALDI-TOF/TOF-MS/MS gave abundant peaks of *m/z* 1516.7 and 1865.9 ($Y_{4\alpha}'$ and $Y_{5\alpha}'$ fragments, Fig. 6b) indicating a Fuc(α1–3) GalNAc(β1–4)[Fuc(α1–3)]GlcNAc (FLDNF) structural motif, as determined in a previous study [26]. The KLH fr. 19–24 contained mainly N-glycans with a common FLDNF structural motif, whereas the Sm egg fr. 10–16 contained mainly DF-LDN-DF and FLDNF motifs [8].

The statistical significance of the differences between PS and NS for these glycoconjugates is more obvious in Fig. 5b and c, where the means of the PS and the NS are compared

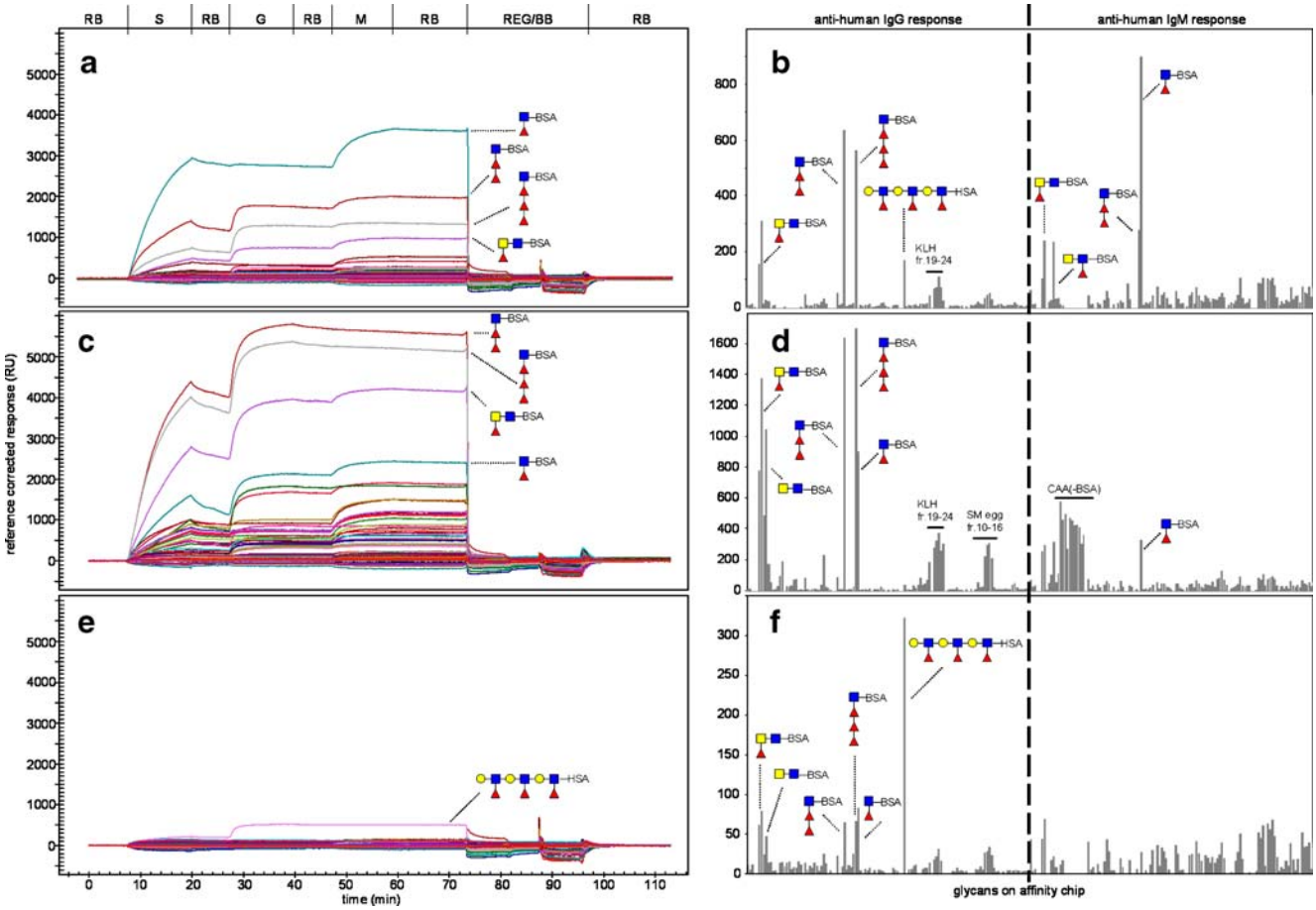

Fig. 4 **a**–**b** SPR screening of PS #2022; **c**–**d** of PS #2052; **e**–**f** of NS #NS21. *RB*, running buffer; *S*, serum; *G*, rabbit anti-human IgG; *M*, rabbit anti-human IgM; *REG*, regeneration solvent; *BB*, blocking buffer; *RU*, resonance units. Reference corrected data was obtained by background subtraction using the reference spots

for a selection of glycoconjugates. Except for BSA-LDN (9) and CAA (14), all of the plotted glycoconjugates have *P*-values smaller than 0.05, which means that the PS contain in general higher amounts of antibodies (IgG and IgM) against these glycoconjugates than the NS. Furthermore, high antibody levels against the *S. mansoni* glycoprotein CAA and all CAA-glycoconjugates (15–24) were only detected for PS #2052, while all other PS and NS revealed hardly antibody binding to these parasite-related glycosylation patterns. To get more information about the (less intense) responses, partial least squares for discriminant analysis (PLSDA) was performed, which sharpens the separation between groups of observations to obtain maximum separation between the classes PS and NS. PLSDA of the data resulted in two distinct groups (Fig. 7a), which demonstrates the ability of this SPR approach to distinguish sera from different groups/backgrounds. Observations that are closely situated are similar and those that are distant differ in many respects. PS #2052 did not cluster within the two groups, which is mainly caused by the high antibody responses against the (BSA-)CAA's. PS #2011 is situated near the NS cluster, which is due to the low antibody (IgG or IgM) responses of this serum to most positions on the glycan chip. The variables carrying the class-separating information are visualized in the PLSDA loading plot (Fig. 7b). Each triangle represents one glycoconjugate. Directions in the score plot correspond to directions in the loading plot, which means that the glycoconjugates responsible for differentiating the PS from the NS are mainly located in the upper right quadrant, with the most influential glycoconjugates situated far from origin. Most of the important variables correspond with the glycoconjugates which were already showing up in the chart in Fig. 5, *e.g.*, BSA-FFGn, BSA-FFFGn, Sm egg fr. 10.1, BSA-LDNF; BSA-FGn, Sm egg fr. 8.2 and KLH fr. 20–23. The PLSDA loading plot shows Sm egg fr. 15.1 (33) as a very suitable variable (FLDNF and DF-LDN-DF structural motif), although the absolute responses against this glycan spot were low (means of anti-IgG plus anti-IgM resonance units: 48 and 27 for PS and NS, respectively). In conclusion, the SPR approach made it possible to monitor in parallel many different anti-glycan antibody patterns/profiles associated with *S. mansoni* infection.

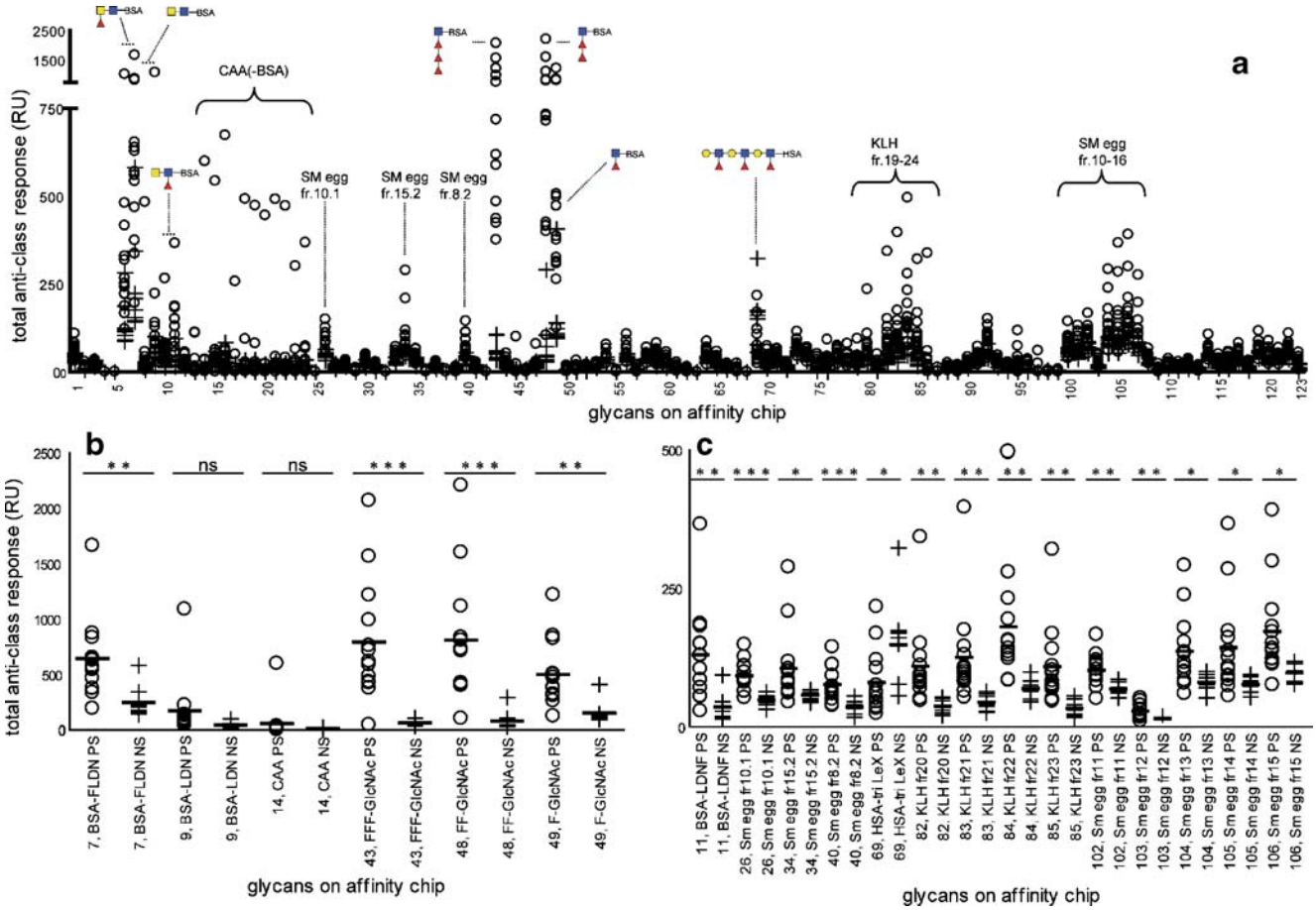

Fig. 5 **a** Scatter plot of SPR response versus glycans on the array. Rabbit anti-human IgG and IgM responses were summed; *plus signs*, NS; *circles*, PS. The compounds BSA-FLDN, BSA-LDN and BSA-LDNF were present at two different concentrations. **b** Selection of high abundant SPR responses; **c** Selection of abundant SPR responses. *Asterisks* indicate significant differences between PS and NS for each glycoconjugate: *triple asterisks*, extremely significant (P value< 0.001); *double asterisks*, very significant ($0.001<P<0.01$); *single asterisks*, significant ($0.01<P<0.05$); *ns*, not significant ($P>0.05$). A *horizontal line* is superimposed at the mean

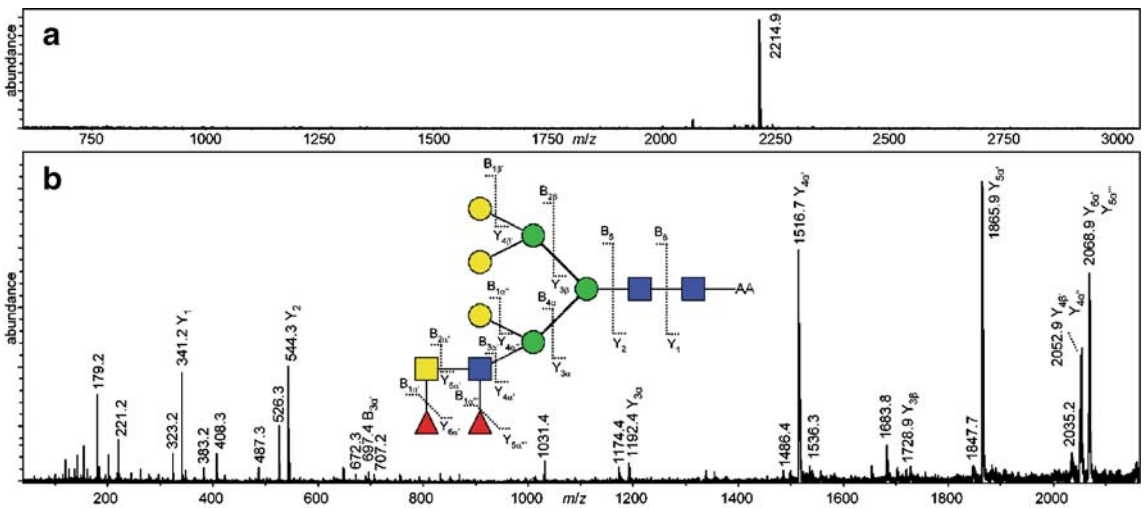

Fig. 6 **a** MALDI-TOF-MS and **b** MALDI-TOF/TOF-MS/MS spectra of KLH fr. 22 (84 in Fig. 1). Spectra were acquired in the negative ion mode. The assignment of fragments is according to Domon and Costello [27]

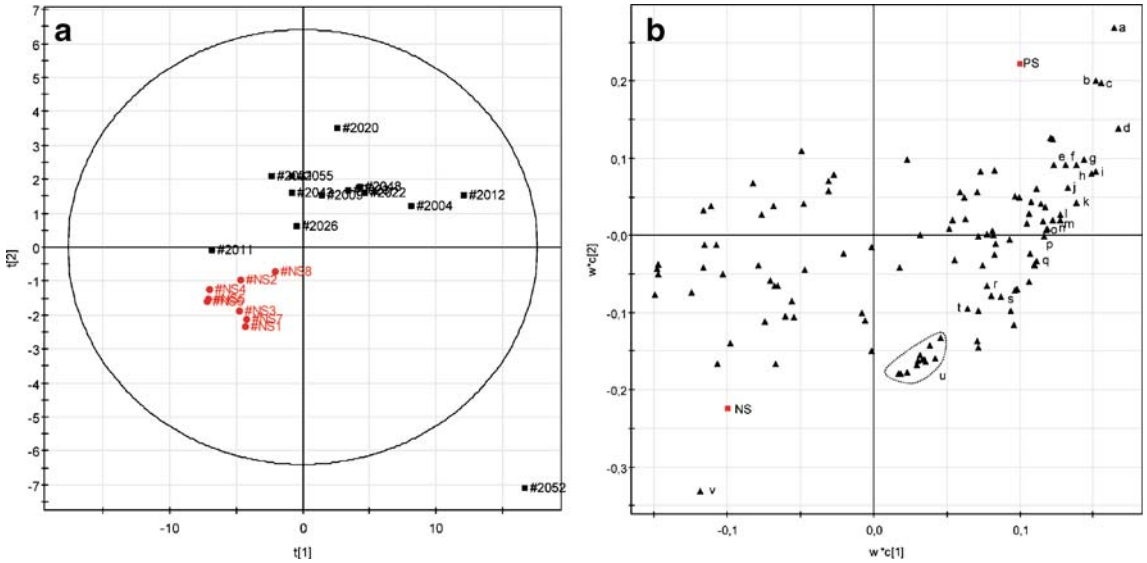

Fig. 7 **a** PLSDA score (*t*) plot of normalized data by blank subtraction; *circles*, NS; *squares*, PS. **b** Corresponding PLSDA loading (*p*) plot, with each *triangle* representing one glycoconjugate: *a*, Sm egg fr. 15.1; *b*, BSA-FFFGn; *c*, BSA-FFGn; *d*, Sm egg fr. 10.1; *e*, BSA-LDNF; *f*, BSA-FGn; *g*, Sm egg fr. 8.2; *h*, Sm egg fr. 8; *i*, Sm egg fr. 10; *j*, BSA-FLDN; *k*, KLH fr. 16; *l*, KLH fr. 22; *m*, KLH fr. 23; *n*, Sm egg fr. 11; *o*, KLH fr. 20; *p*, KLH fr. 21; *q*, KLH fr. 19; *r*, KLH fr. 24; *s*, Sm egg fr. 15.2; *t*, BSA-LDN; *u*, (BSA-)CAA's; *v*, BSA-tri-Le^X; *circles*, NS; *squares*, PS

Conclusions

The feasibility and usefulness of an epoxide-based SPR glycan array for analyzing protein–glycan interactions is demonstrated. SPR allows interaction screening without using fluorescent labels or secondary antibodies. Furthermore, applying additional secondary antibodies adds additional selectivity for monitoring carbohydrate interactions. The combination of SPR and microarrays provides the ability to screen real-time multiple protein–carbohydrate interactions simultaneously. The epoxide affinity chip presented in this paper is very suitable for immobilizing glycans after derivatization with established labels for reductive amination (demonstrated for AA and 2AB) [24, 25]. Reductively aminated glycans possess a secondary amine that is reactive with epoxide and are therefore suitable glycoconjugates for immobilization on the developed epoxide affinity chips. Other labels including 2-aminopyridine (2AP) can presumably be immobilized in a similar way, as it is known that the aromatic amines on these labels are suitable for nucleophilic addition reactions (*e.g.*, during reductive amination of glycans). By applying HPLC separation techniques (high-pH anion-exchange chromatography and graphitized carbon HPLC), capillary electrophoresis, and affinity purification using lectins and antibodies to separate glycan mixtures, the resolution of the glycan array may be increased. For this purpose, the free choice between a variety of fluorescent labels compatible with the separation techniques of choice appears to be highly advantageous. The robustness of the glycan chip was demonstrated by the fact that a single chip could be used for screening of the 21 serum samples included in this study. Efficient regeneration of the glycan affinity chip with an HCl solution followed by a blocking step made it possible to perform multiple runs.

Fmol amounts of glycans were sufficient to obtain protein binding profiles, which is comparable with glycan microarray based on glass slides in combination with fluorescence detection [1, 8]. The glycan microarray is not limited to either synthetic or natural glycans and does not require pure samples. The combination of various kinds of glycans and glycoconjugates on the glycan chip increases the potential of the array in CBP characterization and for screening sera.

In conclusion, this methodology, which combines glycan purification and characterization (HPLC and MS) with interaction screening (SPR), should be applicable (I) to select a set of glycans for diagnostic screening purposes (biomarker discovery), when glycans from healthy and diseased tissue or from pathogens are isolated, spotted on the array and screened with patient body fluids, (II) to obtain diagnostic/clinical profiles by detecting disease-associated anti-glycan antibodies (in sera), (III) to select the best binder in terms of kinetics (k_{on}, k_{off} and the dissociation constant K_d) or specificity from a large set of glycans (HPLC fractions), (IV) to reveal the structural element(s) that are recognized by CBPs, (V) and to identify glycans or glycan fractions (from biological samples) by using well-characterized CBPs, thereby providing structural information on glycans and glycan fractions.

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References

1. Blixt, O., Head, S., Mondala, T., Scanlan, C., Huflejt, M.E., Alvarez, R., Bryan, M.C., Fazio, F., Calarese, D., Stevens, J., Razi, N., Stevens, D.J., Skehel, J.J., van Die, I., Burton, D.R., Wilson, I.A., Cummings, R., Bovin, N., Wong, C.H., Paulson, J.C.: Printed covalent glycan array for ligand profiling of diverse glycan binding proteins. Proc. Natl. Acad. Sci. U. S. A. **101**, 17033–17038 (2004)
2. Feizi, T., Chai, W.G.: Oligosaccharide microarrays to decipher the glyco code. Nat. Rev. Mol. Cell Biol. **5**, 582–588 (2004)
3. Houseman, B.T., Mrksich, M.: Carbohydrate arrays for the evaluation of protein binding and enzymatic modification. Chem. Biol. **9**, 443–454 (2002)
4. Ratner, D.M., Adams, E.W., Disney, M.D., Seeberger, P.H.: Tools for glycomics: Mapping interactions of carbohydrates in biological systems. Chembiochem. **5**, 1375–1383 (2004)
5. Shilova, N.V., Galanina, O.E., Rubina, A.Y., Butvilovskaya, V.I., Huflejt, M.E., Chambers, J., Roucoux, A., Bovin, N.V.: 2-Aminopyridine—a label for bridging of oligosaccharides HPLC profiling and glycoarray printing. Glycoconj. J. (2007) this issue
6. Song, X., Xia, B., Lasanajak, Y., Smith, D.F., Cummings, R.D.: Quantifiable fluorescent glycan microarrays. Glycoconj. J. (2007) this issue
7. Xia, B.Y., Kawar, Z.S., Ju, T.Z., Alvarez, R.A., Sachdev, G.P., Cummings, R.D.: Versatile fluorescent derivatization of glycans for glycomic analysis. Nat. Methods. **2**, 845–850 (2005)
8. de Boer, A.R., Hokke, C.H., Deelder, A.M., Wuhrer, M.: General microarray technique for immobilization and screening of natural glycans. Anal. Chem. **79**, 8107–8113 (2007)
9. Blixt, O., Allin, K., Bohorov, O., Liu, X., Andersson-Sand, H., Hoffmann, J., Razi, N.: Glycan microarrays for screening sialyltransferase specificities. Glycoconj. J. (2007) this issue
10. Culf, A.S., Cuperlovic-Culf, M., Ouellette, R.J.: Carbohydrate microarrays: Survey of fabrication techniques. OMICS. **10**, 289–310 (2006)
11. Karamanska, R., Clarke, J., Blixt, O., MacRae, J.I., Zhang, J.Q., Crocker, P.R., Laurent, N., Wright, A., Flitsch, S.L., Russell, D.A., Field, R.A.: Surface plasmon resonance imaging for real-time, label-free analysis of protein interactions with carbohydrate microarrays. Glycoconj. J. (2007) this issue
12. Nibbeling, H.A.M., Kahama, A.I., Van Zeyl, R.J.M., Deelder, A. M.: Use of monoclonal antibodies prepared against Schistosoma mansoni hatching fluid antigens for demonstration of Schistosoma haematobium circulating egg antigens in urine. Am. J. Trop. Med. Hyg. **58**, 543–550 (1998)
13. van Remoortere, A., Van Dam, G.J., Hokke, C.H., van den Eijnden, D.H., van Die, I., Deelder, A.M.: Profiles of immunoglobulin M (IgM) and IgG antibodies against defined carbohydrate epitopes in sera of Schistosoma-infected individuals determined by surface plasmon resonance. Infect. Immun. **69**, 2396–2401 (2001)
14. Bergwerff, A.A., Van Dam, G.J., Rotmans, J.P., Deelder, A.M., Kamerling, J.P., Vliegenthart, J.F.G.: The immunologically reactive part of immunopurified circulating anodic antigen from Schistosoma mansoni is a threonine-linked polysaccharide consisting of -6)-[b-D-GlcpA-(1–3)]-b-D-GalpNAc-(1- repeating units. J. Biol. Chem. **269**, 31510–31517 (1994)
15. Vermeer, H.J., Van Dam, G.J., Halkes, K.M., Kamerling, J.P., Vliegenthart, J.F.G., Hokke, C.H., Deelder, A.M.: Immunodiagnostically applicable monoclonal antibodies to the circulating anodic antigen of Schistosoma mansoni bind to small, defined oligosaccharide epitopes. Parasitol. Res. **90**, 330–336 (2003)
16. van Roon, A.M., Aguilera, B., Cuenca, F., van, R.A., van der Marel, G.A., Deelder, A.M., Overkleeft, H.S., Hokke, C.H.: Synthesis and antibody-binding studies of a series of parasite fuco-oligosaccharides. Bioorg. Med. Chem. **13**, 3553–3564 (2005)
17. Wuhrer, M., Koeleman, C.A.M., Deelder, A.M., Hokke, C.H.: Repeats of LacdiNAc and fucosylated LacdiNAc on N-glycans of the human parasite Schistosoma mansoni. FEBS J. **273**, 347–361 (2006)
18. Wuhrer, M., Dennis, R.D., Doenhoff, M.J., Lochnit, G., Geyer, R.: Schistosoma mansoni cercarial glycolipids are dominated by Lewis X and pseudo-Lewis Y structures. Glycobiology. **10**, 89–101 (2000)
19. Folch, J., Lees, M., Stanley, G.H.S.: A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. **226**, 497–509 (1957)
20. Bigge, J.C., Patel, T.P., Bruce, J.A., Goulding, P.N., Charles, S. M., Parekh, R.B.: Nonselective and efficient fluorescent labeling of glycans using 2-amino benzamide and anthranilic acid. Anal. Biochem. **230**, 229–238 (1995)
21. Löfås, S., Johnsson, B.: A novel hydrogel matrix on gold surfaces in surface-plasmon resonance sensors for fast and efficient covalent immobilization of ligands. J. Chem. Soc. Chem. Commun. (21), 1526–1528 (1990)
22. Löfås, S.: Dextran modified self-assembled monolayer surfaces for use in biointeraction analysis with surface plasmon resonance. Pure Appl. Chem. **67**, 829–834 (1995)
23. Ro, H.S., Koh, B.H., Jung, S.O., Park, H.K., Shin, Y.B., Kim, M.G., Chung, B.H.: Surface plasmon resonance imaging protein arrays for analysis of triple protein interactions of HPV, E6, E6AP and p53. Proteomics. **6**, 2108–2111 (2006)
24. Anumula, K.R.: Advances in fluorescence derivatization methods for high-performance liquid chromatographic analysis of glycoprotein carbohydrates. Anal. Biochem. **350**, 1–23 (2006)
25. Shilova, N.V., Bovin, N.V.: Fluorescent labels for the analysis of mono- and oligosaccharides. Russ. J. Bioorg. Chem. **29**, 309–324 (2003)
26. Geyer, H., Wuhrer, M., Resemann, A., Geyer, R.: Identification and characterization of keyhole limpet hemocyanin N-glycans mediating cross-reactivity with Schistosoma mansoni. J. Biol. Chem. **280**, 40731–40748 (2005)
27. Domon, B., Costello, C.E.: A systematic nomenclature for carbohydrate fragmentations in FAB-MS/MS spectra of glycoconjugates. Glycoconj. J. **5**, 397–409 (1988)