Multiplex suspension array for human anti-carbohydrate antibody profiling

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Glycan-binding antibodies form a significant subpopulation of both natural and acquired antibodies and play an important role in various immune processes. They are for example involved in innate immune responses, cancer, autoimmune diseases, and neurological disorders. In the present study, a microsphere-based flow-cytometric immunoassay (suspension array) was applied for multiplexed detection of glycan-binding antibodies in human serum. Several approaches for immobilization of glycoconjugates onto commercially available fluorescent microspheres were compared, and as the result, the design based on coupling of end-biotinylated glycopolymers has been selected. This method requires only minute amounts of glycans, similar to a printed glycan microarray. The resulting glycomicrospheres were used for detection of IgM and IgG antibodies directed against ABO blood group antigens. The possibility of multiplexing this assay was demonstrated with mixtures of microspheres modified with six different ABO related glycans. Multiplexed detection of anti-glycan IgM and IgG correlated well with singleplex assays (Pearson's correlation coefficient $r = 0.95$–0.99 for sera of different blood groups). The suspension array in singleplex format for A/B trisaccharide, H_{di} and Le^x microspheres corresponded well to the standard ELISA ($r > 0.94$). Therefore, the described method is promising for rapid, sensitive, and reproducible detection of anti-glycan antibodies in a multiplexed format.

Introduction

Anti-glycan antibodies comprise an important part of naturally occurring and adaptive antibodies in humans. However, their functional roles in both innate and adaptive immunity are still rather unclear. The best studied anti-glycan antibodies in humans are directed against blood group A and B antigens,[1–3] the xenoantigen Galα1–3Galβ1–4GlcNAc,[4,5] and tumor-associated antigens, for example Galβ1–3GalNAcα (TF) and GalNAcα (T_n).[6] Anti-glycan antibodies are also involved in autoimmune inflammatory diseases.[7,8] Autoantibodies against gangliosides (GM1, GM2, GD3) and sulfated glycosphingolipids are found in various neurological disorders, such as Guillain–Barré syndrome, multiple sclerosis, and motor neuron disease.[9–12] In many cases, however, it is unclear whether the antibodies are causative for disease, and more basic knowledge about the biological roles of glycans and anti-carbohydrate antibodies is therefore necessary.

The aim of our study was to develop a practical reliable assay for simultaneous detection of several glycan-binding antibodies in human serum. Two main approaches for the analysis of anti-glycan antibodies are known to date. The first, standard ELISA,[13,14] is a time and material-consuming as well as rather laborious method. A second approach, based on printed glycan array (PGA), was developed recently.[15] Main advantages of PGA technology are high sensitivity, significant reduction of reagent consumption, as well as automated printing of unlimited repertoire of glycans. However, glycochip production requires very expensive equipment and it is "nonflexible", *i.e.* it does not allow for a quick change of the glycan repertoire to be tested.

Multiplex suspension assays, adopted from flow cytometry, are particularly known as tools for the simultaneous quantification of multiple ligands like cytokines.[16–20] This type of assay combines the flexibility of ELISA with the low consumption of key reagents typical for microchips. Optically encoded microsphere-based arrays are known to have significant advantages over flat arrays due to increased control over array preparation and easy reconfiguration (mixing of different pre-coated microspheres) of arrays.[16] Therefore, the suspension array technique might occupy an important niche between ELISA and PGA: the first being more relevant for investigation of limited numbers of ligands and the second for very broad glycan libraries. In between these two, the multiplex suspension array technology is optimally suited for simultaneous detection of up to several dozens of analytes.

In this study, glycopolymers end-capped with biotin were used to produce a glycan suspension array by coupling them to commercial carboxylated microspheres *via* streptavidin. An assay for detection of antibodies against six different glycans was developed, validated with affinity-purified antibodies, and then tested with human serum. The developed technique was also compared with the standard ELISA.

Materials and methods

Fluorescent microspheres

Fluorescence labeled carboxylated microspheres of 5.6 μm diameter were purchased from Luminex Corp., Austin, TX,

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USA. Two types of microspheres were used: first, SeroMap microspheres with spectral "addresses" (bead regions) #28, 38, 48, 58, 68 and 78, and second, MultiAnalyte COOH microspheres, bead regions #24, 26, 28 and 44.

Antibodies, conjugates and serum samples

R-Phycoerythrin conjugated antibodies, goat anti-human IgM and goat anti-human IgG were purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL, USA). Mouse anti-human IgG and mouse anti-human IgM were obtained from Jackson ImmunoResearch (West Grove, PA, USA). Goat anti-mouse IgG_1, labeled with biotin, was purchased from Southern Biotech Associates (Birmingham, AL, USA).

Three types of glycoconjugates were purchased from Lectinity (Moscow, Russia): (1) oligosaccharides conjugated with poly-[*N*-(2-hydroxyethyl)acrylamide], PAA (~30 kDa, 20% mol of saccharide), containing a single biotin as end group (Glyc–PAA–$biot_1$), (2) similar but with several biotins (5% mol) as polymer side groups (Glyc–PAA–biot), (3) biotin-free glycopolymers (Glyc–PAA). Glyc–$O(CH_2)_3NH_2$, Glyc–$O(CH_2)_3CONH(CH_2)_5$-NH–biotin, biotin–$NH(CH_2)_6NH_2$, and adsorbents A_{tri}- and B_{tri}-Sepharose 6FF were obtained from the same source.

Human serum samples from healthy donors of different blood groups were obtained from the Regional Red Cross Blood Donation Center (Bern, Switzerland). Pooled human serum samples were obtained from Sklifosofsky Research Institute of Emergency Medical Aid (Moscow, Russia).

Affinity purification of human anti-glycan antibodies

Polypropylene columns (Bio-Rad) were filled with 1 ml of affinity adsorbent (A_{tri}-Sepharose or B_{tri}-Sepharose), rinsed with distilled water, then with PBS. The serum samples were centrifuged for 10 min at 13 000 × *g* at 4 °C. The supernatant was diluted 1 : 1 with 0.15 M PBS, pH 7.4, containing 0.02% NaN_3 and applied on the column at 20–22 °C, flow rate 0.2–0.3 ml min^{-1}. The ratio (v/v) adsorbent/supernatant was 1/10–1/20. The column was washed with 10–15 volumes of the following solutions: PBS/0.02% NaN_3, then PBS/0.02% NaN_3/0.5% Tween-20, and then again with PBS/0.02% NaN_3. Antibodies were eluted with Tris (0.2 M, pH 10.4) containing 0.02% NaN_3, flow rate of eluting buffer was 60–90 μl min^{-1}. Fractions were neutralized immediately by glycin–HCl (2 M, pH 2.7) on ice. The antibody concentration was measured spectrophotometrically at 280 nm. Antibodies were stored at 4 °C for up to 6 weeks or kept frozen at −20 °C.

Coupling procedure for biotinylated glycopolymers

Modification of microspheres with streptavidin. The stock vial of Luminex COOH microspheres (SeroMap or MultiAnalyte, 1.25×10^7 microspheres ml^{-1}) was vortexed for 30 s, then sonicated for 30 s in a water bath sonicator. 100 μl of bead suspension (1.25×10^6 microspheres, 0.2 nmol –COOH groups in total, according to supplier's information) was centrifuged for 4 min, 14 000 × *g* at RT. 100 μl of microsphere wash buffer (Bio-Plex amine coupling kit, Bio-Rad, cat #171-406001) was added to the microsphere pellet, vortexed, sonicated and centrifuged as above. After gentle removal of the supernatant, the pellet was resuspended in 80 μl of microsphere activation buffer (Bio-Plex amine coupling kit, Bio-Rad), vortexed and sonicated. Sulfo-*N*-hydroxysuccinimide sodium salt (S-NHS) and 1-ethyl-3-[3,3-dimethylaminopropyl]carbodiimide hydrochloride (Pierce Biotechnology, Rockford, IL, USA), both 50 mg ml^{-1} in activation buffer, were prepared immediately prior to use, 10 μl of the carbodiimide, closely followed by 10 μl of S-NHS, were added to the microsphere suspension and vortexed for 30 s. Microspheres were then incubated on a vertical rotor in the dark for 20 min at RT. The activated microspheres were centrifuged and the supernatant removed. The pellet was washed once with 500 μl of activation buffer and resuspended in 150 μl 0.1 M $NaHCO_3$, pH 8.3, containing 1.5 μg (≈3 nmol) of biotin–$NH(CH_2)_6NH_2$. The suspension was rotated with medium speed for 2 h at room temperature in the dark. The obtained biotinylated microspheres were pelleted by centrifugation and resuspended in 150 μl of 0.03% ammonium hydroxide solution. The bead suspension, protected from light, was agitated on a rotator for 30 min at RT. After centrifugation and removal of supernatant, the pellet was washed twice with 500 μl PBS, pH 7.4. Streptavidin (Bio-Rad, 1 nmol in 150 μl PBS) was added to each tube (1.25×10^6 microspheres). The suspension was vortexed and agitated on a rotator for 2 h at room temperature or overnight at 4 °C in the dark. Microspheres were washed twice by centrifuging with 500 μl PBS and the supernatant was carefully removed.

Loading of monobiotinylated glycopolymers to streptavidin-coated microspheres. Aliquots of Glyc–PAA–$biot_1$ solution in water, containing 0.4, 0.8 or 1.6 nmol biotin, were added to reaction tubes with 1.25×10^6 streptavidin-coated microspheres in 150 μl PBS. The mixture was protected from light and agitated on a rotator for 3 h at RT. The modified microspheres were centrifuged and supernatant removed. Microspheres were washed twice with 500 μl storage buffer (Bio-Rad), pelleted by centrifugation and the microspheres resuspended in 100 μl of storage buffer. The bead concentration was determined using a hemocytometer (Roth AG, Karlsruhe, Germany). The Glyc–PAA–$biot_1$ coated microspheres were then stored at 4 °C, protected from light.

Antibody binding assay

A Bio-Plex Suspension Array System (Bio-Rad, Munich, Germany), which identifies and quantitates each specific reaction based on bead color and fluorescence, was used for microsphere-based suspension assays. Data analysis was performed by Bio-Plex Manager 4.1 software.

Antibody binding assays were performed according to the guidelines from Bio-Rad (Bio-Plex Cytokine Assay, Instruction manual).

The wells of 96-well Multiscreen filter plate (Millipore, Billerica, MA, USA) were prewetted for 5 min with 100 μl of antibody diluent (PBS–0.05 M Tris, pH 7.2, 0.5% BSA, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). 2000 microspheres per well in antibody diluent (50 μl per well) were added. The plate was washed three times with 100 μl of washing buffer (PBS–0.05 M Tris, pH 7.2) with a vacuum manifold (Bio-Rad). Human serum in serial dilutions (50 μl

per well) was added to wells, shaken at 1100 rpm for 30 s on a microplate shaker (Millipore) and incubated for 1 h at RT on the shaker (200–300 rpm) in the dark. After incubation, the plate was washed three times with washing buffer. Secondary antibodies labeled with R-phycoerythrin (R-PE), 25 ng per well of goat anti-human IgM-R-PE or IgG-R-PE were added and incubated for 30 min on the plate shaker in the dark. The plate was washed three times with washing buffer, microspheres were resuspended on the shaker and the plate read on the Bio-Plex array reader. Data were acquired in real time by the use of a computer software package (Bio-Plex Manager 4.1; Bio-Rad, Munich, Germany). The results were reported as median fluorescence intensities (MFI) amongst 100 beads per well.

Assay reproducibility

Reproducibility of multiplex suspension assay was assessed by determination of the level of both intra-assay and inter-assay variation. Intra-assay variation was determined from testing 10 sera, each tested on four wells for IgM and on four wells for IgG detection within a plate; thus four values were obtained for each antibody subtype. For each analyte the coefficient of variation in percent (CV) for each quadruplicate was calculated and averaged. Inter-assay variation (variation from assay to assay) was assessed by testing 10 sera in three separate assay runs. The CV between each of these results for each analyte was calculated and averaged.

Inhibition tests

The inhibition tests were performed as follows: 1 : 100 diluted pooled human sera (three donors from A, B and O blood groups) were preincubated for 30 min at room temperature with different concentrations of A_{tri}–PAA (from 1000 to 1 μg ml^{-1}) on a rotor. Sera without inhibitor were used as a control. The aliquots of the incubation mixture were then mixed with microspheres and assayed as described above.

ELISA

96-well NUNC MaxiSorp immunoplates (Thermo Fisher Scientific, Roskilde, Denmark) were coated with oligosaccharide–polyacrylamide conjugates (Glyc–PAA), 10 μg ml^{-1}, 100 μl per well in carbonate buffer (50 mM Na_2CO_3/$NaHCO_3$, pH 9.6) for 60 min at 37 °C or overnight at 4 °C. Plates were blocked by 2% casein in PBS, 200 μl per well for 30 min at 37 °C. Individual human serum samples (initial dilution 1 : 20) or affinity-purified antibodies (initial concentration 10 μg ml^{-1}) were serially diluted in PBS, containing 0.3% BSA, added to the plate in duplicates and incubated for 60 min at 37 °C. Between all of the following steps the plates were washed four times with PBS containing 0.1% Tween-20: incubation with 100 μl per well mouse anti-human IgG or mouse anti-human IgM (diluted 1/4000 in PBS + 0.3% BSA) for 60 min at 37 °C; goat anti-mouse IgG_1–biotin (1/2000 in PBS–0.3% BSA), 60 min at 37 °C; and streptavidin–alkaline phosphatase conjugate (1/2000 in PBS–0.3% BSA), 30 min at 37 °C. The plates were then rinsed with water and

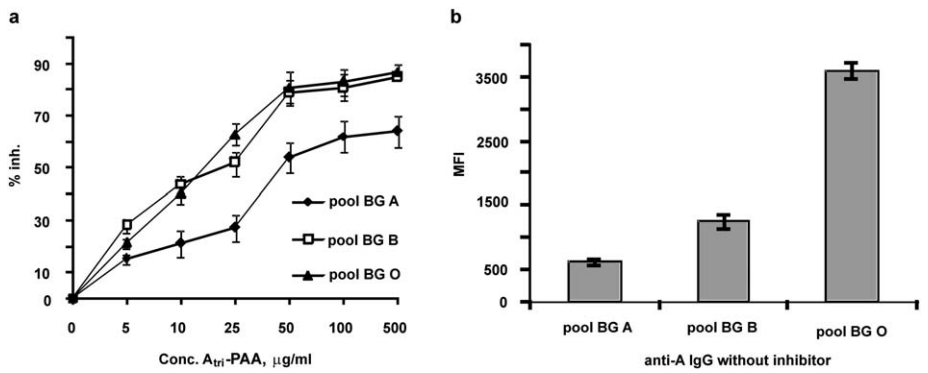

Fig. 1 Inhibition of binding of human IgG antibodies to A_{tri}–PAA–$biot_1$ coated microspheres. Pooled sera from blood groups A, B and O were used at 1 : 40 dilution, binding of IgG was detected with goat anti-human IgG antibody. A_{tri}–PAA was used as an inhibitor at the concentrations indicated on the *x*-axis (a). The initial MFI values for anti-A IgG in three blood group pools are shown separately (b).

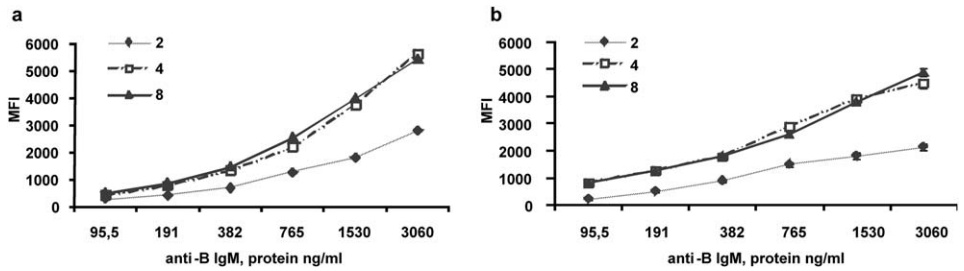

Fig. 2 Binding of affinity purified anti-B antibodies from pooled sera of blood group O to B_{tri}–PAA–$biot_1$ SeroMap microspheres. Curves for 2, 4 or 8 mol of $biot_1$–B_{tri}–PAA per mol of streptavidin are shown. Bound antibodies were detected with secondary anti-IgM (a) or anti-IgG (b) antibodies.

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p-nitrophenyl phosphate (3 tablets per 12 ml of diethanolamine buffer (Sigma)) was added. Color was developed by 20 min incubation at room temperature. Absorbance was read at 405 nm with a TECAN Reader (Tecan Group, Ltd., Männedorf, Switzerland).

Statistics

The groups were graphically described by box plots to define extreme outliers situated outside of the whiskers, using the open source statistical programming language R (The Comprehensive R Archive Network (http://CRAN.R-project.org/)). Differences between groups (average amounts) were calculated by Analysis of Variances (ANOVA) using the Fisher's PLSD as *post hoc* test (Stat View).

Results

Purification of anti-A_{tri} and anti-B_{tri} antibodies

Human polyclonal antibodies against blood group antigens A and B were used primarily as tools for testing of glyco-microspheres. Anti-A and anti-B antibodies were isolated from pooled sera of healthy donors (10 to 50 donors per pool) of blood group O (anti-A_{tri}, two batches; anti-B_{tri}, one batch), blood group A (anti-B_{tri}, one batch) and from blood group O serum of a single donor (anti-A_{tri}) by affinity chromatography on A_{tri}- or B_{tri}-Sepharose as described before.[21] The concentrations of eluted immunoglobulins in different batches varied from 150 μg ml^{-1} to 15 μg ml^{-1}. The affinity purified immunoglobulins were characterized by ELISA with A_{tri}–PAA and B_{tri}–PAA as coating antigens, and secondary anti-IgM and anti-IgG antibodies (data not shown).

Immobilization of glycoconjugates on microspheres

We compared several approaches for coupling of glycoconjugates to commercially available fluorescent microspheres. Firstly, we coupled monomeric 3-aminoalkyl glycosides (or glycine instead of spacered glycan, as a negative control) to pre-activated MultiAnalyte COOH microspheres. In this case, glycine-conjugated microspheres exhibited (very low) MFI values similar to glycoside-coupled microspheres, and the binding of glyco-microspheres with serum antibodies was not inhibited by Glyc–PAA. Glycan immobilization was confirmed by thin layer chromatography. Nevertheless, when attached according to this protocol, the glycans could not be recognized by antibodies. Secondly, biotinylated monomeric glycosides, Glyc–$O(CH_2)_3CONH(CH_2)_5NH$–biotin and glycopolymers with

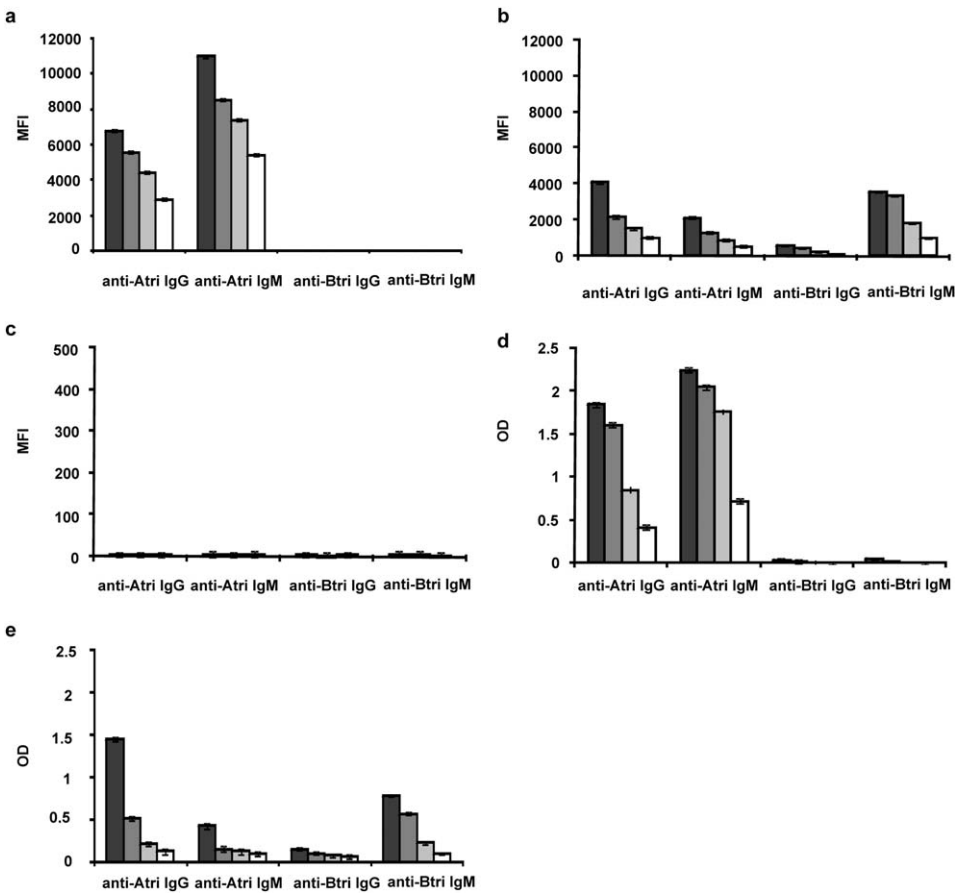

Fig. 3 Binding of anti-A antibodies (affinity purified from pooled sera blood group O) and anti-B antibodies (from pooled sera blood group A), IgG or IgM, to Glyc–PAA–$biot_1$ microspheres in suspension array (a–c) and to Glyc–PAA in ELISA (d, e). (a) A_{tri}-microspheres, (b) B_{tri}-microspheres, (c) Le^x-microspheres, (d) A_{tri} ELISA plate, and (e) B_{tri} ELISA plate. Black bars correspond to 5 μg ml^{-1}, dark grey bars to 2.5 μg ml^{-1}, light grey bars to 1.25 μg ml^{-1}, and white bars to 0.625 μg ml^{-1} of protein in antibody samples.

several or one biotin residue per polymer molecule, Glyc–PAA–biot or Glyc–PAA–$biot_1$, were coupled to avidin microspheres (LumAvidin microspheres, Luminex Corp.). The resulting glyco-microspheres demonstrated inappropriate background levels and low specific binding levels when binding of serum antibodies was tested.

Finally, we used a two-step procedure in which activated COOH groups of carboxylated MultiAnalyte or SeroMap microspheres were first biotinylated with a 10-fold excess of a biotin derivative containing a primary amino group, biotin–$NH_2(CH_2)_6NH_2$, then streptavidin (taken in excess) was anchored. In order to avoid assay noise, supposedly caused by biotin residues, three types of biotinylated glycoconjugates were attached to the surface of streptavidin-covered microspheres: (1) monomeric glycoside, A_{tri}–$O(CH_2)_3CONH(CH_2)_5NH$–biotin, (2) polyacrylamide-based glycopolymer with several (six in average) side biotin groups per molecule, A_{tri}–PAA–biot, and (3) similar polymeric conjugate carrying only one end biotin group per molecule, A_{tri}–PAA–$biot_1$, the synthesis of which was recently reported.[22] No significant fluorescence signals were registered in the case of A_{tri}–spacer–biot (1) coupled microspheres. In the case of A_{tri}–PAA–biot (2) MFI signals were registered, but showed no dependency on serum blood group, and no significant inhibition of serum antibodies binding to the microspheres by A_{tri}–PAA was observed. In contrast, the end-monobiotinylated compound, A_{tri}–PAA–$biot_1$ (3), demonstrated specific binding with appropriate purified human antibodies. Moreover, the binding of serum antibodies to this ligand was dose-dependently inhibited by A_{tri}–PAA (Fig. 1).

Binding of purified polyclonal antibodies to Glyc–PAA–$biot_1$ SeroMap glyco-microspheres

Even in the case of Glyc–PAA–$biot_1$ bound to the surface of regular MultiAnalyte COOH microspheres the background was rather high (data not shown). SeroMap microspheres were therefore used, which are designed by Luminex Corp. to reduce non-specific reactivity in protein-based serological applications. All data presented hereafter were obtained with these microspheres. A_{tri}–PAA–$biot_1$, B_{tri}–PAA–$biot_1$, or Le^x–PAA–$biot_1$ was coated onto SeroMap microspheres differing only by fluorescent "bead region".

Three concentrations of Glyc–PAA–$biot_1$, corresponding to 2, 4 and 8 mol of biotin per mol of streptavidin, were compared with respect to binding of purified polyclonal anti-A or anti-B antibodies. It was found that saturation was reached between 2 and 4 mol of biotin per mol of streptavidin (Fig. 2), which corresponds with the expected value of 3 mol, based on one of the four valences of streptavidin being used for anchoring to the bead.

Suspension array in singleplex format *versus* ELISA

To compare the suspension array data with standard ELISA, glycopolymers A_{tri}–PAA, B_{tri}–PAA and Le^x–PAA were used as coating antigens for ELISA, and the corresponding mono-biotinylated polymers for the suspension array. Both types of glycopolymers (Glyc–PAA–$biot_1$ in suspension array and Glyc–PAA in ELISA) contained 20 mol% of Glyc and had practically the same molecular weights. The same batches of purified polyclonal antibodies from pooled or individual human sera were used in both analyses. Suspension array in this case was applied in a "singleplex version" meaning that separate analyses for each bead sort (A_{tri}-, B_{tri}- and Le^x-microspheres) were performed. The singleplex assays showed a good correlation with ELISA, with a Pearson's coefficient (r), calculated for all values within range of both assays, of >0.94 ($n = 64$, data not shown).

Anti-A and anti-B polyclonal antibodies bound to the cognate trisaccharides in a dose-dependent manner (Fig. 3a and b), and did not bind to Le^x (taken here as a negative control) in both assays. Anti-B antibodies, isolated from pooled blood group A serum, showed almost no cross-reactivity to A_{tri} antigen in suspension array as well as in ELISA. As expected, anti-A antibodies isolated from blood group O sera exhibited cross-reactivity to B_{tri} antigen in both assay formats (Fig. 3d and e).

Singleplex suspension assay of human sera

A_{tri}, B_{tri}, Le^x and H_{di} microspheres were tested in singleplex assays with seven individual normal human serum samples (two each of blood groups A, B and O, and one of blood group AB) diluted 1 : 40 (Fig. 4). We used Le^x as a "negative or low binding" control, because according to the data from printed glycan array from more than 100 healthy donors,[23] this trisaccharide exhibits very low binding to human antibodies. In singleplex suspension assay the low level of binding of human antibodies with Le^x-microspheres was indeed confirmed (Fig. 4). Binding of anti-A and anti-B IgM antibodies was in accordance with the expectations from blood group serology, with only low binding

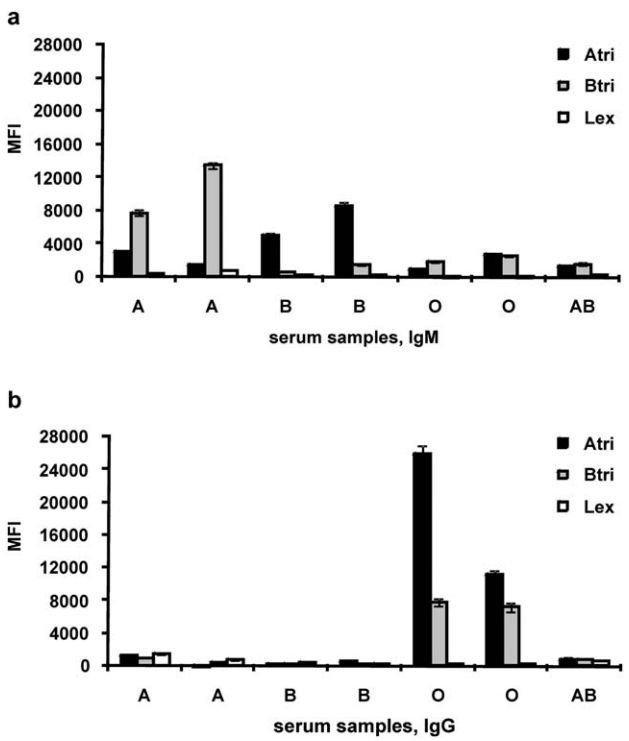

Fig. 4 Binding of IgM (a) and IgG (b) antibodies from individual human sera of blood groups A, B, O and AB to A_{tri}–PAA–$biot_1$, B_{tri}–PAA–$biot_1$ and Le^x–PAA–$biot_1$ microspheres.

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of potentially autoreactive anti-A antibodies in A or AB serum, and anti-B in B and AB serum. Surprisingly, only a low binding of anti-A and anti-B IgM was found in the two O-type sera. In contrast, anti-A and anti-B IgG were almost exclusively detected in the type O sera (Fig. 4).

Multiplex array

To determine how the multiplexing influences the results of the suspension assay, we mixed different glyco-microspheres (A_{tri}, B_{tri}, A_{tetra}, B_{tetra}, H_{di} and Le^x) in one reaction sample and performed a hexaplex assay. Ten human serum samples (three of blood group A, two of B, three of O, and two of AB) were compared in mono- and hexaplex assays. The obtained data correlated well with each other, with Pearson's r values ranging from 0.95 to 0.99 (Fig. 5).

In five out of six types of glyco-microspheres the MFI values in mono- and hexaplex assay formats were practically identical. However, in the case of H_{di}, for which correlation between mono- and hexaplex assays was very good ($r = 0.98$, Fig. 5e), the MFI values in the hexaplex assay were approximately two times lower compared with the singleplex format (Fig. 6). The statistical representation of binding of blood group specific IgM and IgG with six types of glyco-microspheres in hexaplex assay is shown in Fig. 7. Similar distribution of IgM antibody binding to A_{tri} was observed in all blood groups, except AB (Fig. 7a); in

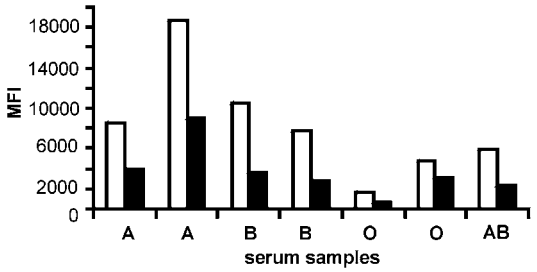

Fig. 6 Binding of IgM from individual sera of A, B, O and AB blood groups to H_{di} microspheres in singleplex (white bars) and hexaplex (black bars) assays. Dilution of sera was 1/40.

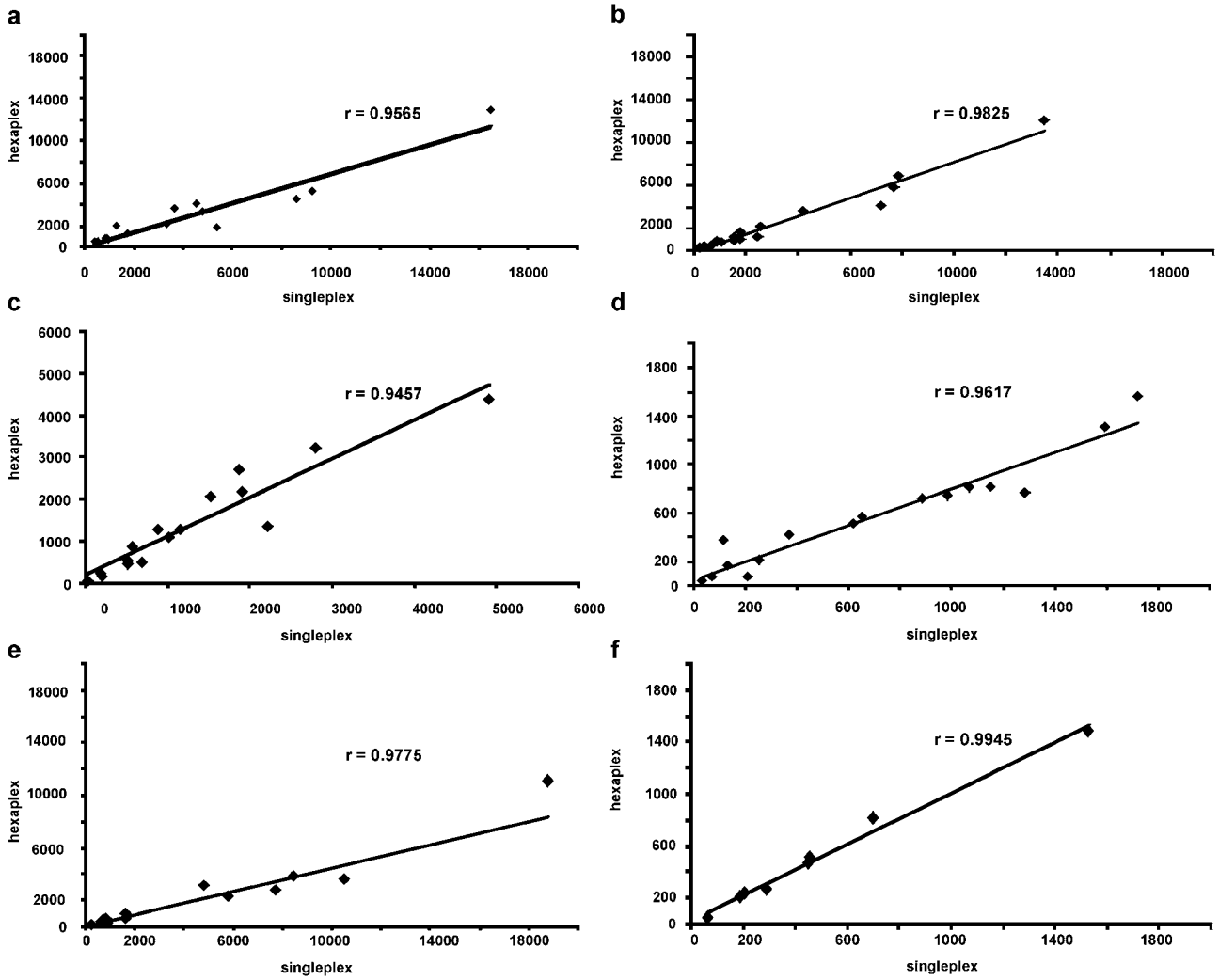

Fig. 5 Correlation of data from singleplex and hexaplex suspension arrays. 10 individual sera were tested both on singleplex and hexaplex for the presence of IgG and IgM antibodies against the following glycans: A_{tri} (a), B_{tri} (b), A_{tetra} (c), B_{tetra} (d), H_{di} (e) and Le^x (f). The serum dilution was 1/40 in all assays. Shown are scatter plots of MFI data with indication of the respective r values.

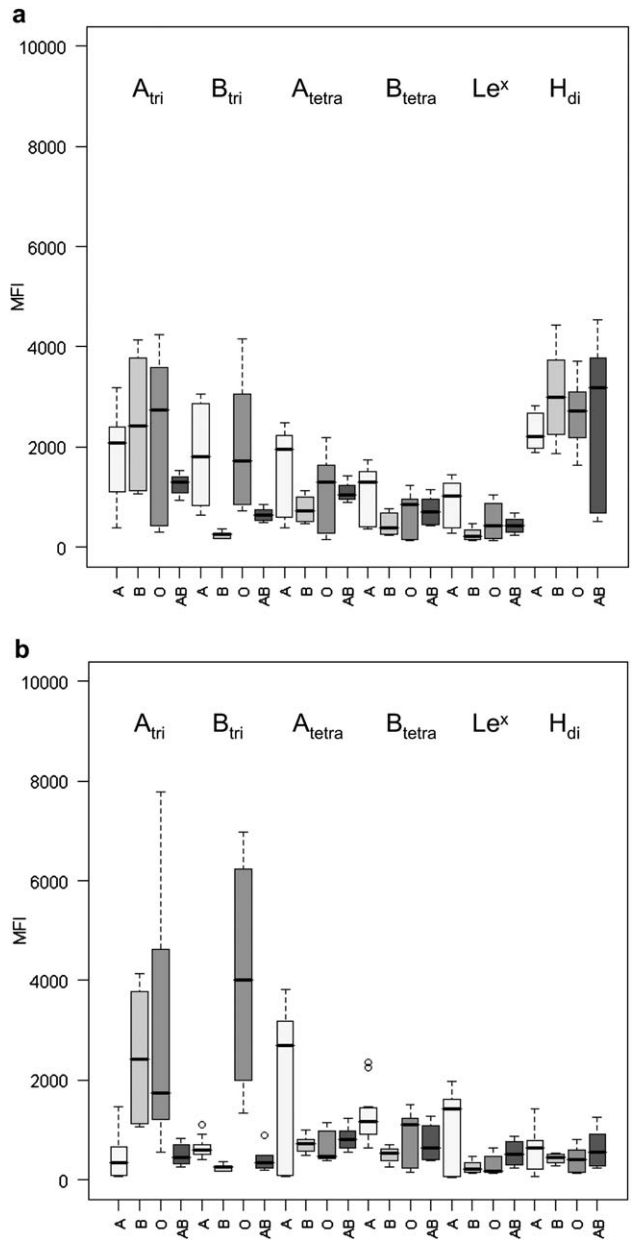

Fig. 7 Binding of serum anti-glycan antibodies to glyco-microspheres in hexaplex assay. Box-and-whisker plots represent the distribution of MFI values for six glycans per blood group (A, B, O, AB). The median per blood group is indicated by the horizontal black bar in a box. The bottom and the top of a box represent the lower and upper quartile, respectively. The length of a box is designated as interquartile range. The length of dotted lines, whiskers, is within 1.5 times of interquartile range. The data outside the whiskers are considered as outliers and are represented by empty circles. (a) IgM and (b) IgG.

contrast, strongly differentiated signals were observed for IgG (Fig. 7b), with lower values in blood groups A and AB, and elevated signals in blood groups B and O sera. However, anti-B_{tri} IgMs demonstrated highly discriminative signals as well (Fig. 7a), with higher values in A and O sera, whereas in the case of IgG binding only anti-B_{tri} from O-type sera were distinctly elevated (Fig. 7b). Compared to trisaccharides, the antibody binding to tetrasaccharides (A_{tetra} and B_{tetra}) was diminished in all blood groups and showed no strong IgM *vs.* IgG difference. IgM binding with H_{di} demonstrated to be highly independent of blood group (Fig. 7a and b).

Assay reproducibility

Intra- and inter-assay variability data were used to determine the reproducibility of the hexaplex assay. The mean %CV obtained from four replicates of 10 serum samples for each immunoglobulin subtype within a plate (intra-assay variation) ranged from 5.7% (A_{tri}, IgM and IgG) to 11.3% (H_{di}, IgM and IgG). Inter-assay variation was calculated by comparison of three experiments with hexaplex assay using 10 sera. The mean inter-assay CVs ranged from 9.1% (IgM) to 18.6% (IgG).

The reproducibility of glycan coupling procedure was determined by comparison of two batches of A_{tri}-microspheres, synthesized independently. The Pearson correlation coefficient for a singleplex assay with these two batches was 0.96.

Hexaplex suspension array *vs.* ELISA

The comparison between hexaplex suspension array and ELISA with the respective glycans, separately for IgM and IgG ($n = 10$), revealed Pearson r values ranging from 0.72 (A_{tri}) to 0.95 (B_{tri}) for IgM, and from 0.51 (B_{tetra}) to 0.97 (A_{tri}, B_{tri}) for IgG (Table 1).

Discussion

Microsphere-based suspension arrays permit rapid quantitative determination of multiple analytes in one sample. This method was first developed for cytokine detection,[19,20] and is now widely applied for protein expression profiling, genomic research, and immunodiagnostics.[24–26] However, to date there are only few references on glycan-based microsphere assays in the area of glycobiology. In the published studies, glycans were chemo-immobilized: group A meningococcal polysaccharide was conjugated with poly-L-lysine;[27,28] monosaccharides mannose and galactose were coupled to maleimide-activated BSA;[29] 13 purified glycopeptides of different types and spacer-armed blood group A trisaccharide were directly immobilized onto beads.[30,31] The binding of resulting glyco-microspheres with various lectins[29,30] and human serum IgG antibodies[27,28] was tested.

In the current study we applied the suspension array technique to develop an assay for the detection of anti-glycan antibodies in human serum, expecting to achieve the advantages shown in literature for other analytes. We tested several methods for immobilization of glycomolecules on the bead surface, demonstrated dose-dependent binding of modified microspheres with human antibodies, compared the suspension assay with standard ELISA, and assessed the assay in the multiplex format.

Table 1 Summary of Pearson's r correlations between hexaplex suspension array and ELISA

Isotype	Comparison	A_{tri}	B_{tri}	H_{di}	A_{tetra}	B_{tetra}
IgG	*Suspension array vs. ELISA*	0.97	0.97	0.94	0.87	0.51
IgM	*Suspension array vs. ELISA*	0.72	0.95	0.92	0.87	0.92

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Our first aim was to elaborate the procedure of glycan coupling to fluorescent microspheres, which is applicable for serum antibodies profiling, *i.e.* in conditions of enhanced noise risk. Immobilization should be simple and quantitative, whereas assay background should allow us to measure low abundance antibodies. The comparison of different immobilization chemistries showed that the attachment of end biotin labeled glycopolymers[22] satisfies both requirements (Fig. 8).

As compared to coupling of Glyc–PAA–biot$_1$, other immobilization techniques had significant drawbacks, supposedly due to the following reasons: first, the coupling of monomeric aminoalkyl glycosides and biotinylated glycans (Glyc–sp–biot) showed generally low signals, which could be caused by short linker and inappropriate density of ligands on the microsphere surface. Second, the high background levels, detected in the case of glycan binding to avidin-coated microspheres, in one-step and two-step detection could be caused by carbohydrate chains of avidin. We therefore replaced avidin with streptavidin, which contains no carbohydrates. SeroMap microspheres, preferentially used for blood sample testing due to reduced background, are not commercially available in streptavidin-coated form; this is why we coupled streptavidin by two-step procedure to standard, commercially available carboxylated beads.

All the used end-biotin labeled glycopolymers have a molecular weight ~50 kDa and the same glycan density (20% mol). Importantly, due to streptavidin–biotin immobilization the loading rate of glycans on the microspheres is identical for all glycans and does not depend on glycan size and charge. A dose-dependent binding of Glyc–PAA–biot$_1$ to the microspheres, detected with the help of polyclonal anti-Glyc antibodies, was achieved. The reproducibility of coupling, tested on the example of two batches of A$_{tri}$–PAA–biot$_1$ microspheres, proved to be very good, with an excellent correlation of the observed MFI values ($r = 0.96$).

Our second aim was to compare standard ELISA with the glycobead-based suspension array. In the singleplex version of the assay, the correlation with ELISA for combined plotting of IgM and IgG was high ($r > 0.94$ for all tested glycans). The comparison between hexaplex assay and ELISA individually for IgM and IgG showed correlations in a range of 0.72–0.95 for IgM and 0.51–0.97 for IgG. The observed variations can be expected, because the difference in the methods' conditions is really dramatic, namely: (1) static conditions in ELISA *vs.* dynamic conditions in suspension assay; (2) related to this competition between IgG and IgM antibodies; (3) opposite stoichiometry between antibodies and antigens; (4) glycans surface density.

The hexaplex version of suspension array was found to be highly reproducible, with low variability from assay to assay (CV < 20%). Intra-assay variation was also shown to be low (CV < 11%). These data are in agreement with ELISA, which commonly shows intra-assay CV 5–10% and inter-assay CV 10–15%. Although stability of the modified microspheres was not explicitly tested, we assume the microspheres to be chemically stable, as no loss of stability of glyco-microspheres, which were stored at 4 °C in the dark, was observed within three months. Modified microspheres were constantly used for different assays within this period and deviations between assays did not exceed inter-assay variability (see Results).

We selected Le^x-coated microspheres as negative control, because of the lack of antibodies to Le^x in PGA.[23,32] Glycan-coupled microspheres seem to be better suited as controls than glycan-free microspheres due to more adequate architecture and hydrophilicity. Indeed, similarly to PGA, serum antibodies demonstrated very low binding to Le^x-microspheres (Fig. 4). The hexaplex assay correlated well with the singleplex version, except for binding of antibodies to H$_{di}$, which was significantly decreased in the hexaplex assay. The reason why these low affinity antibodies showed decreased binding in the multiplex assay is as yet unclear, but a competition for antibody binding by more complex antigens comprising the H$_{di}$ structure (A$_{tri}$ and B$_{tri}$ for example), which are simultaneously present in the multiplex assay, might be an explanation. Slightly decreased correlations of 0.95 and 0.96 in the case of A/B tetrasaccharides might also reflect the influence of some slight cross-reactivity with A/B trisaccharides in hexaplex assay.

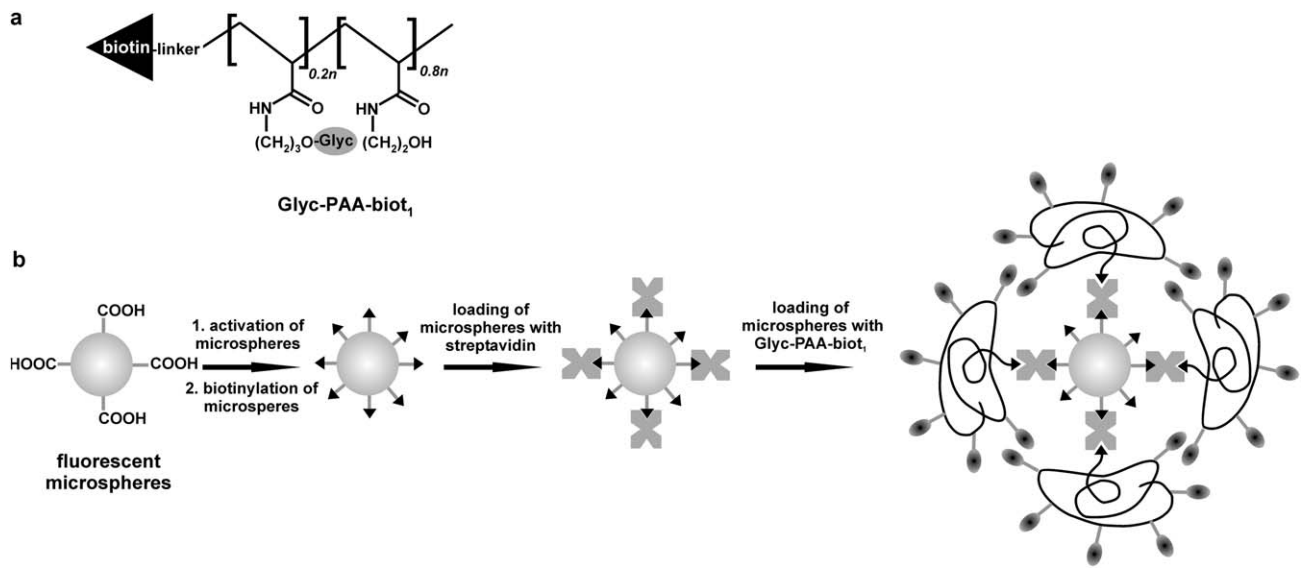

Fig. 8 The structure of end biotin labeled glycopolymer (a) and scheme of its coupling to fluorescent COOH-microspheres (b).

A clear advantage of the current suspension array version over ELISA is significantly lower consumption of glycopolymers (nanomolar *vs.* micromolar amounts per experiment). In addition, the multiplex suspension array combines the flexibility of an ELISA with the possibility for simultaneous detection of multiple ligands in one sample and minimal reagent consumption, typical for high-throughput microarrays. In contrast to printed glycan array, in suspension array the assay reconfiguration could be easily achieved by combining different types of pre-coated microspheres in one sample. Thus, the glycan-based suspension array broadens the methodological flexibility, and could occupy specific application niches.

In conclusion, we demonstrated the potential of a microsphere-based suspension array for fast and reproducible detection of anti-glycan antibodies in human serum. The combination of end-biotinylated glycopolymers with streptavidin-coated fluorescence encoded microspheres allows for easy configuration of arrays in a customizable fashion.

Abbreviations

A_{tetra}	GalNAcα1–3(Fucα1–2)Galβ1–4GlcNAc, A tetrasaccharide type 2
A_{tri}	GalNAcα1–3(Fucα1–2)Gal, A trisaccharide
BG	blood group
BSA	bovine serum albumin
B_{tetra}	Galα1–3(Fucα1–2)Galβ1–4GlcNAc, B tetrasaccharide type 2
B_{tri}	Galα1–3(Fucα1–2)Gal, B trisaccharide
CV	coefficient of variation in percent
ELISA	enzyme-linked immunosorbent assay
Glyc	glycan
Glyc–PAA	glycan–polyacrylamide conjugate
Glyc–PAA–$biot_1$	end biotin labeled oligosaccharide–polyacrylamide conjugate
H_{di}	Fucα1–2Galβ, H disaccharide
Le^x	Galβ1–4(Fucα1–3)GlcNAcβ, Lewis X
MFI	median fluorescence intensity
PAA	poly[*N*-(2-hydroxyethyl)acrylamide]
PBS	phosphate buffered saline

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