Automated glycan-bead coupling for high throughput, highly reproducible anti-glycan antibody analysis

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ABSTRACT

Automation of diagnostic assays generally aims to increase reproducibility and throughput while decreasing human errors and hands-on time. Here, we introduce a protocol for the automated chemical conjugation of glycans to color-coded magnetic beads using the KingFisher Flex magnetic particle processor. The resulting glycan-coupled magnetic beads allow the detection of anti-glycan antibodies of different isotypes from various species. By generating anti-glycan antibody profiles, monoclonal antibodies can be screened for their specificity and cross-reactivity, while anti-glycan antibody profiles from different human body fluids can aid in predicting response to treatment or outcome of disease. This efficient, scalable protocol can also be adapted to attach proteins and other biomolecules to beads, making it useful for a wider range of applications that require bead-based laboratory methods.

1. Introduction

The glycocalyx describes a dense layer of glycosylated biomolecules covering the surface of cells from protozoa to complex multicellular organisms, including humans. These glycosylated biomolecules, also called glycoconjugates, consist of mainly proteins and lipids, and are products of a complex synergy of several sequentially acting glycosyltransferases in the endoplasmic reticulum and the Golgi apparatus. The resulting glycan structures range from simple monosaccharides to complex polysaccharides with important roles in diverse physiological and pathophysiological processes. Amongst others, glycosylation affects the molecules’ folding, stability, activity, as well as intercellular signaling and adhesion [1,2]. Several congenital disorders, autoimmune diseases, and cancer are associated with changes in glycosylation [3]. In addition to protective functions and communication within an organism, glycans are also involved in host-pathogen interactions [1]. Certain species-specific glycosylation patterns are described to be the main triggers for the production of anti-glycan antibodies, with ABO blood group antibodies as one of the most well-known examples. Some antibodies against glycans are thought to be part of the natural antibody repertoire, whereas other anti-glycan antibodies, such as the abovementioned antibodies to the blood group antigens, seem to be induced after birth through exposure to microbes or glycans in food [4,5]. Endogenous anti-glycan antibodies have been shown to retain stable levels over a long time and can have both positive and negative effects on the body’s homeostasis [6,7]. As a benefit, antibodies, including those to glycans, have the ability to opsonize pathogens and aberrant cells, a process in which the binding of an antibody to foreign molecules on the cellular surface induces phagocytosis and the subsequent clearance of the pathogenic cell from the body [8]. On the other hand, antibodies against glycans induced in patients infected with Campylobacter jejuni or Helicobacter pylori were described as pathological contributors to diseases such as Guillain-Barré syndrome and myocardial infarction, respectively [9,10]. Additionally, anti-glycan antibodies have been described to play a role in organ transplants, such as in the case of a heart transplant from an alpha-galactosidase knock-out (“GalSafe”) pig to a human [11]. Antibodies to tumor associated carbohydrate antigens (TACAs) found in the blood have been proposed as non-invasive biomarkers that could be used for diagnosis or prognosis of several types of cancer [12–14]. Examples for common TACAs are the mucin-related Thomsen-nouvelle (Tn) antigen and Core 1, also known as Thomsen-Friedenreich (TF) antigen. As indicated by the name, Core 1
and seven additional core structures (Core 2-Core 8), are described to serve as backbone for further elongation [15]. Depending on the activity of certain glycosyltransferases, larger glycan structures can be formed, including the TACA Lewisβ (also known as stage-specific embryonic antigen (SSEA)-1) and Sialyl-Lewisβ (SLβ, also cancer antigen (CA)19-9) of the Lewis (Le) blood group [16,17]. Moreover, the exclusive presentation of certain TACAs on the surface of tumor cells make them potential targets for monoclonal antibody therapy. While multiple monoclonal anti-glycan antibodies, such as MVT-5873 targeting Sialyl-Leβ (NCT02672917), are currently evaluated in clinical trials, the monoclonal antibody dinutuximab beta (trade name Quazariba) targeting the glycolipid GD2 is already applied as an orphan medicine for second line treatment in neuroblastoma patients [18,19].

To improve the accuracy and efficiency of detecting anti-glycan antibodies for diagnosis, treatment, and research, it is important to continue developing and improving methods. There are several methods currently available for detecting anti-glycan antibodies, each with its own advantages and limitations. ELISA is a widely used technique, but has limited signal range and throughput. Glycan microarrays can be used to analyze reactivities against several hundred glycans, but are less flexible for testing high numbers of samples [20]. Bead-based assay methods are convenient, high-throughput, and require only small sample volumes, with the ability to screen for up to 500 analytes in a single sample depending on the readout device. Different approaches for the manual conjugation of glycans to Luminex beads are described in the literature with variations mainly in chemical conjugation approaches and applied linkers for optimized presentation of the glycans on the bead surface [21,22]. These manual coupling procedures of glycans to beads work well for assay establishment, but get cumbersome when aiming for high-content analysis. Thus, to increase the experimental throughput and particularly reproducibility, an automated process would be of tremendous advantage [23].

We here describe a protocol for the automated coupling of glycans to color-coded magnetic beads for subsequent high-throughput or high-content screening and characterization of anti-glycan antibodies. We demonstrate that the protocol allows a reproducible, accurate, and reliable easy-to-upscale workflow with reduced hands-on time for anti-glycan antibody analysis.

2. Materials and methods

2.1. Human specimens

EDTA-plasma samples from healthy donors were purchased from Bio-Rad Medical Diagnostics GmbH (Dreieich, Germany). Ascites samples were provided by Prof. Dr. Walburgis Brenner and Prof. Dr. Marcus Schmidt (University Medical Center of the Johannes Gutenberg-University Mainz, Germany) and were collected from ovarian cancer patients after written informed consent was given. Cerebrospinal fluid (CSF) of Parkinson’s patients was kindly provided by Prof. Brit Molrenhauser (Paracelsus-Elena-Clinic, Kassel, Germany) after written informed consent was given. All human body fluids used in this study were stored at -80°C.

2.2. Manual coupling of glycans to Luminex beads

For the chemical conjugation of glycans to carboxylated magnetic beads (MagPlex-C microspheres, Luminex Corp., Austin, TX, USA), glycans of the PA series (glycans conjugated to poly-[N-(2-hydroxyethyl)acrylamide] (PAA); GlycoNZ, Auckland, New Zealand; structures described in detail by Tuzikov et al. [24]) were used. MagPlex microspheres are superparamagnetic 6.5 µm beads, which are color-coded into spectrally distinct sets, also called regions. The light sources of the Bio-Plex 200 analyzer (Bio-Rad Laboratories, Hercules, CA, USA) can excite the beads’ dyes and subsequently allocate them to a specific bead region. Individual regions were coated with different glycans (see Table S1 for details), allowing the simultaneous detection of antibodies to multiple glycan structures within a single sample in the subsequent glyco-multiplex assay (GMA). Bead regions were chosen based on their compatibility with the Bio-Plex 200. For the chemical conjugation, the COOH groups on the MagPlex beads were activated through a reaction with the carbodiimide derivative 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) forming an unstable, active O-acetyl-silosure intermediate. A subsequent reaction with N-hydroxysulfosuccinimide (S-NHS) forms a more stable ester, resulting in a covalent bond after adding an amine group-carrying compound. For the conjugation of the applied PAA-glycans to 5 × 10⁶ beads, 40 µL of well-mixed bead suspension were transferred from the stock vial (1.25 × 10⁷ beads/mL) to 60 µL of activation buffer (100 mM NaH₂PO₄, pH 6.2) in a 1.5 mL vial. Apparently, the efficiency of the coupling is strongly dependent on the plastic that is used, and based on our observations, we selected Eppendorf Protein LoBind tubes (Eppendorf, Hamburg, Germany) for optimal performance. All steps, if not stated differently, were performed at room temperature and, if possible, protected from light. The beads were washed twice with 100 µL of activation buffer by positioning the tube in a magnetic separator and removing the supernatant with a transfer pipette. In-between the washing steps, the beads were agitated by vortexing for at least 10 s. Immediately prior to activation of the COOH groups on the beads, EDC and S-NHS sodium salt (both from Sigma-Aldrich, St. Louis, MO, USA) were diluted in activation buffer to a final concentration of 50 mg/mL. An “activation precursor” consisting of 10 µL EDC, 10 µL S-NHS, and 80 µL activation buffer was added to the beads. The COOH groups on the beads were chemically activated for 20 min by overhead mixing on a rotator at a speed of 15-30 rpm with an additional vortexing step after 10 min. Three wash steps with 100 µL of coupling buffer (100 mM 2-(N-morpholino)-ethane sulfonic acid (MES, Carl Roth, Karlsruhe, Germany), pH 6.0) followed the activation. For coupling of glycans to the beads, 1 mg of each glycan was dissolved in 1 mL sterile, nuclease-free, autoclaved DEPC-treated water (Carl Roth), and 25 µL of these 1 mg/mL glycans were mixed with 75 µL of coupling buffer. Next, the mixture was incubated with the activated beads for 2 h by overhead mixing as described above. To deactivate the remaining free binding sites, the beads were washed once with 500 µL quenching buffer (2x tris-buffered saline (TBS, Carl Roth) in ddH₂O), and subsequently incubated with 500 µL quenching buffer by mixing overhead for 30 min as described for the incubation steps above. Afterwards the beads were washed once with 500 µL blocking buffer (1x CarboFree Blocking Solution (Vector Laboratories, Newark, CA, USA) in sterile ddH₂O) and after exchange with fresh blocking buffer, blocked overnight at 4°C by overhead mixing. On the next day, the blocking buffer was exchanged one more time and the beads were adjusted to a concentration of 2.5 × 10⁶ beads/mL in 200 µL of blocking buffer. Until the use in the GMA, the glycan-coupled beads were stored at 4°C in the dark. The used glycans and their structures are listed in Table S1 according to the International Union of Pure and Applied Chemistry (IUPAC) and Symbol Nomenclature for Glycans (SNFG) [25]. As plasma background controls for the subsequent GMA, three regions were conjugated with PAA-aminoglucitol (AG; HOCH₂(HOCH₂)₅CH₂NH₂-PAA; described as open, reduced form of D-glucose by Huflejt et al. [26]) and additional three regions underwent the described coupling procedure with the addition of coupling buffer instead of glycan for the coupling step (“no glycan”).

2.3. Automated coupling procedure

The KingFisher Flex magnetic particle processor (Thermo Fisher Scientific, Waltham, MA, USA), equipped with a 96-well magnet and 96-deep well plates, was utilized for the automation procedure of coupling glycans to Luminex beads. For programming the steps performed by the particle processor, the above described manually operated protocol served as a template. The buffers, volumes, and incubation times were adopted without changes. Detailed descriptions of the protocol
developed with the BindIt software (version 4.0.0.45) are included in the supplementary material. For the run, the instrument was equipped with a bead comb and the plates (Thermo Fisher Scientific) containing the necessary buffers and beads as demanded by the program. An overview of the plate-specific buffer composition and procedure applied is listed in Table 1. The plate containing the activation buffer as well as the plate with the glycan dilutions were prepared fresh immediately prior to their processing. As a final step of the protocol for the automated coupling, the glycan-coupled beads were collected from the first washing step with blocking buffer and added to the plate containing the buffer for the overnight blocking step. The bead solutions were transferred from the 96-well plate to 1.5 mL Protein LoBind tubes (Eppendorf) and processed as described in the manually operated protocol up to the final adjustment for the bead concentration. In brief, the beads were blocked overnight at 4°C, washed once, and re-suspended in 200 µL of blocking buffer for storage.

2.4. GMA for the detection of anti-glycan antibodies

Antibody-containing liquids were mixed with assay buffer (1x CarboFree Blocking Solution in ddH2O) at a dilution of 1:40 (plasma) or 1:20 (ascites). CSF was used undiluted. Prior to the dilution step, thawed human body fluids (plasma, ascites, CSF) were spun at 2,100 g for 2 min to prevent debris from interfering in the assay. In case of the monoclonal antibodies, the following products were used: TAG-72 (5 µg/mL, clone B72.3), mouse IgG; Abcam, Cambridge, UK), SBH-Tn (5 µg/mL, clone 5F4, mouse IgM; SBH Sciences, Natick, MA, USA), anti-A (1:10, clone F98 7C6, mouse IgM; BAG Diagnostics, Lich, Germany) and anti-B (1:10, clones F84 3D6 and F97 2D6, mouse IgM; BAG Diagnostics). The blood group antibodies anti-A and anti-B were kindly provided by Dr. Conradi (University Medical Center of the Johannes Gutenberg University Mainz, Germany). The clone 1116-NS-19-9 (1:10, mouse IgG; Agilent Dako, Santa Clara, CA, USA) was kindly provided by Prof. Dr. Matthias Gaida (University Medical Center of the Johannes Gutenberg University Mainz, Germany). All incubation and wash steps were carried out at room temperature and 750 rpm on a horizontal shaker. 1000 glycan-conjugated beads per region and well were incubated with 100 µL diluted human body fluids or monoclonal antibodies for 1 h. Beads were washed twice with 150 µL wash buffer (PBS-0.1% Tween-20) and incubated for 55 min with 100 µL of species- and isotype-specific R-phycocerythrin (R-PE)-conjugated secondary antibodies in the following concentrations or dilutions: 0.25 µg/mL anti-human IgM-R-PE (2020-09; Southern Biotech), 3 µg/mL anti-human IgA-R-PE (2025-09; Southern Biotech), 3 µg/mL anti-human IgG-R-PE (127; Leinco Technologies, St. Louis, MO, USA), 1:1.000 anti-mouse IgM-R-PE (115-116-075; Jackson ImmunoResearch, Cambridgeshire, UK) and 1:50 anti-mouse IgG-R-PE (115-116-071; Jackson ImmunoResearch). The beads were washed again twice with wash buffer and re-suspended in 150 µL PBS, pH 7.5 for measurement of fluorescence intensity in the Bio-Plex 200 (Bio-Rad Laboratories) instrument. The median fluorescence intensity (MFI) was calculated by the Bio-Plex Manager Software version 6.1 (Bio-Rad Laboratories) based on the data collected of at least 100 beads per region and well. The signals obtainable with the Bio-Plex 200 range from 0 to ~ 33,000 MFI.

2.5. Data analysis

For data processing, analysis, and visualization, Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism Version 9.2.0.332 (GraphPad Software, Boston, MA, USA) were used. To obtain the “normalized” values visualized in the results section, the MFI value from a negative control (no plasma/ascites/CSF/antibody) collected for each bead region was subtracted from the samples’ MFI value of that same bead region. For comparison of different data sets, linear regression was applied to normalized values and the coefficient of determination (R²) was calculated.

3. Results

3.1. Workflow and application of the GMA

We applied the EDC/S-NHS conjugation chemistry for immobilizing a selection of 30 different glycan structures on individual magnetic bead regions. For the measurement of background signals in the subsequent GMA, additional six regions served as background control. The controls consist of three regions that were conjugated with PAA-AG and three regions that underwent the described coupling procedure without the addition of any glycan (“no glycan”). We initially observed that there were high variations of antibody reactivities in the GMA resulting from the type of plastic tubes that were used for the coupling procedure (data not shown). Optimizing the combination of plastic, chemicals, and buffer compositions, the final protocol is visualized in Fig. 1 and described in detail in the methods section. As a quality control for the coupling process, we conjugated PAA-AG and PAA-Tn, which were additionally labeled with a fluorescein, to the beads and could confirm with fluorescence microscopy analysis that the glycan constructs were indeed successfully coupled to the beads (Fig. S1).

The GMA can be used to detect anti-glycan antibodies in human body

| Table 1 Plate IDs, plate names, buffer compositions, and procedures applied to the plates used for the automated coupling procedure. |
|---|---|---|
| Plate ID | Plate name | Buffer composition |
| 1 | pre-wet | 100 µL activation buffer |
| 2 | COOH beads | 40 µL beads (stock: 1.25 × 10⁷ beads/mL) |
| 3, 4 | activation buffer I, activation buffer II | 60 µL activation buffer |
| 5 | activation plate | 100 µL activation buffer activation mix’ |
| 6-8 | coupling buffer I, coupling buffer II, coupling buffer III | 80 µL activation buffer |
| 9 | coupling plate | 10 µL EDC (50 mg/mL in activation buffer) |
| 10 | quenching buffer I | 10 µL S-NHS (50 mg/mL in activation buffer) |
| 11 | quenching buffer II | 100 µL coupling buffer glycan dilution: |
| 12 | blocking buffer I | 75 µL coupling buffer |
| 13 | blocking buffer II | 25 µL glycan (stock: 1 mg/mL in ddH2O) |

* - the chemical reaction taking place between the reagents is the most time-critical step; working time is minimized by preparation of a pre-mix consisting of the diluted chemicals and activation buffer.
Fig. 1. Workflow for measuring anti-glycan antibody reactivities in a bead-based approach. EDC/S-NHS cross-linking is applied for chemical conjugation of glycans to carboxylated, magnetic beads. Individual glycans are conjugated to distinct bead regions that are defined by their unique fluorescent spectral addresses (visualized as differently colored beads). In the first step of the GMA, the bead regions carrying individual glycans are mixed and incubated with antibody-containing liquids (e.g., human plasma). In a subsequent step, bound antibodies are detected by isotype-specific detection antibodies conjugated with a fluorophore (R1-PE). Beads are excited with a red 620 nm and a green 535 nm laser to discriminate the bead regions and to determine the presence or absence and the relative amount of the anti-glycan antibodies in the analyzed sample, respectively.

...fluids such as plasma, ascites, and CSF. One of the main advantages of this technology is that it requires minimal sample volume to generate large data sets. The Luminex xMAP technology is useful for these types of patient samples, which are often scarce and valuable, especially from individuals with diseases. In the GMA, a bead mix of glycan-coupled magnetic Luminex beads is incubated with antibody-containing liquids to generate isotype-specific anti-glycan antibody signatures. The optimal dilutions for the highest values and best signal-to-noise ratios may vary depending on the type of body fluid being analyzed. Dilution studies of plasma (representative data for three plasma samples is depicted in Fig. S2), ascites, and CSF resulted in final dilutions of 1:40, 1:20, and undiluted, respectively (data on dilution studies for ascites and CSF not shown). To compare anti-glycan antibody signatures of different individuals, IgM, IgG, and IgA reactivities to 30 individual glycans were measured in plasma from 10 individual healthy donors (donors 1-10, Fig. 2A-C). Although every donor showed an individual anti-glycan antibody profile, some general trends could be observed. Comparing the different isotypes of the anti-glycan antibodies, all three isotypes recognized similar groups of glycans. Most of the reactivities were found to be of the IgM isotype, which, e.g., showed strong binding to the basic globoside P α antigen (Gb3, CD77) and the core structures Core 3-Core 6, with Core 5 as the most prominent glycan. Also to the well-described Galili (known as α-Gal epitope) antibody reactivities were found in several plasma samples throughout all three isotypes. On the contrary, low antibody reactivity was measured to the Lewis blood group structures Le a and Le b, and no reactivity to Le x, Le x, and Le y, as well as to several sialic acid-containing glycans, independent of the isotype. An exceptional Lewis structure seems to be Le y, a precursor of Le a, to which antibody reactivities of all isotypes could be detected. Both plasma and serum are immunoglobulin-containing products of whole blood processing. The procedures to obtain the two sample materials are different and the choice of material depends on the intended analysis. EDTA-plasma is commonly used for diagnostics, and therefore the GMA was developed for the analysis of human plasma. However, to determine whether in the future serum could also be used for anti-glycan antibody analysis, plasma and serum from the same donor were directly compared in the GMA and showed similar anti-glycan antibody patterns as well as signal intensities (data not shown). In addition to antibodies in plasma, we were able to use the GMA to show the presence of anti-glycan antibodies in ascites from three ovarian cancer patients (donors 11-13) as well as in CSF from three Parkinson’s patients (donors 14-16, Fig. 2A-C). As observed for plasma, anti-glycan antibody reactivities of the ascites samples showed patient- and isotype-specific patterns. In contrast to plasma and ascites, only very low anti-glycan antibody reactivities could be detected in CSF, independent of the isotype that was analyzed. Additionally, the CSF had to be used as pure fluid after dilution studies had revealed dose-dependent signal decrease down to undetectable reactivities (data not shown). Although the low number of samples from ascites and CSF (n=3) are not suited for detailed biological interpretation of the anti-glycan antibody profiles, it demonstrates that the GMA in general facilitates the generation of anti-glycan antibody profiles in human body fluids other than plasma. Besides the analysis of human body fluids, the GMA allows the analysis of the specificity and cross-reactivity of monoclonal anti-glycan antibodies. Five different commercially available monoclonal antibodies (mAbs) to different glycans were screened with the GMA (Fig. 2D). The TAG-72 antibody (clone B72.3) was originally generated against a membrane-enriched extract of human metastatic carcinoma, and was later identified to bind the oncofetal mucin-like tumor-associated glycoprotein (TAG)-72 [27]. We were able to detect binding of the TAG-72 antibody to STn, but also to its precursor Tn and the disaccharide Core 6. The commercial SBH-Tn antibody (clone 5F4) exhibited reactivity towards the Tn monosaccharide as expected. Like the TAG-72 antibody, the SBH-Tn antibody also bound to Core 6. Two anti-glycan monoclonal antibodies that were tested in the GMA, the anti-blood group antibodies anti-A and anti-B, showed binding exclusively to the corresponding trisaccharides (A α or B α) without detectable reactivity to any of the other tested glycans.

With the detection of SLe a using the anti-CA19.9 antibody 1116-NS-19-9, we could demonstrate the reactivity to another sialic acid-containing glycan. This antibody did not react with any other glycan in the panel, not even with the structurally similar tetrasaccharide Sialyl-Le a (SLe a) or with Le a as the precursor of SLe a. Overall, we could demonstrate various applications of the GMA, and with this, its potential for the detection of different anti-glycan isotypes in different body fluids.

3.2. Automation of the coupling procedure
The described GMA allows the analysis of anti-glycan antibody reactivities to 30 glycans at the same time. Depending on the type of read-
out device, this number can be increased to up to 500 different glycans conjugated to distinct bead regions. Furthermore, unlike glycan arrays, for which up to several hundred glycans are printed on one slide, the analytes of interest in the bead-based approach can be easily customized to the aim of the analysis. By addition or removal of glycans from the panel, the platform can be adapted to the detection of anti-glycan

**Fig. 2. Applications of the GMA.** (A-C) Heat maps visualize the signatures of anti-glycan antibodies against the indicated molecules detected in different human body fluids. Signatures from plasma of healthy donors (n=10), ascites of ovarian cancer patients (n=3) and cerebrospinal fluid (CSF) of Parkinson’s patients (n=3) are shown. The relative binding strength of the isotypes (A) IgM, (B) IgG, and (C) IgA were determined in median fluorescence intensity (MFI). (D) Binding profiles of the monoclonal antibodies (mAb) TAG-72 (directed mainly to Sialyl-Tn), SBH-Tn (Tn), two blood group antibodies, anti-A and anti-B, and 1116-NS-19-9 (Sialyl-Leα). undil. – undiluted.

**Fig. 3. Automation of the coupling procedure accelerates the process.** (A) A reverse bead processing principle is applied by the KingFisher system. The 96-well magnet head (dark grey) picks up a comb to protect the magnet pins. The comb is inserted into the 96-deep well plate 1 to collect the magnetic beads. The beads collected on the magnet are subsequently transferred to plate 2. By removal of the magnet from the comb, the beads are released to the solution, and the comb is used for the mixing of the beads in the buffer of plate 2. (B) Estimated hands-on times for manual and automated coupling. The three data points for each coupling procedure are based on our experience. The times necessary for increased numbers of processed bead regions were extrapolated (dashed line). (C-E) Correlation of reactivities obtained for anti-glycan antibodies with beads that were coupled manually or with the automated method. Each graph shows 320 signals obtained from 10 plasma samples for 30 glycans (+ background controls) for the human isotypes IgM, IgG, and IgA as indicated in the graph. Linear regression was applied and the coefficient of determination (R²) was calculated for each isotype as stated in the graphs. MFI – median fluorescence intensity.
antibodies associated with a broad range of diseases. An increase in the number of analytes throughout the establishment of the method did not result in a strong increase of the GMAs’ processing time, whereas the preceding manual coupling procedure (as described in detail in the methods section) reached its limitations at some point. When a sample size of around 30-35 regions was exceeded, it appeared to be difficult to retain the established incubation times for processing all samples identically without additional support. This limitation was one of our motives to develop an automated coupling process. In addition, we aimed to reduce hands-on time and to establish a time-efficient procedure with reproducible output. To establish such an automated procedure, we used the KingFisher Flex magnetic bead processor (KingFisher), which applies a reverse bead processing principle as visualized in Fig. 3A. A magnetic head with 96 individual rods is covered with a plastic comb and inserted into the plate containing the magnetic beads (Plate 1). After collection of the beads, the magnet and the comb collectively transfer the beads into the next plate (Plate 2). By removal of the magnet from the comb, the beads are released into the liquid. The ability to program speed and duration of the vertical movement of the comb allows sufficient agitation for resuspension of the beads. For programming the KingFisher protocol, the BindIt software for KingFisher instruments was used. Throughout the optimization process of the KingFisher protocol, we experienced some difficulties that affected the output of the run. Low bead recovery was one of the most noteworthy problems we encountered. When performing the original manual coupling protocol, the respective buffers are exchanged but the beads remain in the vials at all times. In contrast, when using the automated KingFisher procedure, the beads are transferred several times between plates during the run, owed to the fact that the instrument performs reverse bead processing. At first, this resulted in the retention of a clearly visible amount of beads in the plates accompanied with a low bead recovery. As the process is intended for high-throughput, we refrained from determining the bead count of each individual region. A visual inspection of the plates after the run of the protocol served to determine the success of the bead transfer between the plates. We addressed low bead recovery by introducing a second collection step after each bead transfer. In short, the beads are collected from plate 1 and after their release into plate 2, the magnet returns to plate 1 for an additional bead collection. Herewith, we could obtain comparable bead recovery between the automated and manual bead processing.

Additionally, we observed that a well-mixed bead suspension was necessary at any time during the KingFisher run to ensure good bead recovery. Because the beads start to settle relatively quickly, it is essential to mix the beads shortly before starting the KingFisher run, especially when handling a high number of bead regions in parallel. A mixing of the bead plate by horizontal shaking revealed to be inefficient. Agitation of the beads by manually pipetting up and down with a multichannel pipette is inevitable. Moreover, the protocol includes programmed pauses to manually insert, e.g., the plate containing the activation mix or the prepared glycan dilutions. These pauses should be kept as short as possible. The required programmed pause for adding the activation plate originates from the need for a freshly prepared activation mix, which contains the moisture sensitive cross-linking agent EDC. This requirement makes the activation step the most time critical and, in case of the manually performed protocol, also the most limiting factor. While the activation mix can be added to the 96-well KingFisher plate with a manual multichannel pipette fast and efficiently, each vial has to be handled individually when performing the fully manual coupling procedure. Therefore, when the number of 30-35 parallel processed bead regions is exceeded, the protocol for manual coupling requires repetitive executions. The consequential increase in hands-on time is considerable. Hands-on time for both procedures raises linearly with the sample number, but the slope of the line for the automated procedure is much smaller (Fig. 3B). Therefore, the benefit for just 32 bead regions is rather small (160 min vs. 120 min) but increases to a difference of 210 min (460 min vs. 250 min) for 96 regions. For this calculation, we did not take into consideration the gradually occurring mental exhaustion of the operator, which occurs faster when performing the procedure manually, and the additional time gained by the operator during the automated coupling run. Both facts are harder to quantify than hands-on time, but should not be disregarded. To assess the quality of the beads coupled with the KingFisher, a GMA was performed with glycan-coupled beads resulting from both methods, manual and automated. Plotting the data from the manual coupling procedure against the data from the automated coupling procedure resulted in R² values of 0.97, 0.93, and 0.95 for IgM, IgG, and IgA respectively, indicating a very high correlation between both methods for coupling of glycans to magnetic beads (Fig. 3C-E; data representation with individual bar graphs for each glycan and donor from manual and automated couplings is depicted in Figs. S3–S5).

3.3. Automation results in reproducible anti-glycan antibody reactivities

In addition to the benefit of a reduced hands-on time, the automated coupling is potentially less error-prone than the manual handling, as a programmable device carries out the major steps. This contributes to the outperformance of automated over manual processes regarding reproducibility. To evaluate if the established automated procedure results in reproducible data output, we repeatedly performed the automated coupling of glycans to magnetic beads. A GMA was performed with the beads from the different couplings to measure anti-glycan antibody reactivities of the isotypes IgM, IgG, and IgA (Fig. 4). This included beads from performances of the protocol in different runs on the same day (intra-day comparison, Fig. 4A-C) and in individual runs on different days (inter-day comparison, Fig. 4D-F). For the intra-day comparison, we conjugated one set of 36 bead regions with individual glycans in one run (Fig. 4A-C, set #1). In a succeeding second run, two sets of 36 bead regions, and with that 72 regions in total, were processed in parallel (Fig. 4A-C, set #2 and set #3). Black circles represent data points from the GMA with glycan-coupled beads generated in separate KingFisher runs (set #1 vs. set #2), and grey circles from the GMA with beads coupled during the same run (set #2 vs. set #3). Very high reproducibility of the protocol is outlined by coefficients of determination > 0.99 when correlating data from intra-day coupleings, regardless of whether the beads were processed during the same run or succeeding runs (Fig. 4A-C). A similar high reproducibility was observed for the entirely independent inter-day comparison (black squares), represented by R² values between 0.96 and 0.94 for all isotypes (Fig. 4D-F).

4. Discussion

In this work, we report the automation of a procedure to chemically conjugate glycans to color-coded magnetic beads. The resulting glycan-coupled beads can subsequently be used for the detection of anti-glycan antibodies in different body fluids as well as for the analysis of the specificity and cross-reactivities of mAbs directed to glycan targets. A great advantage of the bead-based approach is the flexibility to upscale the GMA depending on the scientific question and experimental requirements. While the coupling of glycans to Luminex beads for analysis of anti-glycan antibodies has been described by others [21,22], to our knowledge, no protocol for the automation of the coupling process has been published so far.

With the application of different methods, such as ELISA, microarray, and bead-based approaches, polyclonal anti-glycan antibodies can be detected in the plasma or serum of both healthy and diseased individuals. Despite the fact that anti-glycan antibody profiles are known to be very donor-specific, observations regarding common occurrence of antibodies to certain glycans have been described in literature. Amongst them are antibodies to the core structures, Le₄, GalI, and the P₄ antigen, which are all glycans to which we also observe reactivities in most donors [26,28,29]. In addition, the dominance of IgM amongst the isotypes of antibodies has been described [7]. This is in
and advanced stage Parkinson antibody patterns larger cohorts would need to be analyzed. Of course, for the interpretation of individual anti-glycan our knowledge, the analysis of anti-glycan antibodies in CSF has not with CSF/serum IgG ratios of 0.0027 and 0.0039, respectively [33]. To detect antibodies to glycans with the GMA in other body fluids could contribute to new discoveries of prognostic and diagnostic tools in this field. With the analysis of ascites and CSF, we demonstrated the feasibility to detect antibodies to glycans with the GMA in other body fluids than plasma. Of course, for the interpretation of individual anti-glycan antibody patterns larger cohorts would need to be analyzed.

Next to human body fluids, we tested monoclonal anti-glycan antibodies in the GMA. The mAbs served as potential positive controls for individual glycans but also allowed their characterization regarding specificity and cross-reactivity. While the blood group antibodies anti-A and anti-B as well as the SLeα antibody 1116-NS-CA19-9 showed very high specificities, we detected some cross-reactivities of the TAG-72 and SBH-Tn antibodies. The mAb TAG-72 was originally generated against a membrane-enriched extract of human metastatic carcinoma, and was identified to bind TAG-72, an oncofetal mucin-like glycoprotein [27]. Early studies of the clone describe a preferred binding to Tn, but additional binding to Tn has also been reported [34]. Several additional saccharides have been identified to be (patho)physiologically conjugated to TAG-72, including the Lewis structures Leα, Leβ, Lea, and SLeα. Published analysis of the TAG-72 antibody did not show binding to purified glycolipids with any of the specified Lewis structures [35]. In our analyses, we also did not detect any antibody reactivity to these Lewis structures. To our knowledge, binding of the TAG-72 antibody to the disaccharide Core 6 has not been reported before. The cross-reactivity to Core 6 might be caused by the fact that the disaccharide Core 6 is composed of the Tn antigen with an additional monosaccharide (Table S1). The structural similarities between Core 6 and Tn might also explain the cross-reactivity to Core 6 that we observed for SBH-Tn. In general, most of the core structures included in the GMA panel (Core 2 to Core 6, and Core 8) have not yet been studied as TACAs like TFα (Core 1), Tn, and STn, making it difficult to interpret our detected antibody reactivities. However, our findings that monoclonal antibodies to certain glycans show cross-reactivities to other glycan structures are in agreement with bead-based- and glycan microarray analyses performed by other groups [21,36]. These observations are highly valuable and contribute to the characterization of monoclonal antibodies, which is especially important for those antibodies that are intended for clinical applications.

Both the characterization of monoclonal antibodies and the presence of anti-glycan antibodies in three different body fluids show the potential broad applications of the GMA. As the assay was established with the aim to screen cancer patient cohorts, the current glycan panel includes mainly TACAs or structurally similar saccharides. However, there are numerous additional and overlapping human and non-human glycan structures associated with infectious, neurological, and other diseases.
To be able to further increase the throughput of the assay, the coupling procedure for the conjugation of glycans to Luminex beads was automated. Furthermore, we aimed to reduce hands-on time and to establish a time-efficient procedure with reproducible output. High intra-day and inter-day reproducibility could be demonstrated, thereby emphasizing the advantage of reduced error rates in automated protocols (Fig. 4). In addition, this protocol allows a simple upscaling of the panel to up to 96 bead regions (equal to 96 different glycans) in one run. Two subsequent coupling runs can theoretically be performed within the timeframe of a regular workday, resulting in a throughput of 192 individually glycan-coupled bead regions per day. One limitation of the present study is the small number of individual glycans that were analyzed. It is very likely that both the mAbs as well as the polyclonal antibodies in the body fluids bind to glycans that are not included in the panel. Since we have now successfully established the automated coupling procedure, our next step is to expand the glycan panel to increase the content of the GMA.

As part of the continuous development and deeper characterization of the GMA, we are constantly obtaining and testing additional monoclonal antibodies, which are described to bind glycans that are included in the GMA panel. Next to monoclonal antibodies, lectins are well-known and broadly applied glycan-binding proteins. There are great attempts to gain detailed insights into the binding specificities of single lectins by combining glycan microarray data with machine-learning approaches [40]. The individual binding specificities are, however, not yet fully explored and unanticipated cross-reactivities cannot always be excluded. As the GMA was developed for the investigation of anti-glycan antibodies, we believe the application of monoclonal antibodies to be the most suitable tool for the validation of the successful glycan-to-bead coupling.

The manual as well as the automated protocol are not only limited to the conjugation of glycans but also allows the coupling of proteins, such as antibodies, as well as peptides to the carboxylated Luminex beads. Wakeinan et al., e.g., conjugated different peptides to differently color-coded Luminex beads applying the EDC/S-NHS crosslinking approach for subsequent detection of antibody responses to Plasmodium falciparum, the parasite responsible for malaria, in human plasma [41]. Minor adaptations to those types of protocols would most likely allow straightforward automation supported by the protocol described here. Altogether, we report for the first time the successful process automation for the coupling of glycans to carboxylated magnetic beads. The method is highly flexible and reproducible and can be used with small sample volumes to generate large data sets. The automated coupling process can be adapted for the conjugation of additional types of amine-carrying biomolecules, making this protocol valuable for additional research fields.

Contributors
A.K.H. – planned and carried out the experiments and wrote the manuscript in consultation with S.M.M.H., C.V., A.R., and U.S.
S.M.M.H. – contributed to the design and implementation of the research, to the analysis of the results, and to the writing of the manuscript
C.V. – aided in interpreting the results and worked on the manuscript
A.R. – contributed to the design and implementation of the research, to the analysis of the results, and to the writing of the manuscript
U.S. – aided in interpreting the results and worked on the manuscript
All authors discussed the results and commented on the manuscript

Declaration of Competing Interest
The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ugur Sahin reports a relationship with BioNTech SE that includes: board membership, employment, and equity or stocks. Sanne Maria Mathias Hensen reports a relationship with BioNTech Diagnostics GmbH that includes: employment and equity or stocks.

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Supplementary materials
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