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Research paper

PEGylation of microbead surfaces reduces unspecific antibody binding in glycan-based suspension array

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ABSTRACT

Glycan-based suspension array (SGA) is an "in-house" developed multi-target immunoassay, employing commercially available fluorescent microbeads as a solid support for unique chemically synthesized glycopolymers which capture naturally occurring human anti-glycan antibodies. SGA is a sensitive and reliable tool for the high-throughput screening of anti-glycan antibody alterations characteristic for a vast number of human diseases including cancer. However, unspecific background binding, for instance binding of non-target antibodies, is a common obstacle in such immunoassays. In an attempt to reduce unspecific background binding of serum (or plasma) antibodies, we prepared glycosylated microbeads modified with linear poly(ethylene glycols) (PEGs) of different lengths. We compared several kinds of PEG modifications: (a) partial side-chain substitution of glycopolymers by PEGs of different lengths, (b) end-point addition of biotin-linked PEGs to glycopolymer-coupled beads, and (c) linking of heterobifunctional PEGs to the bead surface prior to glycopolymer immobilization. Among the various modifications investigated, the direct modification of the bead surface with linear heterobifunctional PEGs, consisting of 23- and 60 PEG-units significantly reduced the background binding. The end-point addition of biotin-linked PEGs, especially in the case of PEG consisting from 50 PEG-units, helped to repel non-target binding caused by endogenous biotin. We observed unspecific binding predominantly for antibodies of IgG but of IgM class. The novel design of fluorescent microbeads allows the detection of human anti-glycan antibodies with increased specificity and opens new horizons for practical application of SGA as a diagnostic tool.

1. Introduction

Ovarian cancer is the fifth most common cause of death from all cancers occurring in women and the leading cause of death from gynecological malignancies (Ozols, 2006). This poor outcome (overall survival of less than 20%) results from the lack of early disease-specific symptoms and reliable tools (e.g. tumor markers) for early diagnosis,

Abbreviations: A_{tri}, A trisaccharide; B_{tri}, B trisaccharide; biot, biotin; biot-PEG_m, biotinylated PEG, where $_m$ = number of PEG units; Glyc, glycan; ELISA, enzyme-linked immunosorbent assay; LacNAc, lactosamine; MFI, median fluorescence intensity; PAA, polyacrylamide; PEG_{23}, biotin-PEG_{23}-NH_2; PEG_{50}, biotin-PEG_{50}-NH_2; PGA, printed glycan array; α-Rha, α-rhamnose; R-PE, R-phycoerythrin.

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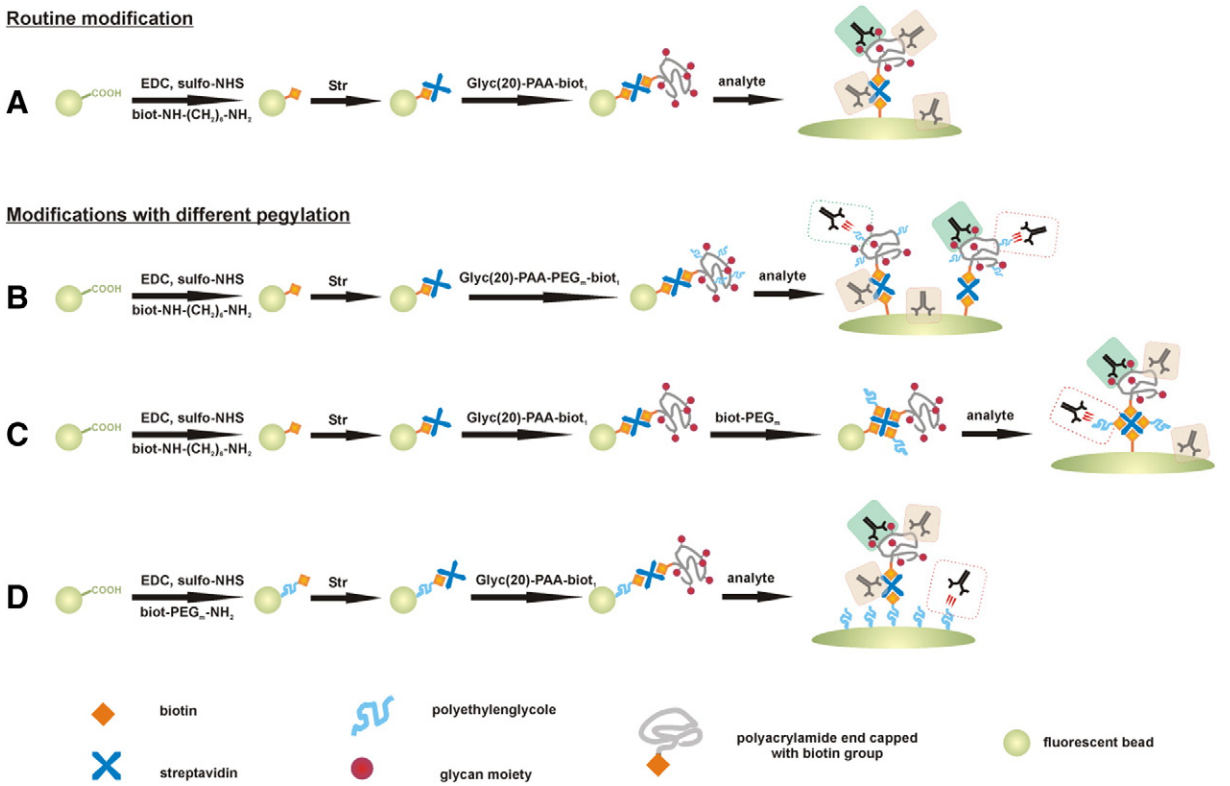
from ineffective therapy for advanced disease, and from the limited understanding of the early-initiating events and early stages of ovarian cancer development.

For the search and the identification of sensitive and ovarian cancer-specific tumor biomarkers we have previously developed "in-house" and then validated a multiplex suspension array specifically designed for the high throughput detection and profiling of anti-glycan antibodies naturally occurring in human blood serum/plasma for diagnostic purposes (Pochechueva et al., 2011a).

Our first version of the SGA employed chemically synthesized glycans in the form of end-biotinylated polyacrylamide conjugates (Chinarev et al., 2010) coupled to commercially available fluorescence-labeled microbeads, allowing the specific multivalent binding of anti-glycan antibodies or lectins to the immobilized glycopolymers. The set of glycans included P_1 (Galα1–4Galβ1–4GlcNacβ), a trisaccharide which we have previously identified using PGA as significantly associated with ovarian cancer (Jacob et al., 2012). We found that the SGA, when compared to the PGA, exhibited a similar or, in some cases, even higher sensitivity and specificity in the detection of plasma anti-glycan antibodies (Pochechueva et al., 2011b; Jacob et al., 2012), which is one benefit of SGA. The other benefits of SGA are the opportunity to assess multiple analytes in a single sample, the wide dynamic range, the feasibility of the assay reconfiguration, and the minute consumption of glycans and glycan-binding proteins, making SGA an attractive tool for biomedical and diagnostic applications.

A crucial step for the quality/performance of the SGA is the immobilization of the glycoconjugates to the fluorescent microbeads. In our previous study (Pochechueva et al., 2011a) we have compared several approaches for the immobilization, and found that the multi-step coupling procedure, i.e. the anchoring of streptavidin to the beads and the subsequent attachment of the polyacrylamide-based glycopolymer end-capped with single biotin, was the most appropriate strategy for the specific binding of serum/plasma-derived antibodies. This "sandwich construct" (Scheme 1A) is rather complex but stable and well-suited for highly sensitive detection of specific interactions between glycans and glycan-binding antibodies (Pochechueva et al., 2011a,b).

However, unspecific or non-target interactions between analytes and glycopolymers (and even microbeads) can naturally occur in immunoassays such as SGA due to the high complexity of the analyte sample of interest (human serum/plasma or other body fluids) and the characteristics of the employed microbeads. For instance, in addition to binding to glycans, serum/plasma antibodies may also recognize other sites on the surface of beads or even adhere to beads in a purely unspecific way. Due to the large heterogenic interface antibodies may bind to unmodified portions of the bead



Scheme 1. Schematic presentation of the routine "sandwich construct" (A), the three kinds of PEG-modified glycobead (B–D) preparations, and the various types of antibody-bead interactions. Specific (filled turquoise rectangles) binding and unspecific (filled red rectangles) binding of antibodies to glycan moieties are shown. Prevention of unspecific binding is highlighted by a red-framed transparent rectangle.

surface or to on-surface non-carbohydrate, i.e. streptavidin and polyacrylamide, molecules in a non-immunological fashion, i.e. via hydrophobic or electrostatic interactions. So-called heterophilic antibodies (Kricka, 1999; Martins et al., 2006; Waterboer et al., 2006) may be engaged in the fine regulation of the immune system and are believed to bind, though with low affinity, to a variety of antigens such as self-antigens or even purely synthetic molecules. Unspecific interactions, in particular those arising by heterophilic antibodies (Levinson and Miller, 2002; Bjerner et al., 2005; Preissner et al., 2005), are likely to increase the background signal and to fail in the detection of low-affinity interactions between glycans and anti-glycan antibodies. This negatively affects the SGA outcome (specificity and sensitivity) and complicates the interpretation of the SGA results, eventually producing false-positive and negative or over- or understated results and therefore compromising the reliability of SGA.

Avoiding or at least minimizing unspecific interactions/binding of antibodies is considered essential for the design of glyco-analysis tools. Two common strategies can be utilized (Ratner, 2005). (i) The analytical platform (e.g. bead surface) is covered with a dense monolayer of antigens or glycans. However, antibodies may be incapable of tight binding to target glycans constituting such monolayers due to the suboptimal surface density of glycan residues and to the length of their bonds to the surface. (ii) Parts of the analytical platform remain unoccupied by the glycans and are blocked (masked) by a detergent, a protein or a synthetic polymer such as poly(ethylene glycol) (PEG), a linear or branched polyether terminated with hydroxyl groups. This strategy is based on the protein-repelling effect of PEG due to the low free energy at PEG–water interface, incapability of hydrogen bonding or electrochemical interaction of PEG with proteins, and to the high mobility of PEG chains (Kingshott and Griesser, 1999). The particular characteristics of PEG, including its water like-structure, absence of charges, resistance to protein adsorption, variation in molecular weight, size (length) and shape, and low immunogenicity make PEG not only suitable for biomedical and therapeutic applications (Desai and Hubbell, 1991; Prime and Whitesides, 1991; Bergstrom et al., 1992; Roberts et al., 2002; Caliceti and Veronese, 2003; Larsson et al., 2007; Fishburn, 2008; Wattendorf et al., 2008a,b; Jain and Nahar, 2010; Jokerst et al., 2011), but also ideal molecules in the design of SGA and related tools.

In the latter context, bifunctional PEG tags were recently used as protein-repelling spacers for glycan primers. These glycoPEG tags were conjugated to latex fluorescent beads and these glycoPEG-functionalized beads were shown to bind to a lectin array with higher sensitivity and selectivity than glycan beads without PEG tag (Etxebarria et al., 2013). PEG linking appears to be a suitable strategy, although it can in some circumstances (PEG of inappropriate length, density or orientation) also cause spatial masking (steric interference) of specific antigen epitopes and therefore result in the loss of binding potency (Fishburn, 2008).

The main objective of the present study was the optimization of our current/first version of SGA through the reduction/minimization of unspecific (background) antibody binding. We explored (i) how the modification of the beads with linear PEGs of different lengths can influence both specific and unspecific antibody binding and (ii) which of these modifications reduce unspecific binding. We demonstrate that unspecific antibody binding was significantly reduced by the direct modification of the bead surface with linear heterobifunctional PEG consisting of 23- and 60 PEG-units and by avoiding the employment of IgG-class antibodies.

2. Materials and methods

2.1. Antibodies

The following antibodies from the indicated suppliers were used: anti-P_1 human monoclonal IgM antibody (clone P3NIL100, Immucor Gamma GmbH, Rödemark, Germany, dilutions used: 1/200; 1/500; 1/1000; 1/2000; 1/5000; and 1/10,000); anti-Gb3 (CD77, P^k) murine IgG2b (clone BGR23, Seikagaki Biobusiness Corp., Tokyo, Japan, dilution of 1/100); anti-Gb3 (CD77, P^k) rat monoclonal IgM (AbD Serotec, Oxford, England, dilution of 1/100); biotin mouse anti-rat IgM (BD Pharmingen™, BD Biosciences, Allschwil, Switzerland, dilution of 1/1000); R-phycoerythrin (R-PE)-streptavidin (LubioScience GmbH, Luzern, Switzerland, dilution of 1/200); goat anti-human R-PE conjugated Ig (H + L), IgM or IgG antibodies (dilution of 1/1000) and goat anti-mouse Ig (H + L) antibodies (dilution of 1/1000, Southern Biotechnology Associates, Inc., Birmingham, AL, USA).

2.2. Human plasma samples

Blood samples were collected prospectively from healthy women at the Department of Gynecology, University Hospital Zurich after informed consent was granted (ethical approval to V.H.S., SPUK Canton of Zurich, Switzerland). Specimens were processed and stored as described previously (Pochechueva et al., 2011b; Jacob et al., 2012). Human plasma samples were used in all the experiments in the dilution of 1/40, as described previously (Pochechueva et al., 2011a). Plasma samples from healthy donors of blood groups A, B and O (five donors each group) were pooled in equal volumes and used similarly to individual plasma samples.

2.3. Glycopolymers with end-biotin group

The glycopolymers, Glyc(20)–PAA–$biot_1$, Glyc(20)–PAA–$PEG_4(80)$–$biot_1$, and Glyc(20)–PAA–$PEG_m(5)$–$biot_1$, used for coupling to fluorescent beads were produced in-house as previously described (Chinarev et al., 2010); their chemical structures are presented in Fig. 1. The glycopolymers are composed of a polyacrylamide carrier (PAA, number of the average polymerization degree, n = 220) provided with end biotin groups and side-pendant Glyc residues, either Galα1–4-Galβ1–4GlcNAcβ– (referred to as P_1) or Galα1–3(Fucα1–2)Galβ– (referred to as B_{tri}) that are statistically distributed along the polymer backbone. The content of monomer units with glycan substitution is 20 mol%. Non-glycosylated monomer units are substituted either with an ethanolamine residue for the regular Glyc(20)–PAA–$biot_1$ or with an tetraethylene glycol residue for Glyc(20)–PAA–$PEG_4(80)$–$biot_1$. For Glyc(20)–PAA–$PEG_m(5)$–$biot_1$, 5 mol% of non-glycosylated monomer units are conjugated with long PEG chains, m ~ 50 (MW ~ 2.2 kDa) or

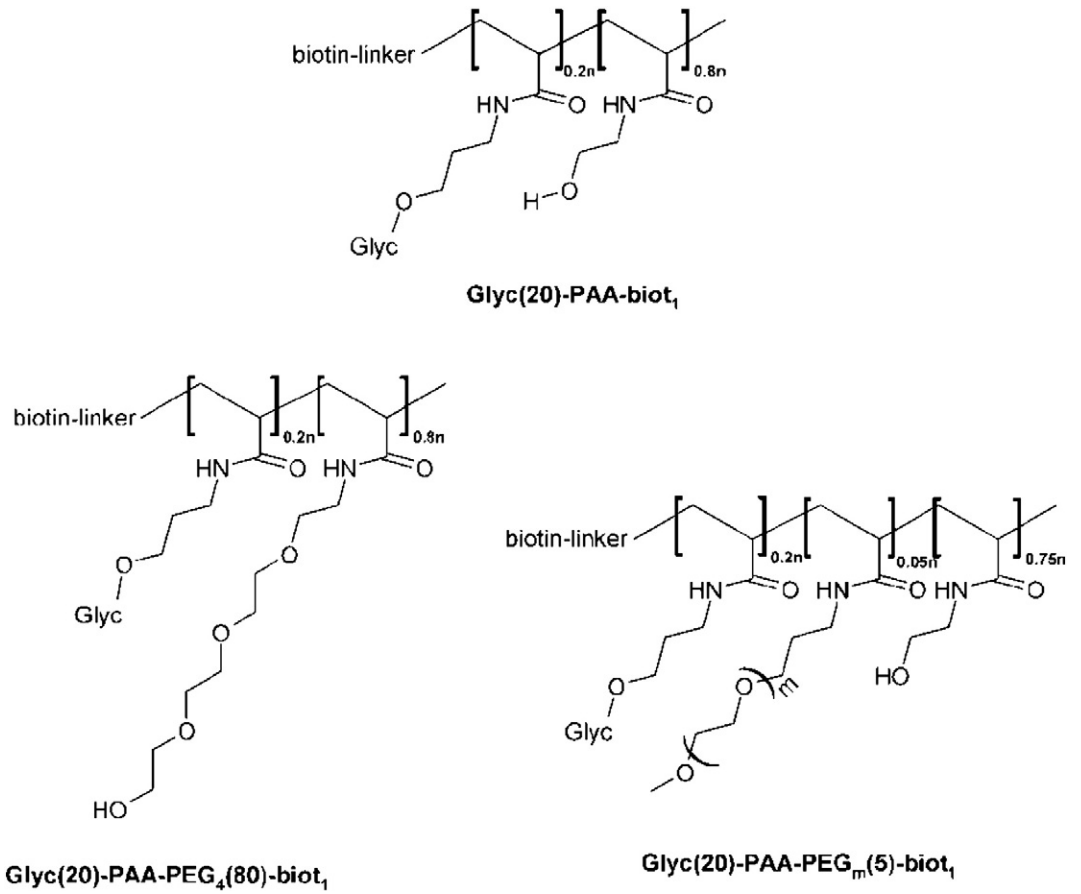


Fig. 1. Chemical structure of the regular glycopolymer (top) and its newly synthesized PEGylated analogs (bottom).

280 (MW ~ 12.2 kDa), whereas the all the other units are substituted with ethanolamine.

2.4. PEGs used for glycopolymer and microbead modifications

Biot-PEG_m were produced in-house by ligation of biotin–$NH(CH_2)_5COONp$ (Lectinity Holdings, Moscow, Russia) with the PEG-amines, $NH_2CH_2CH_2CH_2(OCH_2CH_2)_mOCH_3$, m ~ 50 (MW ~ 2.5 kDa) or 280 (MW ~ 12.5 kDa), which were purchased (NDF Corp, Tokyo, Japan). The chemical structure of biot-PEG_m is presented in Fig. 2A.

Hetero-bifunctional PEGs (biot-PEG_m-NH_2) were purchased (Iris Biotech GmbH, Marktredwitz, Germany). Biot-PEG_{23}-NH_2 was the individual compound (MW = 1300), whereas biot-PEG_{60}-NH_2 was a polymer with MW ~ 3.0 kDa (Fig. 2B).

2.5. Modification of fluorescent microbeads

2.5.1. The regular coupling procedure

Biotinylated glycopolymers were coupled to fluorescent Bio-Plex Pro™ magnetic COOH beads of 6.5 μm diameter with distinct spectral "addresses" (Bio-Rad Laboratories Inc., Hercules, CA, USA). Each bead's region was embedded with a precise ratio of red and infrared fluorescent dyes allowing its identification using a Bio-Plex 200 suspension array system (Bio-Rad Laboratories Inc., Hercules, CA, USA). Coupling of biotinylated glycopolymers was accomplished similarly to the procedure developed for non-magnetic Bio-Plex carboxylated beads (Pochechueva et al., 2011b). Briefly, the stock vial of microspheres (1.25×10^7 microspheres/ml) was vigorously vortexed for 30 s and sonicated for 15 s in a water bath prior to its use. The tube with bead suspension (1 scale reaction: 100 μl; 1.25×10^6 microspheres) was placed into a magnetic separator (DynaMag™-2, Life Technologies, Zug, Switzerland) for 30–60 s and the supernatant carefully removed. The pellet was resuspended in bead wash buffer (100 μl; Bio-Plex amine coupling kit, Bio-Rad Laboratories Inc., Hercules, CA, USA) by vortexing and sonication, and applied for magnetic separation as described above. After gentle removal of supernatant, the pellet was resuspended in 80 μl of bead activation buffer (Bio-Plex amine coupling kit, Bio-Rad Laboratories Inc., Hercules, CA, USA), vortexed and sonicated. Sulfo-N-hydroxysuccinimide sodium salt (S-NHS) and 1-ethyl-3-[3,3-dimethylaminopropyl]carbodiimide hydrochloride (EDC; Pierce Biotechnology, Rockford, IL, USA, both 50 mg/ml in activation buffer) were prepared immediately prior to use, and 10 μl of each solution was added to the bead suspension, followed by vortexing for 30 s. Beads were agitated in the dark on a rotator at room temperature for 20 min. The activated beads were applied for magnetic separation and supernatant was removed. The pellet was resuspended in 150 μl biotin-solution (0.1 M $NaHCO_3$, pH 8.3,

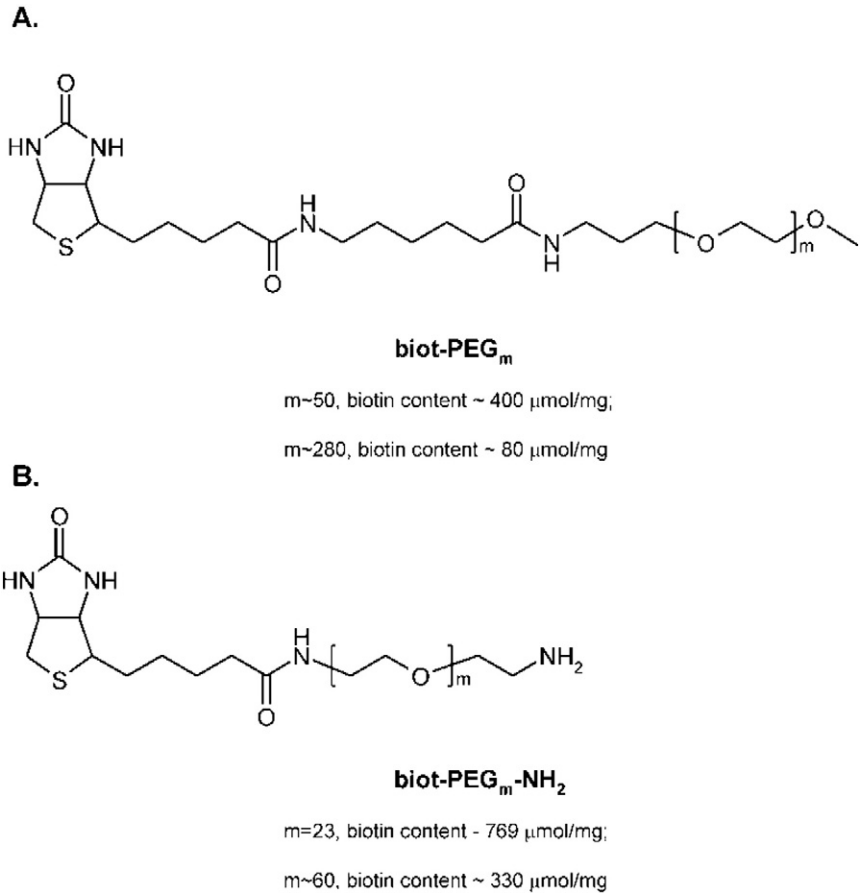


Fig. 2. Biotinylated PEGs (biot-PEG$_m$, where m = 50 or 280) (A) and heterobifunctional PEGs (biot-PEG$_m$-NH$_2$, hereafter PEG$_{23}$ and PEG$_{50}$) (B) used for coupling to fluorescent beads.

containing 1 μg (≈2 nmol) of biotin–NH(CH$_2$)$_6$NH$_2$, Lectinity Holdings, Moscow, Russia) and agitated in the dark on a rotator at room temperature for 2 h. Obtained biotinylated beads were pelleted by magnetic separation and resuspended in 150 μl of 50 mM ethanolamine (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) in 0.1 M NaHCO$_3$, pH 9.0 to quench unbound activated groups. Beads were agitated in the dark on a rotator at room temperature for 30 min. After magnetic separation the pellet was washed twice with 500 μl PBS, pH 7.4 and resuspended in streptavidin-solution (400 pmol streptavidin in 150 μl PBS; Bio-Rad Laboratories Inc., Hercules, CA, USA). Suspended beads were vortexed and agitated in the dark on a rotator at room temperature for 2 h. Beads were washed twice with 500 μl PBS using a magnetic separator. Glyc–PAA–biot$_1$ solutions, regular (Chinarev et al., 2010), or PEG-modified (20 pmol per 1 scale coupling reaction in 150 μl PBS, for details see (Pochechueva et al., 2011a,b)) were added to the reaction tubes with streptavidin-coated beads. The mixture was protected from light and agitated on a rotator at room temperature for 6 h or overnight at 4 °C. Modified microspheres were applied to a magnetic separator, supernatant was removed and beads were washed twice with 500 μl of bead storage buffer (Bio-Rad Laboratories Inc., Hercules, CA, USA). Beads were resuspended in 100 μl of bead storage buffer and concentration determined using a hemocytometer (Roth AG, Karlsruhe, Germany) before storing at 4 °C, protected from light.

2.5.2. Coupling of the biotinylated PEGs

An excess of biot-PEG$_m$ (m = 50 or 280) was taken to saturate the binding sites of streptavidin, which still remain vacant after immobilization of biotinylated glycopolymer on beads. Namely, 1 μl of 1 mg/ml solution of biot-PEG$_m$ was added to 1.25×10^6 glycopolymer-covered beads (resuspended in 150 μl PBS) and the resulting suspension was agitated on a rotator at room temperature for 2 h. Afterwards the beads were washed twice with 500 μl of bead storage buffer, resuspended in 100 μl of bead storage buffer and stored as described above.

2.5.3. Coupling of the heterobifunctional PEGs

After the standard activation procedure, bead pellets were resuspended in 150 μl of biot-PEG$_m$-NH$_2$ solution (10 mg/ml, 0.1 M NaHCO$_3$, pH 8.3), agitated in the dark on a rotator at room temperature for 2 h. The obtained PEGylated beads with biotin groups on their surface were applied for further coupling to streptavidin and glycopolymers as described above.

2.6. Suspension glycan array (SGA)

2.6.1. The regular procedure

The Bio-Plex 200 suspension array system (Bio-Rad Laboratories, Hercules, CA, USA) is a multiplex analysis system that permits the simultaneous analysis of up to 200 different biomolecules in a single microwell plate. The constituents of each well are drawn up into the flow-based Bio-Plex array reader, which quantifies each specific reaction based on its bead color using fluorescently labeled reporter molecules specific for each target protein followed by Bio-Plex Manager software data analysis. Antibody diluent (125 μl PBS, pH 7.2, 1% BSA (w/v), Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) incorporating 2500 beads of each region per well (50 μl/well) was added to a Bio-Plex Pro 96-well flat bottom microplate (Bio-Rad Laboratories Inc., Hercules, CA, USA). The plate was washed twice with washing buffer (PBS—0.02% Tween-20 (v/v), pH 7.2) using a Bio-Plex Pro II Wash Station (Bio-Rad Laboratories Inc., Hercules, CA, USA). Various anti-glycan antibody dilutions or human serum samples, diluted to 1/40 (in accordance to (Pochechueva et al., 2011a)), or antibody diluent alone as a negative control were added to wells (in antibody diluent, 50 μl/well) and vigorously agitated for 30 s on a microplate shaker before incubation on a shaker with medium speed for 1 h at room temperature in the dark. After incubation, the plate was washed thrice with washing buffer using the Bio-Plex Wash Station. Secondary antibodies (R-PE conjugated goat anti-human IgM or IgG, 25 ng/well in antibody diluent, 50 μl/well) or antibody diluent alone as a negative control were added and incubated for 30 min on the plate shaker in the dark. The plate was washed thrice with washing buffer; beads were resuspended and shaken for 30 s vigorously in 125 μl of washing buffer before being analyzed on the Bio-Plex array reader. Data were acquired in real time, analyzing 100 beads by their median fluorescence intensity (MFI) using computer software package (Bio-Plex Manager 5.1; Bio-Rad Laboratories, Hercules, CA, USA). The technical cut-off of the method, defined using a validation kit was 10 MFI. If not otherwise denoted SGA experiments were performed with triplicate experimental samples three times in an independent manner.

2.6.2. Modification of SGA protocol used for the detection of biot-PEG$_m$ binding

As primary anti-glycan antibody anti-P^k rat monoclonal IgM was applied (dilution of 1/100; incubation for 1 h), followed by secondary biotinylated mouse anti-rat IgM (dilution of 1/1000; incubation for 30 min) and streptavidin-R-PE (dilution of 1/200; incubation for 10 min). All the other experimental details were the same as described above.

2.7. Purification of anti-glycan antibodies

Anti-A (A_{tri}), anti-B (B_{di}) and anti-αRha antibodies were affinity purified from pooled plasma of blood group O individuals as described previously (Obukhova et al., 2007; Pochechueva et al., 2011a). Anti-P_1, anti-LacNAc and anti-3′-sulfo-LacNAc antibodies were affinity purified from ascites (exudative fluid from peritoneal cavity) of an ovarian cancer patient and processed by centrifugation at 3000 ×g for 15 min at 4 °C. Supernatant was aliquoted and kept frozen at −80 °C. Thawed ascites (50 ml) was filtered through a 0.22 μm filter (Millipore, Billerica, USA) and diluted in PBS (pH 7.4). Pre-processed ascites was affinity purified against 10 ml of equilibrated PBS glycan-PAA-Sepharose. A constant flow rate of 1 ml/min was controlled by an auxiliary pump (model EP-1 Econo Pump, Biorad, Hercules, USA). Protein content was recorded by UV at 280 nm (BioLogic DuoFlow™ Workstation, Biorad, Hercules, USA). The column was washed with PBS containing 0.05% (v/v) Tween 20, until no protein was detected. Bound anti-glycan antibodies were eluted using 0.2 M TrisOH (pH 10.2) and neutralized by 2.0 M glycine HCl (pH 2.5). Remaining eluted anti-glycan antibodies were concentrated using the Amicon® Ultra-0.5 centrifugal filter (Millipore, Billerica, USA) and concentration was determined spectrophotometrically at 280 nm. The following dilutions of affinity purified antibodies were used: anti-P_1 — 1/10 and 1/50; and anti-B_{tri} — 1/100, 1/200 and 1/500. All the other antibodies were used in the dilution of 1/10.

Additional depletion of ascites fluid after removal of anti-P_1 antibodies was performed as follows. After affinity purification of anti-P_1 antibodies, ascites fluid was additionally incubated with P_1-adsorbent, taken in a 1/1 ratio (v/v), for 1 h on a shaker at RT, then centrifuged for 10 min, at 13,000 g. The supernatant (diluted to 1:10 with PBS, 1% BSA) was assayed with P_1-regular and PEG-modified beads.

2.8. Statistical analysis

Statistical analysis was performed with GraphPad Prism 6 software using the repeated-measures ANOVA followed by the Tukey posttest. Adjusted p-values < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Design of various PEG-modifications

Expanding on the strategy of PEG linking we designed and investigated several kinds of PEG-modifications in order to mask sites on the glycobeads at which unspecific binding of antibodies may occur: partial PEG substitutions with PEGs of different lengths within end-biotinylated glycopolymers (Scheme 1B); attachment of biotin-modified PEGs to presumably unsaturated streptavidin binding sites next to coupling of end-biotinylated glycopolymers (Scheme 1C); covalent binding of amino-functionalized biotin-PEGs (heterobifunctional PEGs) directly onto the bead surface prior to glycopolymer coupling (Scheme 1D).

3.2. PEG substitutions within the glycoprobes (Scheme 1B)

Glycoconjugates based on linear polyacrylamides (PAAs) with side-attached carbohydrate groups are widely used in bioanalytical research as multivalent glycoprobes (Bovin, 1998, 2003). However, serum antibodies may bind not only to the pendant glycan residues but also to the polymer backbone. The latter effect can be reduced by the substitution of the side groups (e.g. *N*-(2-hydroxyethyl)) within the non-glycosylated monomer units by PEG. Three types of PEGs were used for this purpose: “short” (m = 4, substitution rate −80%),

"medium" or "long" (m ~ 50 or 280, substitution rate −5%, see Glycopolymers with end-biotin group and Fig. 1). In addition, two different glycopolymers were included: B_{tri} belongs to the ABO blood group system and served as a "reference glycan". P_1 trisaccharide is our top candidate as potential ovarian cancer marker (Pochechueva et al., 2011b; Jacob et al., 2012).

The binding of corresponding affinity purified antibodies and healthy donor plasma antibodies (analytes) to these different PEGs with our regular (Scheme 1A) B_{tri}- and P_1-glycoprobes was compared with SGA.

The results showed that the MFI values, representative for antibody binding, were lower for all three PEGylated compared to the regular glycopolymers. This was true for the B_{tri} (Fig. 3A) as well as the P_1 (Fig. 3B) glycopolymers. Even more interesting, the binding of the antibodies to both PEGylated glycopolymers, i.e. B_{tri} and P_1, progressively decreased with the increase of PEG chain length in a statistically significant manner ($p < 0.05$) for all analytes. These results indicate that (i) PEGylation reduces antibody binding to these glycopolymers and that (ii) this decrease is PEG chain length-dependent.

This observation can unambiguously be explained by the shielding of the glycan residues by the PEG molecules, which is stronger with longer PEG chains attached to the polymer backbone. However, this shielding effect is likely to affect specific binding and presumably also unspecific binding of the antibodies to these glycopolymers, and the distinction whether or not unspecific binding of antibodies occurred to non-glycosylated parts of polyacrylamide backbone was not possible with these kinds of PEGylations.

To determine the potential contribution of unspecific binding we assayed the beads modified with the regular or with PEGylated P_1-glycoprobes with native ascites fluid and with ascites fluid depleted of anti-P_1 antibodies. As expected the results showed (ESM, Fig. 1) substantially lower MFI values in the depleted than in native ascites setting. More importantly, antibody binding decreased with the length of the PEGs in both settings, comparable to the setting for affinity purified and plasma anti-glycan antibodies presented in Fig. 3B. The finding that this PEG chain length-dependent decrease in binding occurred in both settings, i.e. also in the native ascites, indicates that these types of PEGs (different chain lengths) were not sufficient to avoid unspecific antibody binding.

3.3. Biotinylated PEGs (biot-PEG_m) bound to glycopolymer-modified microbeads (Scheme 1C)

The next PEG modification considered was the attachment of biotinylated PEGs (biot-PEG_{50} and biot-PEG_{280}) to glycopolymer pre-treated beads (see PEGs used for glycopolymer and microbead modifications and Fig. 2A). The idea was that these biot-PEGs may bind streptavidin binding sites that may have been left unbound after the antecedent coupling of the glycopolymers. We assayed the binding of the analytes, i.e. three different human antibodies (commercial anti-P_1 monoclonal IgM antibody, affinity purified anti-P_1 antibodies, and plasma antibodies) to regular and biot-PEG_m-modified P_1-conjugated beads.

Fig. 4A demonstrates that the MFI values for the regular and the two biot-PEG_m-modified P_1-conjugated beads were comparable for each of the analytes (differences in MFI values among three types of beads were less than the inter-assay variability (from 8.5 to 18.5%) previously described (Pochechueva et al., 2011a)). This result indicates that the attachment of these two biot-PEG_m did not affect the binding of anti-glycan antibodies to P_1-beads. Possible explanations are that either all streptavidin binding sites were saturated with biotinylated glycopolymer prior to biot-PEG_m coupling or the influence of non-target binding to streptavidin was negligible.

To determine whether streptavidin binding sites were indeed saturated by biotinylated glycopolymers and whether biot-PEGs were able to block potentially remaining/unbound sites we

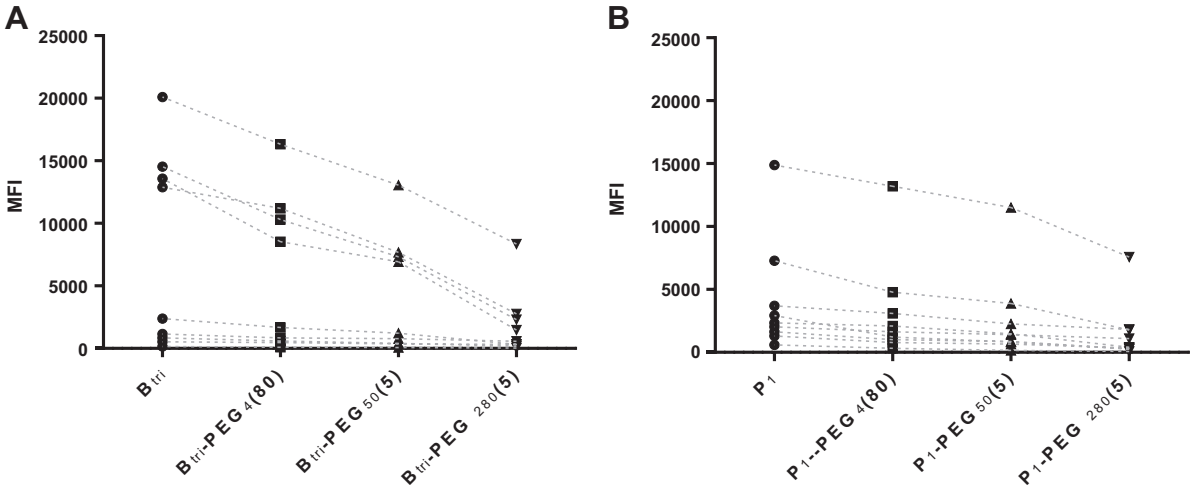


Fig. 3. Binding (given as MFI values) of the analytes (n = 18; affinity purified antibodies, antibodies in healthy plasma donors) is presented for the different microbeads modified with the regular (spheres) and the three PEGylated polyacrylamide (squares and triangles) glycopolymers B_{tri} or P_1. For the B_{tri} setting (A) affinity-purified anti-B_{tri} antibodies and plasma pools of blood groups A, B and O and for the P_1 setting (B) affinity-purified anti-P_1 antibodies and seven individual plasma samples were used as analytes (dilutions: see "Materials and methods"). The binding was detected using R-PE labeled goat anti-human Ig (M + G + A) antibodies. Each data point is the mean of three independent MFI measurements performed in triplicates: identical analytes are connected by dotted lines. The mean MFI values and standard deviation for each point are shown in Tables 1 and 2 in the electronic Supplementary material.

assayed the commercial monoclonal rat anti-P^k IgM antibody for its binding to the regular and to the biot-PEG_m-modified P_1-beads. P^k (also referred to as Gb3 or CD77) shares the terminal Galα1–4Galβ1 motif with P_1 trisaccharide, and the anti-P^k antibody may thus cross-react to some extent with P_1. The secondary biotinylated anti-rat IgM antibody was used for binding detection, followed by streptavidin-R-PE. The contribution of direct binding of the secondary biotinylated antibody to the beads was determined in the absence of the primary anti-P^k antibody.

The results are shown in Fig. 4B. For the regular P_1 beads the MFI values were comparable, irrespective of the presence or absence of the anti-P^k antibody. This indicates that the secondary antibody binds directly to streptavidin on these beads. In contrast, the MFI values in the absence of anti-P^k antibodies were lower for both biot-PEG_m (to a greater extent with biot-PEG_{50}). This demonstrates that direct binding of secondary biotinylated antibody to streptavidin was almost completely abolished (30-fold reduction) for biot-PEG_{50} and intermediately (2-fold) reduced for biot-PEG_{280}, suggesting that the remaining streptavidin binding sites were almost completely saturated by biot-PEG_{50} and partially saturated by biot-PEG_{280}.

These results indicate that (i) not all biotin-binding sites on streptavidin were occupied by regular glycopolymers initially, (ii) unspecific binding due to these remaining free biotin-binding sites did not have any influence in our standard experimental setup in the absence of secondary biotinylated antibodies, (iii) the use of secondary biotinylated antibodies is feasible and still allows for the correct detection of analyte binding in the case of end-point addition of biot-PEG_{50} (or to a lesser degree of biot-PEG_{280}) to block the remaining free streptavidin binding sites, and (iv) we can minimize the risk of unspecific binding often caused by endogenous biotin in serum and cell and tissue lysate samples by using biot-PEG_{50}.

3.4. Microbead surface modification with heterobifunctional PEGs (Scheme 1D)

The heterobifunctional PEG_{23} and PEG_{60} (see PEGs used for glycopolymer and microbead modifications and Fig. 2B for structure and details) were coupled to the beads prior to the anchoring of streptavidin and the immobilization of the glycopolymers. In this setup the two versions of biot-PEGs-NH_2 were bifunctional linkers between the bead and streptavidin. The binding of human monoclonal anti-P_1 antibodies as well as plasma antibodies from healthy donors to modified beads was assayed by SGA. The results (Fig. 5A) showed that binding of monoclonal anti-P_1 antibodies and plasma antibodies to all three types of beads, i.e. regular P_1-beads and P_1-beads modified with both heterobifunctional PEG, was comparable, indicating that neither the bead modification with heterobifunctional PEGs in general nor the PEG length affected antibody binding to P_1. This is in contrast to the PEGylated (different PEG chain lengths) glycopolymers (Fig. 3) where the binding decreased with longer PEG chains.

In order to explore whether and how unspecific binding can be detected we used anti-human IgG instead of anti-human IgM as secondary antibodies for the detection of human monoclonal anti-P_1 IgM antibody binding to P_1. In this setting we assayed the binding to the regular P_1-beads or the P_1-beads

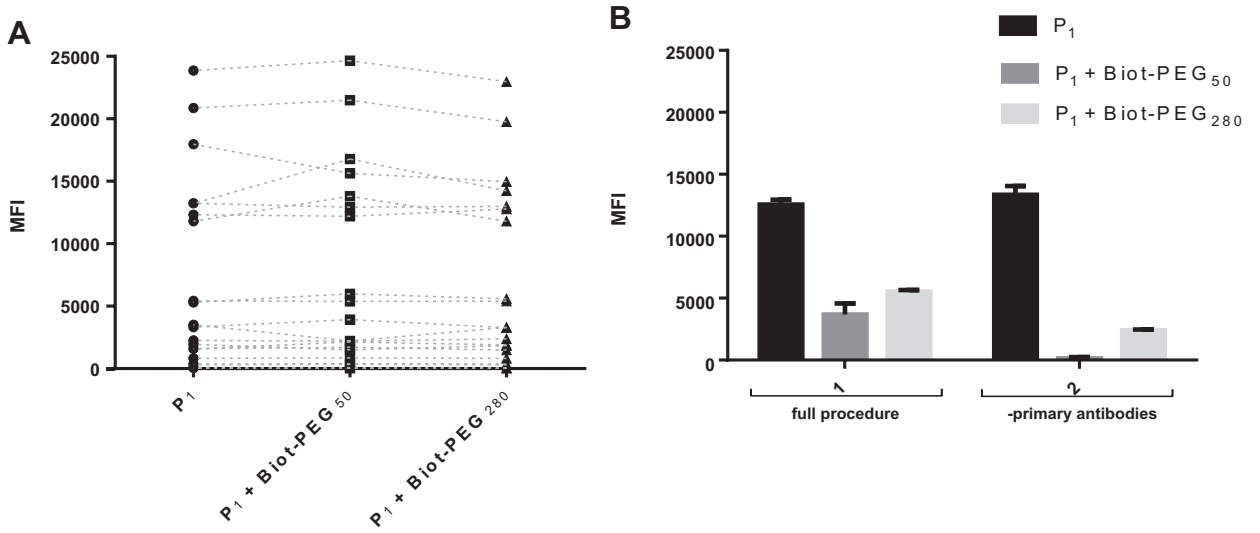


Fig. 4. (A) Comparison of the binding (given as MFI values) of analytes (n = 20: anti-P_1 IgM antibodies, affinity-purified anti-P_1 antibodies, five individual plasma samples; for dilutions see "Materials and methods") to regular P_1 beads (spheres) and P_1 bead modified with biot-PEG_{50} (squares) or biot-PEG_{280} (triangles). The binding was detected using R-PE labeled goat anti-human (both IgM and IgG) antibodies. Each data point is the mean of three independent MFI measurements performed in triplicates: identical analytes are connected by dotted lines. The mean MFI values and standard deviation for each point are shown in Table 3 in the electronic Supplementary material. (B) Bar chart presenting the binding of the secondary anti-rat IgM biotinylated antibody (detected by streptavidin-R-PE) to regular P_1 beads (black) and P_1 bead modified with biot-PEG_{50} (gray) or biot-PEG_{280} (light-gray) either in the presence (1, full procedure) or absence (2, primary antibodies) of the primary rat monoclonal anti-P^k IgM antibody (detailed explanation: see text). Data are the mean ± SD of three independent MFI measurements performed in triplicates.

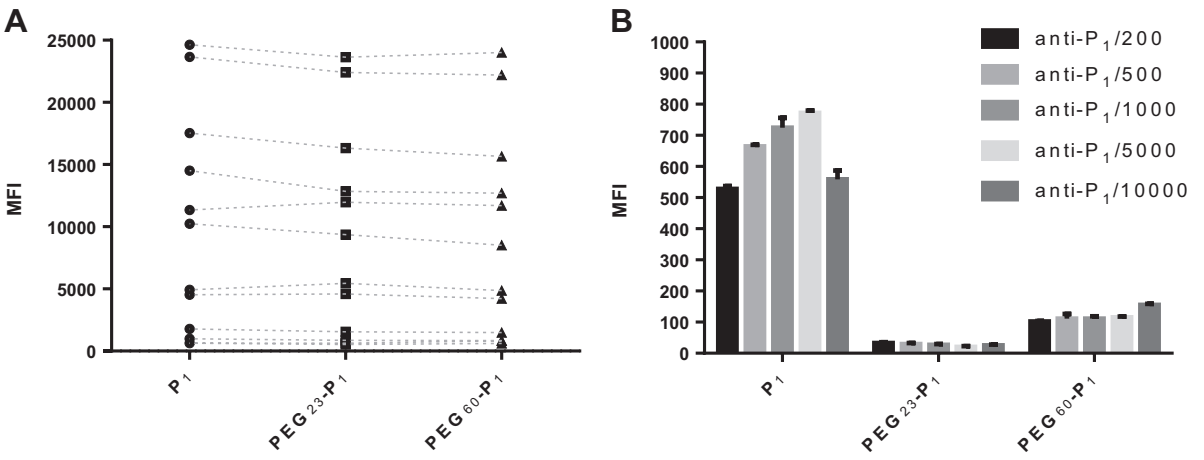


Fig. 5. (A) Comparison of the binding (given as MFI values) of analytes (n = 12: anti-P_1 IgM antibody, four individual plasma samples; for dilutions see "Materials and methods") to regular P_1 beads (spheres) and P_1 beads modified with heterobifunctional PEGs: PEG_{23}-P_1 (squares) or PEG_{60}-P_1 (triangles). The binding was detected using R-PE labeled goat anti-human IgM (anti-P_1 monoclonal human IgM) or R-PE labeled anti-human IgM and IgG (plasma samples). Each data point is the mean of three independent MFI measurements in triplicates: identical analytes are connected by dotted lines. The mean MFI values and standard deviation for each point are shown in Table 4 in the electronic Supplementary material. (B) Detection of non-target binding to regular P_1 and heterobifunctional PEG-modified beads. Data are the mean ± SD of three independent MFI measurements in triplicates as a function of increasing antibody dilution (as indicated) for P_1-, PEG_{23}-P_1- or PEG_{60}-P_1-beads.

modified with heterobifunctional PEGs by using progressively higher dilutions of the anti-P_1 IgM antibody. The results (Fig. 5B) essentially showed that regular P_1-beads exhibited substantial unspecific binding which was notably substantially reduced near to the technical cut-off level of the method (approx. 10 MFI) with both heterobifunctional modified PEG P_1-beads (30-fold for PEG_{23} and 6-fold for PEG_{60}). A decrease of binding to regular P_1-beads with progressively decreasing anti-P_1 IgM antibody concentrations was not observed. These results suggest that commercial anti-P_1 IgM antibodies may contain traces of unspecific (heterophilic) antibodies of IgG class which bind directly to the bead. We also performed a similar experiment with biot-PEG_{50}- and biot-PEG_{280}-modified beads (see above), but did not observe any difference in unspecific binding between these biotinylated PEG-modified and regular P_1-beads (data not shown). This indicates that only bead modifications with heterobifunctional PEGs but not the end-point addition of biot-PEG prevented unspecific binding of non-target antibodies.

Because the nature of this unspecific IgG-mediated binding was unknown we assayed P_1-, PEG_{23}-, and PEG_{60}-P_1 beads with several non-related anti-glycan antibodies of IgM class (anti-A_{tri}, anti-B_{di}, anti-LacNAc, anti-3′-su-LacNAc, anti-α-Rha) which we purified from human ascites fluid and plasma. Regardless of the bead type we found that LacNAc (Galβ1–4GlcNacβ) antibodies cross-reacted with P_1 to some extent (MFI from 300 to 450) whereas the other antibodies cross-reacted to P_1 only minimally (MFI of less than 100) (ESM, Fig. 2). However, in a similar setting with the respective IgG class antibodies (and also a commercial monoclonal mouse anti-P^k IgG antibody) we found substantial cross-reactivity of these IgG class antibodies to P_1 with the regular beads (MFI from 700 to 900) but not with the heterobifunctional PEG P_1-beads (MFIs below 200) (Fig. 6A). These results indicate that the observed cross-reactivity (unspecific binding) may be largely attributed to the IgG class of the anti-glycan antibodies. For comparison the binding of the monoclonal anti-P^k IgG antibody to the P_1-beads is included, showing the extent of the cross-reactivity of the anti-P^k antibody to P_1 (Fig. 6A). The cross-reactive binding of anti-P^k IgG to P_1 may, even with monoclonal antibodies, not be surprising, because P^k and P_1 share the same terminal disaccharide motif. The vast number of naturally occurring anti-glycan antibodies generally are not monospecific and exhibit some degree of polyreactivity: they are able to recognize more than one glycan structure but bind them with differing affinities. The latter may serve to "fine tune" their actions in vivo (Schwartz-Albiez, 2012). These results also indicate that this IgG class-dependent cross-reactivity can be reduced by the introduction of PEGs, and this is considered important for the accurate detection of analytes in particular in an artificial array system as the SGA.

In order to determine the contribution of non-target binding we assayed P_1-, PEG_{23}-, and PEG_{60}-P_1 modified beads with fetal calf serum-derived and presumably heterophilic antibodies. The results (Fig. 6B) demonstrate that no substantial binding to all three types of beads was observed for IgM (MFI of around 20) whereas some binding (MFI of around 500) was detected for IgG for the regular P_1-beads. This observation is in concordance to the previous experiments (Figs. 5B and 6A). These IgG signals were reduced for the PEG_{60}-P_1 beads to about 100 MFI and for the PEG_{23}-P_1 beads to 15 MFI.

In summary, unspecific binding was observed almost exclusively when IgGs, but not IgMs were applied as glycan-binding antibodies or as secondary detection antibodies. These data seem to be consistent with existing evidence regarding anti-glycan antibodies. It is known that naturally occurring anti-glycan antibodies are predominantly of IgM class and are produced by CD5 positive B1 cells expressing a distinct pattern of surface markers, but not conventional B cells (Viau and Zouali, 2005; Vollmers and Brandlein, 2009; Griffin

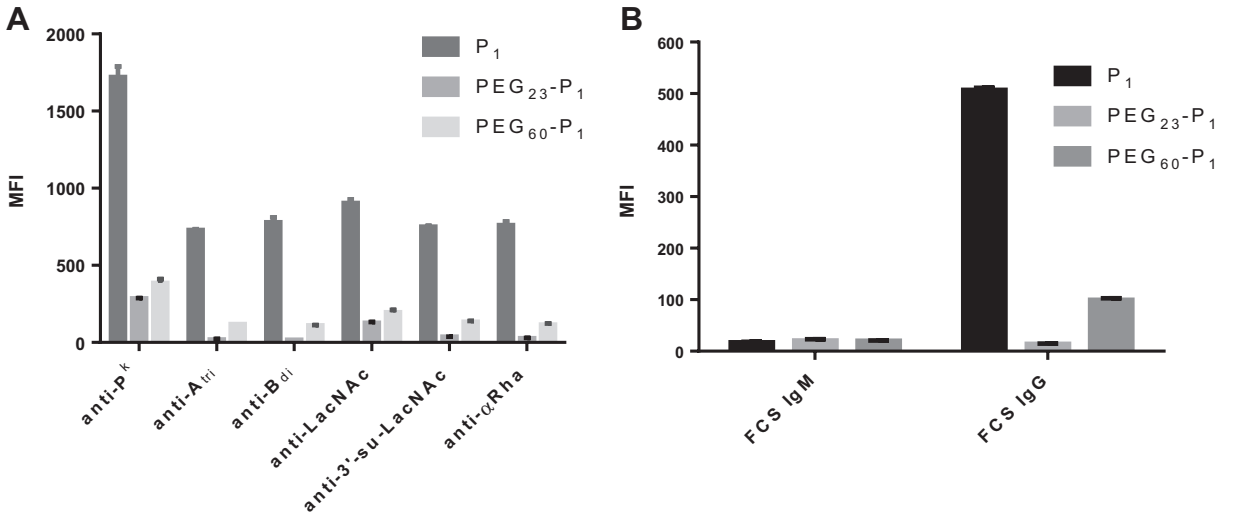


Fig. 6. Bar graph presentation of antibody cross-reactivity/background binding of polyclonal anti-glycan antibodies. (A) Binding of anti-glycan affinity-purified human antibodies and anti-P^k mouse monoclonal IgG antibody to regular P_1-beads and to PEG_{23}-P_1- and PEG_{60}-P_1-modified beads. Detection with R-PE labeled anti-human IgG and R-PE labeled anti-mouse Ig (G + M + A). Antibody dilutions are indicated in "Materials and methods". (B) Detection of background binding to the respective beads using undiluted fetal calf serum (FCS). Detection with R-PE labeled goat anti-human IgM or IgG. Data are the mean ± SD of three independent MFI measurements in triplicates.

et al., 2011; Bovin, 2013). Despite their polyreactive nature anti-glycan IgMs appear to be highly specific in terms of affinity distinctions. Specific recognition of certain glycan structure strongly depends on its natural molecular context (Bovin, 2013). Pentameric IgMs have ten Fab regions and therefore possess a theoretical valency of 10. Multivalent recognition is very important for glycan–protein interaction, providing stable and affine binding to multiple oligosaccharide structures. On the contrary, IgGs are only divalent, their interactions with glycans may be weaker that is why this antibody class is typically not ascribed to recognize glycans in nature. Due to the same reasons IgGs may be more predisposed to unspecific binding than IgMs upon profiling with glycoarrays.

To further exploit the possibility to reduce anti-glycan antibody cross-reactivity by using heterobifunctional PEGs, we linked PEG_{23} and PEG_{60} to the bead surface, coupled P^k trisaccharide to these beads, and compared the binding of monoclonal human anti-P_1 IgM either to P_1-coupled beads or to P^k-coupled beads (without or with heterofunctional PEGs) as a function of the antibody dilution (Fig. 7). The results showed that the binding of the anti-P_1 IgM antibodies, regardless of the dilution, to P^k-beads was several-fold lower than to P_1-beads, indicating that indeed anti-P_1 antibodies bind to P^k trisaccharide with much lower affinity than P_1 trisaccharide. The binding signals were even further lowered (about 2-fold) with both kinds of heterobifunctional PEGs.

4. Conclusion

In this study we designed and evaluated various kinds of PEG-modifications, exploiting unique chemically synthesized end-biotinylated glycopolymer capture molecules in combination with a simple and affordable PEG-linking, to optimize the current version of our previously "in-house" developed SGA in order to reduce the experimental background (essentially unspecific and non-target binding) of this glycan-based assay. This background reduction may minimize the risk of occurrence of false-positive/negative results and may improve the diagnostic performance (increased sensitivity and specificity) of SGA. The following conclusions may be drawn from the findings: (i) The most significant decrease of background binding was achieved when PEG molecules bearing two functional groups, biotin and amine (hence heterobifunctional), were covalently attached directly to microbeads. This modification may be beneficial because it decreases "experimental noise" at low detection signals and does not compromise specific binding of the cognate anti-

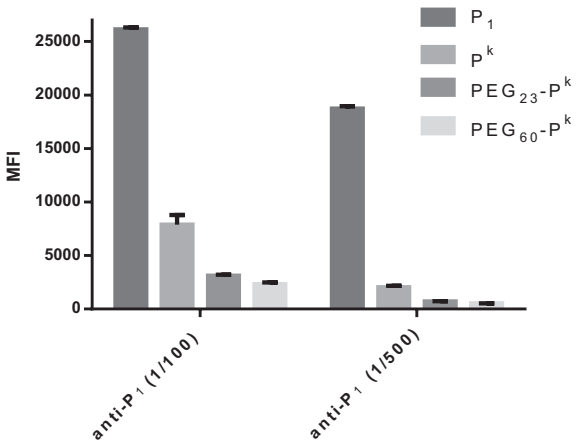


Fig. 7. Bar graph presenting the binding of anti-P_1 monoclonal human IgM to regular and heterobifunctional PEG_{23}/PEG_{60}-modified P^k-beads. The binding to regular P_1-beads is given for comparison. Detection with R-PE labeled goat anti-human IgM. Data are the mean ± SD of three independent MFI measurements in triplicates.

glycan antibodies. Interestingly, the shorter version of these two heterobifunctional PEGs, biot-PEG_{23}-NH_2, exhibits a more pronounced repelling effect, namely the capacity to block binding of non-target antibodies, than the respective longer version and therefore may preferably be used in an advanced version of SGA. (ii) The end-point addition of biot-PEG_{50} can be used to repel unspecific binding caused by endogenous biotin potentially present in the analyte (e.g. plasma samples) or in secondary antibody samples and can be easily combined with bead surface PEGylation. (iii) A considerable extent of unspecific binding can be attributed to the IgG class of the antibodies whereas the contribution of IgM class antibodies to unspecific binding signals is low. It is therefore recommended to use IgM class rather than IgG class antibodies in glycan-based immunoassays. (iv) Background binding was not reduced when glycopolymers were PEG-modified at their side-chains, possibly because the PEG-chains that are attached to polyacrylamide backbone of glycopolymer preclude specific binding of anti-glycan antibodies to the glycan epitopes. Taken together, the combination of the appropriate PEG-modifications, i.e. the bead modification with PEG_{23} and the end-point addition of biot-PEG_{50} is a promising advancement in the optimization of the current version of our SGA. These or similar modifications probably could be also recommended to be included in experimental protocols of related bead-based immunoassays for the improvement of their diagnostic performance.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jim.2014.06.015.

References

Bergstrom, K., Holmberg, K., Safranj, A., Hoffman, A.S., Edgell, M.J., Kozlowski, A., Hovanes, B.A., Harris, J.M., 1992. Reduction of fibrinogen adsorption on PEG-coated polystyrene surfaces. J. Biomed. Mater. Res. 26, 779.

Bjerner, J., Bormer, O.P., Nustad, K., 2005. The war on heterophilic antibody interference. Clin. Chem. 51, 9.

Bovin, N.V., 1998. Polyacrylamide-based glycoconjugates as tools in glycobiology. Glycoconj. J. 15, 431.

Bovin, N.V., 2003. Neoglycoconjugates as probes in glycobiology. Nato Sci Ser Ii Math, 129, p. 207.

Bovin, N.V., 2013. Natural antibodies to glycans. Biochem. Mosc. + 78, 786.

Caliceti, P., Veronese, F.M., 2003. Pharmacokinetic and biodistribution properties of poly(ethylene glycol)-protein conjugates. Adv. Drug Deliv. Rev. 55, 1261.

Chinarev, A.A., Galanina, O.E., Bovin, N.V., 2010. Biotinylated multivalent glycoconjugates for surface coating. Methods Mol. Biol. 600, 67.

Desai, N.P., Hubbell, J.A., 1991. Biological responses to polyethylene oxide modified polyethylene terephthalate surfaces. J. Biomed. Mater. Res. 25, 829.

Etxebarria, J., Serna, S., Beloqui, A., Martin-Lomas, M., Reichardt, N.C., 2013. Three-dimensional arrays using glycoPEG tags: glycan synthesis, purification and immobilisation. Chemistry 19, 4776.

Fishburn, C.S., 2008. The pharmacology of PEGylation: balancing PD with PK to generate novel therapeutics. J. Pharm. Sci. 97, 4167.

Griffin, D.O., Holodick, N.E., Rothstein, T.L., 2011. Human B1 cells in umbilical cord and adult peripheral blood express the novel phenotype $CD20^+CD27^+CD43^+CD70$. J. Exp. Med. 208, 67.

Jacob, F., Goldstein, D.R., Bovin, N.V., Pochechueva, T., Spengler, M., Caduff, R., Fink, D., Vuskovic, M.I., Huflejt, M.E., Heinzelmann-Schwarz, V., 2012. Serum antiglycan antibody detection of nonmucinous ovarian cancers by using a printed glycan array. Int. J. Cancer 130, 138.

Jain, N.K., Nahar, M., 2010. PEGylated nanocarriers for systemic delivery. Methods Mol. Biol. 624, 221.

Jokerst, J.V., Lobovkina, T., Zare, R.N., Gambhir, S.S., 2011. Nanoparticle PEGylation for imaging and therapy. Nanomedicine 6, 715.

Kingshott, P., Griesser, H.J., 1999. Surfaces that resist bioadhesion. Curr. Opin. Solid State Mater. 4, 403.

Kricka, L.J., 1999. Human anti-animal antibody interferences in immunological assays. Clin. Chem. 45, 942.

Larsson, A., Ekblad, T., Andersson, O., Liedberg, B., 2007. Photografted poly(ethylene glycol) matrix for affinity interaction studies. Biomacromolecules 8, 287.

Levinson, S.S., Miller, J.J., 2002. Towards a better understanding of heterophile (and the like) antibody interference with modern immunoassays. Clin. Chim. Acta 325, 1.

Martins, T.B., Augustine, N.H., Hill, H.R., 2006. Development of a multiplexed fluorescent immunoassay for the quantitation of antibody responses to group A streptococci. J. Immunol. Methods 316, 97.

Obukhova, P., Rieben, R., Bovin, N., 2007. Normal human serum contains high levels of anti-Gal alpha 1-4GlcNAc antibodies. Xenotransplantation 14, 627.

Ozols, R.F., 2006. Challenges for chemotherapy in ovarian cancer. Ann. Oncol. 17 (Suppl. 5), v181.

Pochechueva, T., Chinarev, A., Spengler, M., Korchagina, E., Heinzelmann-Schwarz, V., Bovin, N., Rieben, R., 2011a. Multiplex suspension array for human anti-carbohydrate antibody profiling. Analyst 136, 560.

Pochechueva, T., Jacob, F., Goldstein, D.R., Huflejt, M.E., Chinarev, A., Caduff, R., Fink, D., Hacker, N., Bovin, N.V., Heinzelmann-Schwarz, V., 2011b. Comparison of printed glycan array, suspension array and ELISA in the detection of human anti-glycan antibodies. Glycoconj. J. 28, 507.

Preissner, C.M., Dodge, L.A., O'Kane, D.J., Singh, R.J., Grebe, S.K., 2005. Prevalence of heterophilic antibody interference in eight automated tumor marker immunoassays. Clin. Chem. 51, 208.

Prime, K.L., Whitesides, G.M., 1991. Self-assembled organic monolayers: model systems for studying adsorption of proteins at surfaces. Science 252, 1164.

Ratner, D.M., 2005. Advancing the burgeoning field of glycomics. Biotechnol. Int. 17, 8.

Roberts, M.J., Bentley, M.D., Harris, J.M., 2002. Chemistry for peptide and protein PEGylation. Adv. Drug Deliv. Rev. 54, 459.

Schwartz-Albiez, R., 2012. Naturally occurring antibodies directed against carbohydrate tumor antigens. Adv. Exp. Med. Biol. 750, 27.

Viau, M., Zouali, M., 2005. B-lymphocytes, innate immunity, and autoimmunity. Clin. Immunol. 114, 17.

Vollmers, H.P., Brandlein, S., 2009. Natural antibodies and cancer. New Biotechnol. 25, 294.

Waterboer, T., Sehr, P., Pawlita, M., 2006. Suppression of non-specific binding in serological Luminex assays. J. Immunol. Methods 309, 200.

Wattendorf, U., Coullerez, G., Voros, J., Textor, M., Merkle, H.P., 2008a. Mannose-based molecular patterns on stealth microspheres for receptor-specific targeting of human antigen-presenting cells. Langmuir 24, 11790.

Wattendorf, U., Kreft, O., Textor, M., Sukhorukov, G.B., Merkle, H.P., 2008b. Stable stealth function for hollow polyelectrolyte microcapsules through a poly(ethylene glycol) grafted polyelectrolyte adlayer. Biomacromolecules 9, 100.