

GlycoChip: multiarray for the study of carbohydrate-binding proteins

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Biotinylated glycoconjugates which were designed as oligosaccharides attached to 30 kDa polyacrylamide were coated on a microarray platform XNAonGOLD™, which was developed earlier for an oligonucleotide assay. The specificity of antibodies to carbohydrate antigens was analyzed using the glyco-microarray. Comparison of the obtained results with those of common 96-well plate ELISA completely coincided with the found antibody specificities. However, parameters such as the analytical sensitivity of the method and the amount of biotinylated material coated on the microarray platform proved to be worse than expected.

Introduction

The microarray concept that was introduced at the beginning of the 1990s seems quite interesting in respect of possible practical applications. Its significant points are the following: (1) the possibility of achieving high sensitivity using ultra low amounts of a ligand, (2) a low background level due to the high regularity of the active layer, usually self-assembled monolayers, SAM,¹ (3) a practically unlimited number of measured parameters within one chip due to the small size of well (dot); each of them can contain a particular specific ligand, thus a microarray is also a multiarray. The concept of such arrays is especially attractive for the analysis of biological samples (particularly blood) and high throughput screening during the search for new lead candidates.

Besides actively developed oligonucleotide and peptide chips a GlycoChip is also of great importance. The GlycoChip is a tool to provide information about the presence and the level of clinically significant anti-carbohydrate antibodies, such as a blood group-, xeno-, autoimmune-, cancer-associated, and other pathology-associated, diagnostic and prognostic antibodies. The data obtained with the help of the GlycoChip should be a passport for all individuals before transfusion, transplantation, administration of blood preparations, blood donation, motherhood, as well as extracorporeal treatment with glycoadsorbents or therapeutic antibodies, industrial sorting of blood preparations, as well as for basic research. As the objectives of functional glycomics² are principally the same as those of proteomics, we have attempted to transfer this approach to the study of carbohydrate–protein interactions, *i.e.*, the screening and study of lectins and carbohydrate-specific antibodies utilizing oligosaccharide multiarrays. At the first stage we have chosen the determination of specificity of monoclonal antibodies as a model. The pool of antibodies was obtained from the 7th Workshop and Conference on Human Leukocyte Differentiation Antigens (Harrogate, UK, 2000); the antibodies had been previously described as being anti-carbohydrate.³ In sum, 21 synthetic oligosaccharides as biotinylated conjugates (Syntesome GmbH, Germany) were used as carbohydrate ligands for the design of the corresponding array. Streptavidin-coated 192-well chips XNAonGOLD™ (Interactiva Biotechnologie GmbH, Germany), that were originally designed for the

attachment of biotinylated oligonucleotides,⁴ were utilized as a platform for a glycoarray. The same antibodies were correspondingly analyzed using a similar set of biotin-free glycoconjugates Sug-PAA that are in common with ELISA on 96-well polystyrene plates.

Materials and methods

Materials

Phosphate buffer tablets, Tween-20 and BSA were purchased from Sigma (USA). Polyacrylamide glycoconjugates Sug-PAA and Sug-PAA-biot (soluble, 30–40 kDa, saccharide ligand content is 20 mol.%, biotin content is 5 mol.%) were from Syntesome GmbH (Munich, Germany). Anti-mouse IgG+IgM (H+L)-alkaline phosphatase (AP) conjugate, ECF-fluorescent substrate for AP and Cy5-labeled anti-mouse IgG (H+L) were the products of AP Biotech (UK), α -galactosidase and conjugate anti-mouse Ig-HRPO were purchased from Boehringer Mannheim (Germany). Mouse monoclonal antibodies (mABs) B8 (anti-B_{tri}, against the blood group trisaccharide B) and A16 (anti-A_{tri}, against the blood group trisaccharide A) used as cultural fluid diluted 1:10 by PBS containing 0.3% BSA were obtained from the All-Russian Hematology Research Center (Moscow). A set of mouse monoclonal antibodies was obtained from Carbohydrate Panel submitted for the 7th Workshop and Conference on Human Leukocyte Differentiation Antigens (Harrogate, UK, 2000). Polystyrene 96-well microtiter MaxiSorp immunoplates were purchased from Nunc (Denmark). All other analytical grade chemicals were purchased from Fluka (Switzerland).

96-well plate ELISA

Plates were coated with Sug-PAA conjugates, 10 μ g ml⁻¹ in 0.05 M Na-carbonate buffer, pH 9.6 for 1 h at 37 °C. Plates were blocked with 3% BSA in PBS for 1 h at 37 °C and washed three times with PBS containing 0.1% Tween-20 (washing buffer). Twofold serial dilutions of mABs (100 μ l) were added and the plates were incubated for 1 h at 37 °C, washed three times with

washing buffer, incubated with anti-mouse Ig-HRPO conjugate (1/1000 in PBS containing 0.3% BSA) and washed. Finally, the color was developed by 30 min incubation with 0.1 M sodium phosphate–0.1 M citric acid buffer, containing 0.04% *o*-phenylenediamine and 0.03% H₂O₂, the reaction was stopped with the addition of 50 μ l 1 M H₂SO₄. Absorbance was recorded at 492 nm in a microtiter plate reader (Multiskan MCC/340, Labsystems, Finland). The blank reaction was performed by omitting the mABs. The blank reading was subtracted from the final reaction optical density to provide the corrected absorbance values.

GlycoChip Assay

Streptavidin 192-spot XNA on Gold™ platform (Interactiva GmbH, Ulm, Germany) was coated manually, using a pipettor, with a set of Sug-PAA-biot glycoconjugates in PBS, 1 μ l per spot, 200 pmol (on carbohydrate ligand) per spot, in duplicate. The platform was incubated in a wet chamber for 60 min at 37 °C and washed twice (1–2 min on a shaker at room temperature) with washing buffer. The obtained glycoarrays are herein referred to as GlycoChips. The second sort of GlycoChip was prepared using A_{tri}-PAA-biot and B_{tri}-PAA-biot, in twofold serial dilutions, from 5 mM to 150 nM of Sug per spot.

Fluorescence detection of bound mABs directed to carbohydrate antigens

The GlycoChip was covered with a ground-glass microscopic slide. 100 μ l mABs in appropriate dilution were introduced between the chip and the covering glass using the capillary effect (drop suction between two ground-glasses). The covered GlycoChip was incubated in a wet chamber for 90 min at 37 °C and then uncovered and washed twice with the washing buffer. Two fluorescent techniques were used for the quantification of bound antibodies.

Firstly, the GlycoChip was incubated with Cy5-labelled anti-mouse antibodies. 100 μ l of labeled antibodies, in an appropriate dilution (1:500), were introduced between the chip and covering glass as described above. The covered GlycoChip was incubated in a wet chamber for 60 min at 37 °C, uncovered and washed twice with washing buffer. Fluorescence intensity of the dots was measured with a Fluorescent Image Analyzer FLA-200 (Fuji film) at an emission wavelength of 670 nm. Blank reaction was performed by omitting the mABs. A blank reading was subtracted from the final reaction of fluorescent intensity in order to produce the corrected values.

Secondly, the GlycoChip was incubated with anti-mouse antibodies–AP conjugate (1:10 000 in PBS with 0.3% BSA). 100 μ l of labeled antibodies solution in appropriate dilution (1:500 in PBS with 0.3% BSA) were introduced between the chip and covering glass. A covered GlycoChip was incubated in a wet chamber for 60 min at 37 °C. One μ l of ECF substrate was added per each spot, and after incubation for 5 min fluorescence intensity was measured with a Fluorescent Image Analyzer at an emission wavelength of 550 nm. Blank reaction was performed by omitting the mABs. A blank reading was subtracted from the final reaction of fluorescent intensity in order to produce the corrected values.

Values for all measurements were the means of at least triplicate determination

Results

The XNAonGOLD™ platform (see Fig. 1 and legend for a description of its design) was used for the construction of GlycoChip. Sug-PAA-biot (PAA is a 30 kDa soluble poly-

acrylamide, biot is a biotin residue, and Sug ligands are listed in Table 1) conjugates were immobilized on the streptavidin-coated platform surface. The obtained GlycoChip was used for the study of anti-carbohydrate antibodies.

Concentration dependency and background level. Antibodies with known specificity

First of all, we have tested specificity of mABs A16 and B8 with the help of a standard 96-well polystyrene plate ELISA (Fig. 2). Unlabeled conjugates A_{tri}-PAA and B_{tri}-PAA⁵ (see saccharide structures in Table 1) were physically adsorbed on polystyrene plates⁶ followed by incubation with anti-A_{tri} and anti-B_{tri} mABs and the further development of bound antibodies using an anti-mouse–peroxidase conjugate. It can be seen from Fig. 2 that both mABs recognized only their ‘own’ specific antigen, not displaying cross-reactivity with a related structure, *i.e.*, the antibodies were truly highly specific.

An analogous experiment using the same antibodies A16 and B8 was performed with a GlycoChip. Polyacrylamide conjugates of the same trisaccharides as biotinylated derivatives A_{tri}-PAA-biot or B_{tri}-PAA-biot, were coated on the Str-platform in different concentrations (4–500 pmole per dot). It should be noted first that the molecular weight and carbohydrate content in the biotin-labeled and the label-free conjugates were the same and, second, that ELISA with Sug-PAA-biot instead of Sug-PAA demonstrated practically the same ODs and background levels (data not shown). After incubation the GlycoChips were treated with antibodies and the bound antibodies were devel-

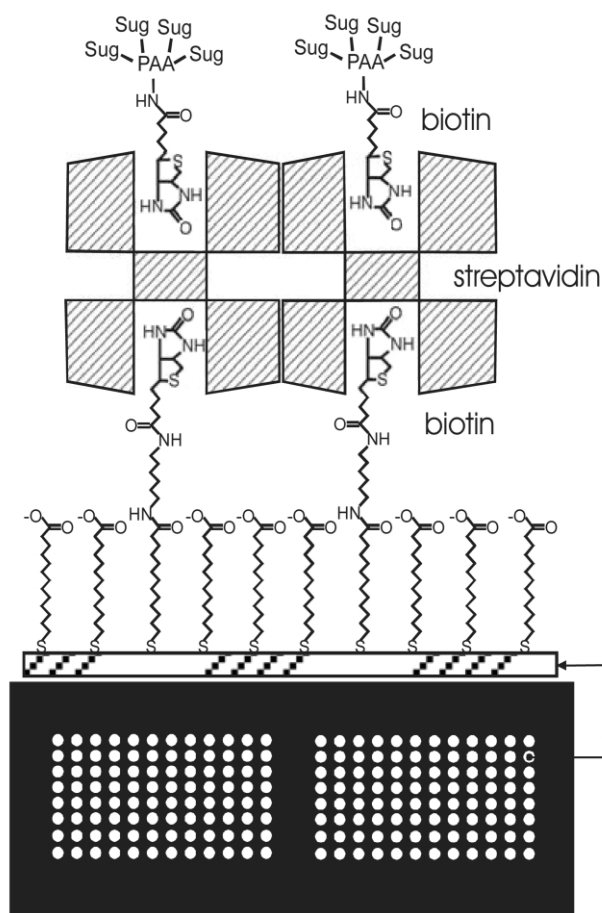


Fig. 1 Design of the GlycoChip. The XNAonGOLD™ platform designed in 2 × 96-spot format using a microscope slide (75 × 26 mm) footprint. A Teflon thick film was used to pattern the 50 μ m deep flat-wells, 1.5 mm in diameter. Self-assembled monolayers of long-chain biotinylated alkanethiol were chemisorbed onto a gold layer. Biotin residues were saturated with streptavidin. Biotinylated carbohydrates were attached to streptavidin.

oped using an anti-mouse-Cy5 conjugate (Fig. 3), the picture was taken directly from the chip (note the absence of a background in the wells with negative control); the obtained results are also presented in chart form. It can be seen that only trisaccharide A is revealed on the GlycoChip with anti-A antibodies analogously to common ELISA and that any

Table 1 List of carbohydrate ligands used for the construction of the GlycoChip

Structure of saccharide ligands	Designation or trival name
Fucα1-2 Galβ-sp GalNAcα1-3	A-trisaccharide (A _{tri})
Fucα1-2 Galβ-sp Galα1-3	B-trisaccharide (B _{tri})
Fucα1-2Galβ-sp	H-disaccharide (H _{di})
Fucα1-2Galβ1-4GlcNAcβ-sp	H (type 2)
Fucα1-2Galβ1-3GlcNAcβ-sp	Le ^d (H type 1)
Fucα1-4 GlcNAcβ-sp Galβ1-3	Le ^a
Fucα1-3 GlcNAcβ-sp Galβ1-4	Le ^x
Fucα1-4 GlcNAcβ- sp Fucα1-2Galβ1-3	Le ^b
Fucα1-3 GlcNAcβ-sp Fucα1-2Galβ1-4	Le ^y
Fucα1-3 GlcNAcβ-sp Neu5Acα2-3Galβ1-4	SiaLe ^x
Fucα1-3 GlcNAcβ-sp 3-O-Su-Galβ1-4	3'-O-sulfo-Le ^x
Fucα1-3 Su-6-GlcNAcβ-sp Neu5Acα2-3Galβ1-4	6(GlcNAc)-O-sulfo-SiaLe ^x
GalNAcα-sp	T _n
Galβ1-3GalNAcα-sp	TF
Galβ1-3GalNAcβ-sp	T _{ββ} -disaccharide
GlcNAcβ1-6 GalNAcα- sp Galβ1-3	"core 2"
GlcNAcβ1-3 Galβ1-4Glcβ-sp GlcNAcβ1-6	"core 4"
Neu5Acα2-3Galβ1-4Glcβ-sp	3'-sialyllactose
Neu5Acα2-6Galβ1-4Glcβ-sp	6'-sialyllactose
GlcAβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-sp	desulfated HNK-1
3-O-Su-GlcAβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-sp	HNK-1

sp = -OCH₂CH₂CH₂NH- or -OCH₂CH₂NH-, Su = HSO₃⁻

background binding, even at high concentrations of an 'alien' antigen, B_{tri}, is absent.

An alternative method was used for improvement of the assay sensitivity, *i.e.*, a conjugate anti-mouse-AP in combination with a fluorescent substrate for AP (Fig. 4). Due to the cascade mechanism of signal enhancement, the sensitivity of this development method was one order of magnitude higher than that of Cy5 experiments. For comparison the ELISA and GlycoChip results are presented within one figure, see Fig. 5; concentration ranges for glycoconjugates used for the coating on solid phase are identical. It can be seen from Fig. 5 that slopes of the plots are also identical.

Study of monoclonal antibodies obtained from the Workshop

To perform the specificity characterization of a panel consisting of 12 mABs (7th Workshop and Conference on Human Leukocyte Differentiation Antigens, Harrogate, UK, 2000) obtained against human leukocytes (see Materials and methods), we prepared a GlycoChip coated with 21 carbohydrate antigens. We chose typical human cell surface antigens, which included the blood group antigens ABH and Lewis, tumor-associated and differentiation antigens, and adhesion molecule ligands. The full list of carbohydrate ligands used for the design of the GlycoChip is shown in Table 1. In practice one antibody was studied using one chip. Correspondingly, specificity of the Workshop mABs was verified with the help of a 96-well plate

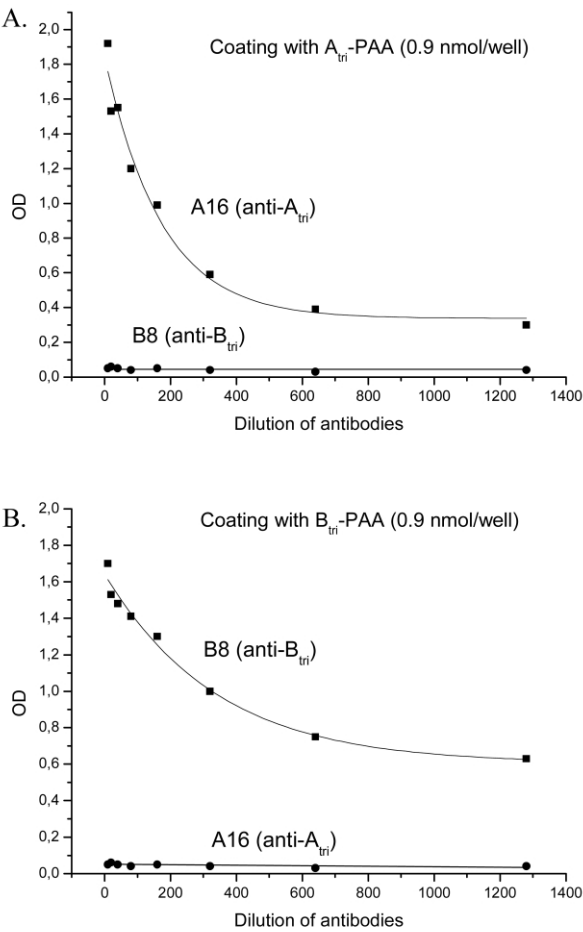


Fig. 2 Concentration dependence of anti-A_{tri} and anti-B_{tri} monoclonal antibodies binding to Sug-PAA coated onto 96-well polystyrene plates (conventional ELISA, see Materials and methods). (A) coating with A_{tri}-PAA; (B) coating with B_{tri}-PAA.

ELISA. The results obtained with the GlycoChip and conventional ELISA (Table 2) are practically identical. It should be noted that the working concentration of antibodies in the GlycoChip tests was one order of magnitude *higher* than that in a standard 96-well plate ELISA.

Discussion

The advantages of the microarray concept can be divided into two groups. The first one relates to miniaturization and concerns technical improvements, such as the decrease of reagent consumption, minimization of reaction volume, decrease of test duration, and the possibility of screening automation, *etc.* The second group comprises test efficiency improvement related to principal change of a solid phase surface and the replacement of an irregular physically adsorbed ligand to regularly oriented complexes.^{7–9}

In this study we have tested whether these expected advantages can be realized in practice by the transfer of the ELISA technique to the microarray format: a GlycoChip containing oligosaccharide antigens–ligands was immobilized using the streptavidin–biotin system. A limited number of papers^{10–13} is related to the design of glycochips. With one exception,¹² the cited works are based on the very limited array of simple saccharides, mainly monosaccharides and thus they should be considered as a preliminary model rather than research systems. One paper¹² describes polysaccharides that

were coated onto nitrocellulose. No papers were found in which an avidin–biotin system was used for the immobilization of a saccharide array.

It is known that the way of ligand immobilization on a solid phase determines to high extent the efficacy of a further test: theoretically, according to the law of mass action, the more ligand is coated onto a solid phase, the higher is the sensitivity, *i.e.*, a lesser amount of the protein in the study can be detected. Passive physical adsorption of the ligand on polystyrene (standard 96-well plates) is widely used due to the simplicity of the method. However, a serious disadvantage of physical adsorption is low efficiency. Thus, it has been demonstrated using a radioassay¹⁴ that only 2% of the taken conjugate is adsorbed on polystyrene, whereas the rest of the 98% is actually wasted (washed off) in the case of Sug-PAA conjugates. Similar results have also been documented for proteins, as cluster formation was observed during their passive adsorption, the most part of the polystyrene surface was resting antigen-free.¹⁵ Homogenous coating is principally possible in the case of chemical (covalent) ligand immobilization on a surface; moreover, strict ligand orientation towards the surface becomes tangible.^{8,9} If the surface is uniformly coated with streptavidin, the attachment of biotinylated ligands should additionally be even. Streptavidin–biotin ligand immobilization was used for the GlycoChip prepared for this study (see Fig. 1). We expected that in the case of applying the biotinylated ligands as a Sug-PAA-biot to a streptavidin-coated surface, a denser filling of the surface by the ligand than in the case of physical adsorption (common ELISA) would be possible.

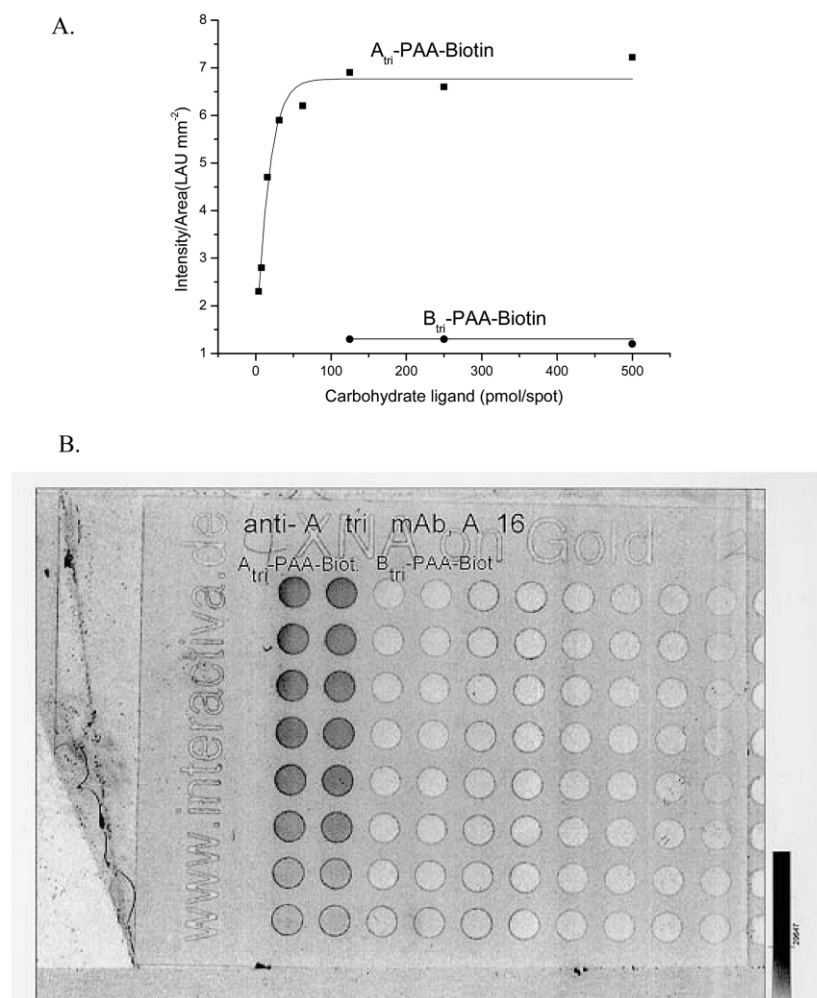


Fig. 3 Fluorometric detection of antibodies A16 binding with trisaccharides A and B immobilized on GlycoChip. (A) dependence of signal on the amount of coated trisaccharide; (B) GlycoChip scanned on Fluorescent Image Analyzer FLA-200. Bound antibodies were detected using anti-mouse Cy5 conjugate.

However, the GlycoChip and ELISA showed in practice almost identical results (Fig. 5), *i.e.*, it was necessary to coat the same amount of carbohydrate ligand to the dot (chip) as to the well (96-well plate) for the work at a maximally available sensitivity (plateau on the plots). Though the solution volume was less in the case of the GlycoChip, the glycoconjugate concentration was proportionally higher. Taking into consideration the above-mentioned low yield of Sug-PAA physical adsorption on polystyrene, it can be concluded that the immobilization yield of Sug-PAA-biot on XNAonGOLD™ is also rather low. Moreover, a one order of magnitude higher concentration of antibodies (*i.e.* protein in this study) was necessary to achieve a maximum level of further ligand binding with antibodies. This should be interpreted as the result of an even smaller (paradoxically) ligand density on the carrier surface in the case of the chip. The unexpectedly low degree of adsorption cannot be explained by generic particularities of Sug-PAA-biot, this being evidenced by the high yields during the modification of other avidin- or streptavidin-coated surfaces with this material,¹⁶ and by the fact that monomeric Sug-linker-biot did not produce better results with XNAonGOLD™ as compared to polymeric conjugates (data not shown). According to the manufacturer's data,⁴ the density of streptavidin residues on XNAonGOLD™ was close to what was theoretically possible. If one mole of streptavidin had bound one mole of Sug-PAA-biot, they would have also formed a continuous monolayer, as the molecule size is close to that of streptavidin. In this case binding with antibodies would have been principally better than in ELISA (where only 10–20% of the surface is coated with Sug-PAA)¹⁴ on polystyrene, however this was not the case. This means that the most part of the streptavidin

immobilized on XNAonGOLD™ is inaccessible for the binding of biotin in a composition of biotinylated glycoconjugates. Thus, not all the streptavidin-coated surfaces, including those designed on self-assembled monolayers and working successfully in an oligonucleotide hybridization assay, are suitable for the development of a GlycoChip with expected parameters, *i.e.*, the maximal possible density of carbohydrate ligand.

One should compare the GlycoChip and ELISA with other parameters besides sensitivity. The GlycoChip is more suitable with respect to consumed amounts of a tested sample (mABs in this particular case) due to a general miniaturization of the test. Though in the case of the GlycoChip the used antibody

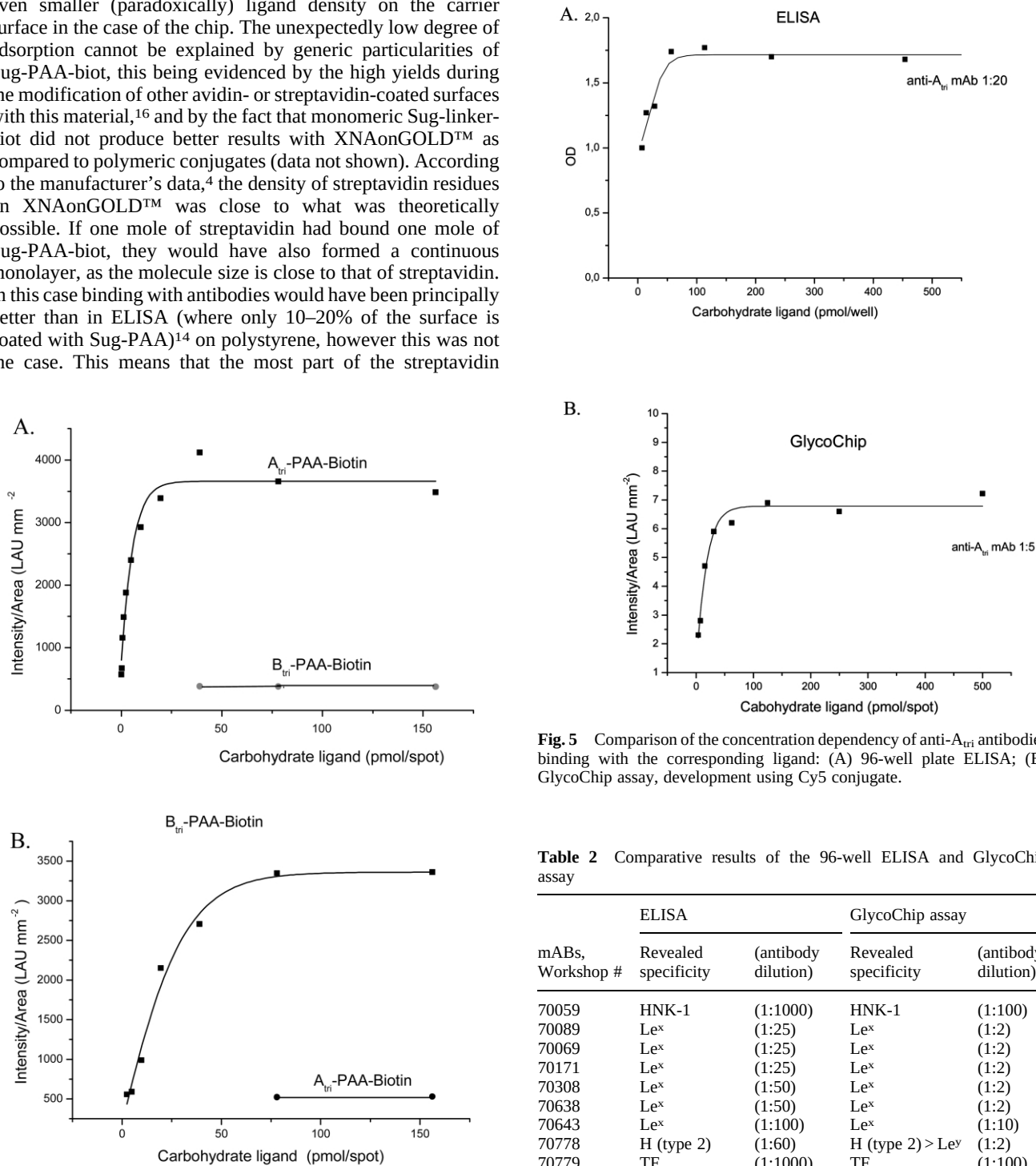


Fig. 4 Detection of antibodies bound to the GlycoChip using anti-mouse Ig-alkaline phosphatase conjugate: (A) anti-A_{tri} antibodies; (B) anti-B_{tri} antibodies. See details in Materials and methods.

Fig. 5 Comparison of the concentration dependency of anti-A_{tri} antibodies binding with the corresponding ligand: (A) 96-well plate ELISA; (B) GlycoChip assay, development using Cy5 conjugate.

Table 2 Comparative results of the 96-well ELISA and GlycoChip assay

mABs, Workshop #	ELISA		GlycoChip assay	
	Revealed specificity	(antibody dilution)	Revealed specificity	(antibody dilution)
70059	HNK-1	(1:1000)	HNK-1	(1:100)
70089	Le ^x	(1:25)	Le ^x	(1:2)
70069	Le ^x	(1:25)	Le ^x	(1:2)
70171	Le ^x	(1:25)	Le ^x	(1:2)
70308	Le ^x	(1:50)	Le ^x	(1:2)
70638	Le ^x	(1:50)	Le ^x	(1:2)
70643	Le ^x	(1:100)	Le ^x	(1:10)
70778	H (type 2)	(1:60)	H (type 2) > Le ^y	(1:2)
70779	TF	(1:1000)	TF	(1:100)
70780	Le ^y	(1:50)	Le ^y	(1:10)
70782	T _{ββ} > B _{tri} > TF	(1:400)	T _{ββ} > B _{tri}	(1:10)
70791	T _{ββ}	(1:50)	T _{ββ}	(1:10)

concentration was one order of magnitude higher than that in ELISA, an absolute amount of consumed antibodies was lower; consumption of the rest reagents was so much the lower in the case of the GlycoChip. As for the time necessary for the completion of the process from the ligand coating to the result reading, it proved to be comparable. However, it should be noted that the described experiments were in fact model ones with the use of a limited number of ligands and analyzed protein samples. It is obvious that in the case of large-scale screening, chip technology gets considerable odds.

The main positive point of this study is that the qualitative results of the antibody specificity test on the GlycoChip and in routine ELISA coincided. Though the polymeric conjugate bears several biotin residues per molecule, one of which serves the purpose of linking to the platform and remainders, which are rather hydrophobic ones, in essence stay free, there were no cases of false positive results (non-specific antibody binding). Thus, although the GlycoChip, which is based on a XNAon-GOLD™ platform, is far from optimal, the future prospects for further studies in this field are optimistic due to our results.

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