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Glycoprofiling with micro-arrays of glycoconjugates and lectins

S. Angeloni[1,2,5], J.L. Ridet[1,3,6], N. Kusy[6], H. Gao[2,5], F. Crevoisier[2,5], S. Guinchard[2,5], S. Kochhar[6], H. Sigrist[2,5], and N. Sprenger[4,6]

[5]CSEM S.A., Centre Suisse d'Electronique et de Microtechnique, Rue Jaquet-Droz 1, CH-2000 Neuchâtel, Switzerland, and [6]Nestlé Research Center, Nestec Ltd., Vers-chez-les-Blanc, CH-1000 Lausanne 26, Switzerland

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To facilitate deciphering the information content in the glycome, thin film-coated photoactivatable surfaces were applied for covalent immobilization of glycans, glycoconjugates, or lectins in microarray formats. Light-induced immobilization of a series of bacterial exopolysaccharides on photoactivatable dextran-coated analytical platforms allowed covalent binding of the exopolysaccharides. Their specific galactose decoration was detected with fluorescence-labeled lectins. Similarly, glycoconjugates were covalently immobilized and displayed glycans were profiled for fucose, sialic acid, galactose, and lactosamine epitopes. The applicability of such platforms for glycan profiling was further tested with extracts of Caco2 epithelial cells. Following spontaneous differentiation or on pretreatment with sialyllactose, Caco2 cells showed a reduction of specific glycan epitopes. The changed glycosylation phenotypes coincided with altered enteropathogenic *E. coli* adhesion to the cells. This microarray strategy was also suitable for the immobilization of lectins through biotin-neutravidin-biotin bridging on platforms functionalized with a biotin derivatized photoactivatable dextran. All immobilized glycans were specifically and differentially detected either on glycoconjugate or lectin arrays. The results demonstrate the feasibility and versatility of the novel platforms for glycan profiling.

Key words: exopolysaccharides/glycoarray/glycomics/glycosylation/lectins

Introduction

Carbohydrates (glycans) cover the surface of most (if not all) living cells and organisms in the form of diverse glycoconjugates. Glycans create a landscape of recognition sites, barriers, and carriers that help control the rhythms of metabolism from conception to catabolism. Generally, glycans represent the first and crucial interface to the cell's biotic and abiotic environment, mediating recognition and communication processes. Thus they control immunological recognition, cell–cell adhesion, pathogen attack, and protein folding and placement (Bertozzi and Kiessling, 2001; Karlsson, 1995; Varki, 1993).

Glycans are generally assembled of diverse monosaccharide building blocks that are glycosidically linked to each other at different positions. Consequently glycans show a high structural diversity reflecting in general functional diversity. Thus it is a multidisciplinary challenge to decode the information content displayed by glycan structures in various biological contexts (Fukui *et al.*, 2002; Gabius *et al.*, 2002; Nam *et al.*, 2003).

Lactic acid bacteria produce a wide spectrum of secreted high-molecular-weight exopolysaccharides (EPSs). These glycans can be formed of structurally diverse building blocks. In milk processing, EPSs are of significant interest because of their texturizing properties on milk fermentation. However, they might not only contribute to structural and biofilm-related properties but also serve as a decoy for unwanted agents such as enterotoxins or adhesins of pathogens. Moreover, EPSs might contribute to the establishment of a favorable matrix for beneficial microbial communities in natural environments, such as the intestine.

The surface glycosylation pattern of enterocytes changes during intestinal development. Most prominently there is a shift from a high sialic acid to high fucose displaying phenotypes (for review see Biol-N′garagba and Louisot, 2003). Concurrently the enterocyte microbial cross-talk is likely affected by such changes, leading to changed bacterial community structures and resistance or vulnerability of the host to pathogen insults.

EPSs and intestinal surface glycosylation patterns obviously encode information implicated in physiological and ecological processes. Deciphering this information content is thus an important prerequisite for an educated selection and design of strategies to promote either protection from unwanted noxious agents or promote wanted beneficial microbes.

One route to facilitate decoding efforts is the establishment of analytical platforms for robust and high-density display of glycan structures. Recent years have seen increasing needs for such platforms to investigate protein–carbohydrate interactions and specifically to identify ligands and test specificities of bacterial, plant, and animal lectins (for review see Drickamer and Taylor, 2002; Wang, 2003). Different types of microarray platforms were described for oligosaccharide, glycan, glycolipid, glycoprotein, and

[1]These authors contributed equally to this work.
[2]Present address: Arrayon Biotechnology, Jaquet-Droz 1, CH-2000 Neuchâtel, Switzerland.
[3]Present address: University of Fribourg, Dept. of Medicine, Rue du Musee 5, CH-1700 Fribourg, Switzerland.
[4]To whom correspondence should be addressed; e-mail: norbert.sprenger@rdls.nestle.com

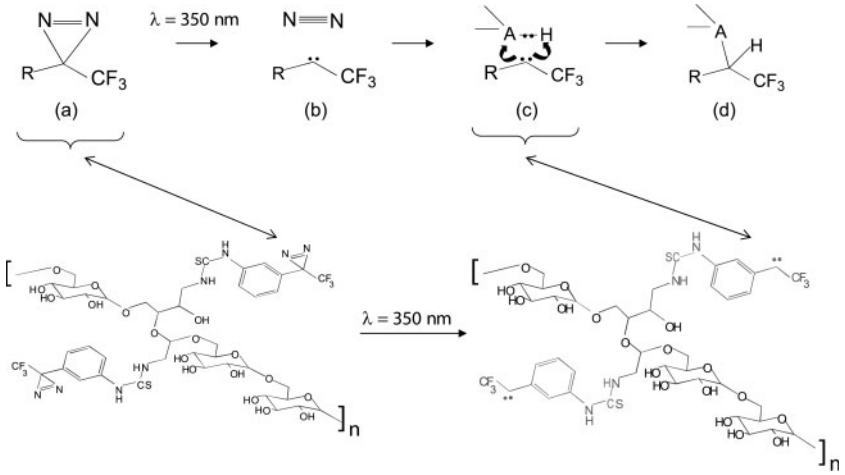

Fig. 1. Reaction mechanism of light-induced covalent immobilization of bio/molecules on a surface. Aryl-trifluoromethyl-diazirines (**a**), photoactivated at 350 nm generate highly reactive carbenes and release molecular nitrogen (N_2) (**b**). Carbenes spontaneously undergo insertion reactions (**c**) and form covalent bonds with the surface and/or biomolecules (**d**). Lower half of panel illustrates dextran derivatized with the photoactivateable aryl-trifluoromethyl-diazirines.

glycoconjugate display with most approaches based on noncovalent bonding (Espina *et al*., 2003; Fukui *et al*., 2002; Nam *et al*., 2003; Qian *et al*., 2002; Schwarz *et al*., 2003; Seong and Choi, 2003; Templin *et al*., 2003). Characteristics of the different platforms for glycoprofiling were recently reviewed (Feizi *et al*., 2003). Microarray-based platforms in microformats are of particular interest because they require small sample quantities, allow high-density sample display, and provide reliable assay sensitivity. Microarray platforms have an exceptional content capacity and permit the identification and quantification of thousands of molecules in one single experiment.

Despite the exorbitantly high theoretical number of possible molecular glycan configurations, the actual number of different glycan structures found in nature is likely only a minute fraction. For endogenous mammalian glycan structures, it is estimated to be in the range of 500 (Drickamer and Taylor, 2002). Thus, by having an appropriate surface at hand it is possible to display all natural glycan structures on a single microarray.

Here we present the use and applications of versatile microarray platforms based on dextran-coated glass slides (PhotoChips) suitable for (1) covalent glycan and (2) glycoconjugate immobilization and for (3) ligand-mediated bridging of functionlized biomolecules, such as lectins or oligosaccharides on OptoDex-biotin platforms. The dextran-based polymer OptoDex is derivatized with aryl-trifluoromethyl-diazirine groups (Barie *et al*., 1998; Caelen *et al*., 2002; Gao *et al*., 1994). On illumination, aryl-trifluoromethyl-diazirine groups form reactive carbenes (Figure 1), which eventually react with any vicinal molecule to form covalent bonds (Liu, 1992; Sigrist *et al*., 1995). As expected, this approach leads to covalent linkage of any molecule of interest to any surface of interest in one step. In combination with other functional groups such as biotin, the photoactivatable dextran polymer yields a higly versatile tool for surface (bio)engineering. The presented results show the feasibility of biomolecule binding to such microarray platforms and illustrate the versatility of the microarray based tools with different applications.

Results

EPS microarray

Bacterial EPSs from seven different lactic acid bacteria were covalently immobilized in microarray format aiming at the screening of functionally displayed glycan structures. To this end, the seven EPSs of known structures were dissolved in water and printed onto PhotoChips. On illumination, the aryl-trifluoromethyl-diazirine groups formed reactive carbenes and covalently linked the EPS to the dextran surface in one step. The structures of the repeating molecular features of the EPS are shown (Figure 2), together with the respective peanut agglutinin (PNA) and *Maackia amurensis* agglutinin (MAA) binding profiles. PNA bound preferentially to the EPS structure of *L. bulgaricus* 291 harboring a β-D-Galp-(1-4)-β-D-Glcp- side chain (Figure 2E, H), and also bound to the *L. helveticus* 59 EPS with a β-D-Galp-(1-6)-α-D-Glcp- side chain (Figure 2C, H). PNA did not bind to EPS with branches of single α- or β-Gal moieties or with terminal Gal in the furanose form (Figure 2A, B, D, F, G, H). Equally, the sialic acid–specific lectin MAA did not bind to any of the presented EPS structures. Therefore PNA bound preferentially to β-Gal residues on EPS, when they are spaced by a Glc from the EPS backbone (Figure 2).

These results illustrate the specific ligand binding of PNA lectin and, more important in the context of this investigation, show the feasibility of EPS immobilization on microarrays for functional screening and identification of the epitopes displayed on unknown EPS structures. Thus bacterial EPS structures displayed in microarray formats can in the future be screened for their ability to serve as a decoy for viruses and microbial adhesins and toxins.

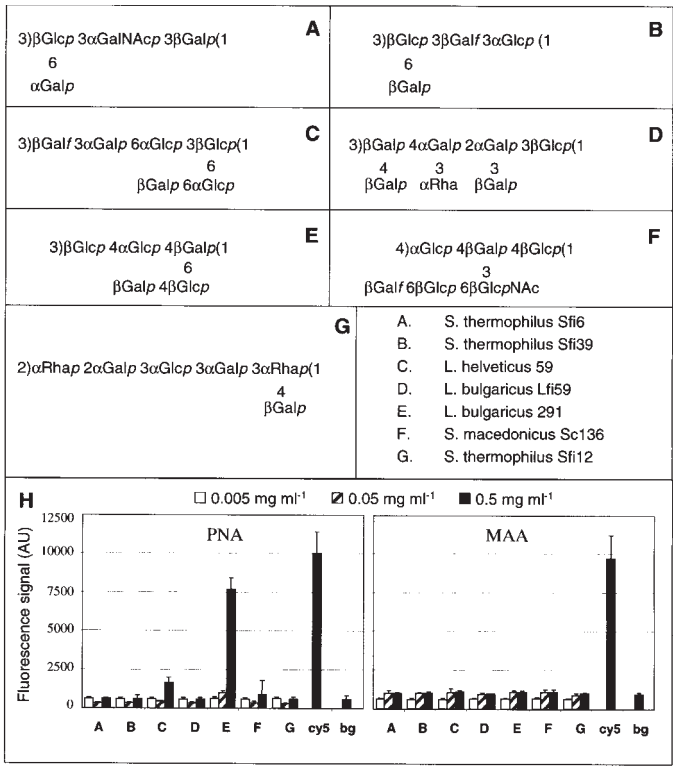

Fig. 2. Structures of the EPS repeating unit from different lactic acid bacteria (**A–G**) as dubbed in the figure, and detection of different concentrations of immobilized EPS by the lectins PNA and MAA (negative control) (**H**). Cy5-OptoDex (Cy5) is an internal fluorescent standard and bg indicates the measured background values. Mean signals and SDs are shown.

Glycoprotein microarray characteristics

As for glycans, a series of reference glycoproteins were immobilized covalently in microarray formats, again taking advantage of the carbene reaction. Resulting glycoprotein microarrays were probed with fluorophore-conjugated lectins to demonstrate feasibility of differential glycan profiling. Figure 3 depicts the detection of five glycoconjugates together with bovine serum albumin (BSA) as control protein with six different plant lectins. All tested lectins primarily recognized specific glycan structures as predicted from their respective binding specificities (Table I, Figure 3). All lectins showed excellent signal-to-background ratios. Virtually no binding to the BSA control protein was detected with either lectin (Figure 3).

MAA recognized glycans displayed on fetuin as predicted and to a much lower extent on asialofetuin. The latter might be due to incomplete desialylation (Figure 3A). PNA recognized best glycans displayed on asialofetuin, and also recognized lactose and 2′fucosylated lactose linked to BSA (Figure 3A). The former two results are expected from the known PNA ligand specificity. *Ulex europaeus* agglutinin (UEA) and *Tetragonobolus* lectin (TPA) bound only to fucosylated lactose (Figure 3CE). *Datura stramonium* agglutinin (DSA) showed preferred binding to asialofetuin as predicted but also measurable binding to fetuin and fucosylated lactose (Figure 3D). Finally, *Sambucus nigra* agglutinin (SNA) recognized transferrin and fetuin (Figure 3F), which terminally expose α-2,6-linked sialic acid residues.

The presented findings indicate that glycoprotein display and respective glycan profiling on PhotoChips are (1) possible. Glycan arrays yield (2) appreciable signal-to-noise ratios, and (3) quantifiable differential glycan profiles. The display of oligosaccharide structures in the form of neoglycoconjugates (e.g., 2′fucosyllactose on BSA, lactose on dextran) represents a facile approach allowing for covalent oligosaccharide immobilization on PhotoChips.

Different concentrations of OptoDex-lactose (a photactivatable dextran-based neoglycoconjugate; Caelen *et al.*, 2002; Chevolot *et al.*, 2001), were immobilized on PhotoChips to assess signal quantitation, glycoconjugate binding capacities, and detection limits. The displayed glycans were detected and quantified with fluorescence-labeled PNA. The response is dose-dependent (Figure 4) and does not reach saturation at the concentrations used. Linearity of response is best achieved at print solution concentrations between 0.5 and 1 mg/ml. The detection limit is somewhere below 0.25 mg/ml print solution dispensed, corresponding to about 12 pg probe molecule applied per spot. In parallel, a dilution series of fluorophore-conjugated dextran was printed and quantified. The results showed linearity of the recorded fluorescence signal and demonstrated that the surface binding capacity did not saturate at the concentrations used. Moreover, the experiments illustrated good spot-to-spot reproducibility.

Similarly, different concentrations of reference glycoproteins were printed, and their glycans were detected and quantified with specific lectins (Figure 5). PNA and DSA detection of asialofetuin showed saturation (Figure 5A, C) at 2.5 pg asialofetuin applied (50 pl of 0.05 mg/ml), and the detection limit was below 0.5 pg per spot. Detection of fetuin-linked glycans with MAA did not saturate at the concentrations employed, and the detection limit was around 2.5 pg (Figure 5B). The results suggest that fetuin can be immobilized at still greater amounts (>12.5 pg) than asialofetuin (2.5 pg) or, more likely, the results reflect the different binding affinities between MAA, DSA, and PNA. For DSA and PNA, response linearity is best achieved at asialofetuin print solution concentrations between 0.01 and 0.05 mg/ml. For MAA, linearity of response is attained with fetuin print concentrations between 0.075 and 0.25 mg/ml. The reference glycoproteins were selected to serve as positive controls when assaying glycoprotein arrays.

Application: glycan profiling of model cell extracts

Extracts of Caco-2 intestinal cells were arrayed and immobilized on PhotoChips to test the applicability of the glycoprotein microarray platform for glycan profiling. Displayed glycans were profiled and quantified using a series of plant lectins (Figure 6).

Caco-2 cells at 7 days postseeding (i.e., nondifferentiated) were compared with cells grown for 21 days (i.e., differentiated), either mock-treated or treated with 3′sialyllactose for 48 h prior to harvest. Relative glycan expression levels

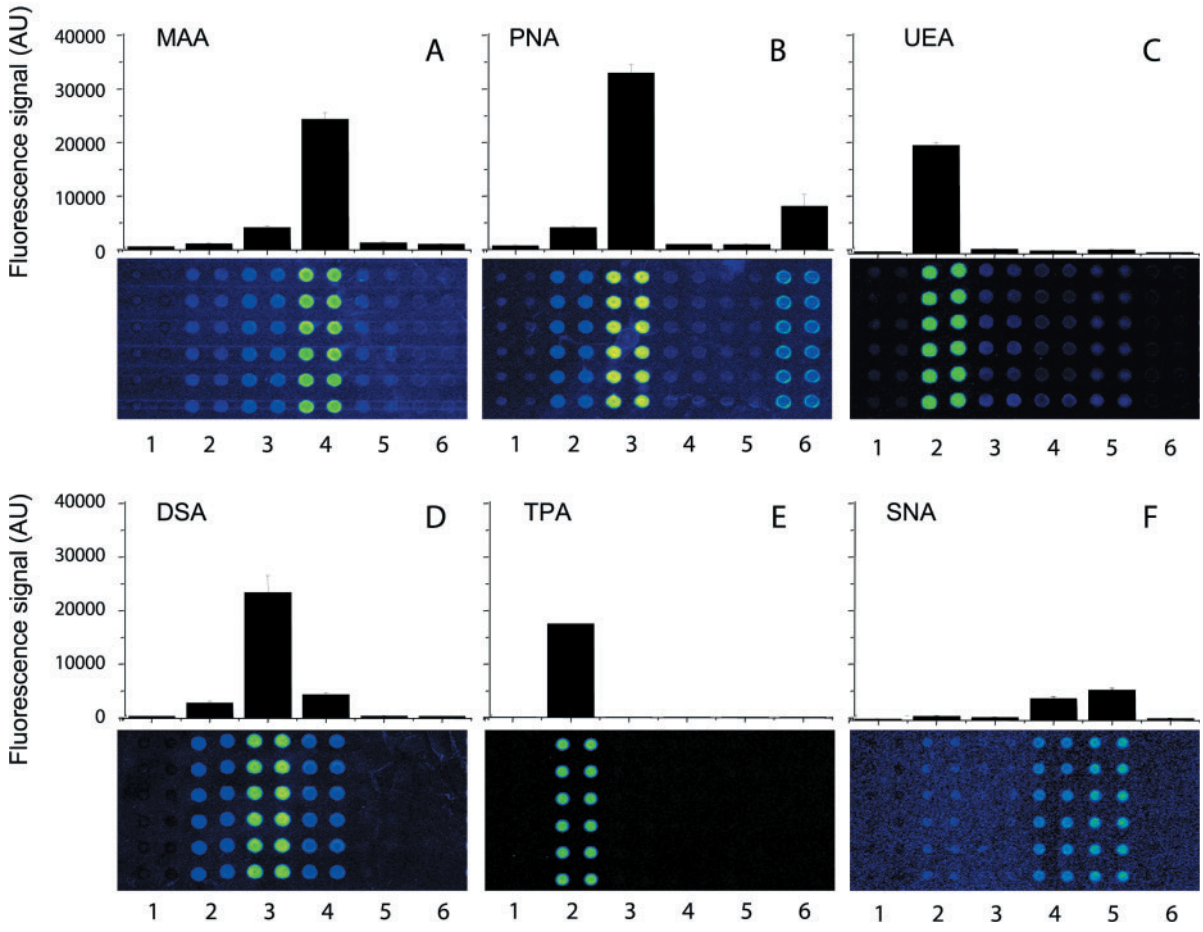

Fig. 3. Printed BSA (1), reference glycoproteins 2′fucosyllactose-BSA (2), asialofetuin (3), fetuin (4), transferrin (5), and the neoglycoconjugate OptoDex-lactose (6) detected by lectins MAA (**A**), PNA (**B**), UEA (**C**), DSA (**D**), TPA (**E**), and SNA (**F**). The lower panel of each part shows the actual spot image, false colored with yellow, green, blue in the order of decreasing signal intensity. Mean signals and SDs are shown.

Table I. Known glycan recognition specificities of plant lectins used in this study

Abbreviation	Full name	Glycan recognized	Protein/ glycoconjugate control
PNA	Peanut agglutinin	Gal-β-1,3-GalNAc-R	Asialofetuin
		Gal-R	OptoDex-lactose
UEA	*Ulex europaeus* agglutinin	Fuc-α-1,2-Gal-R	2FL-BSA
TPA	*Tetragonolobus purpureas* agglutinin	Fuc-R	2FL-BSA
SNA	*Sambucus nigra* agglutinin	SA-α-2,6-Gal-R	Fetuin, transferrin
MAA	*Maackia amurensis* agglutinin	SA-α-2,3-Gal-R	Fetuin
DSA	*Datura stramonium* agglutinin	Gal-β-1,4-GlcNAc-R	Asialofetuin
		GlcNAc-R	Asialofetuin

Data complied from datasheets of respective lectins.

recognized by MAA (i.e., α-2,3 linked sialic acid) decreased most prominently between the 7 and 21 days cultured cells (Figure 6A). Further, lower glycan expression in 21 days as compared to 7 days cultured cells was also found for α-2,6-linked sialic acid containing glycans seen by SNA, fucose-containing glycans seen by UEA and TPA, and galactose-ending glycans seen by PNA (Figure 6B–E). In contrast, glycan moieties targeted by DSA (i.e., lactosamine) did not markedly change over time (Figure 6F).

Interestingly, 3′sialyllactose treatment of 7 days cultured cells moved the expression of glycans seen by MAA, SNA, UEA, TPA, and PNA from elevated levels down to levels comparable to those found for mock-treated 21 days cultured cells (Figure 6A–E). Thus, α-2,3- and α-2,6-linked sialic acid residues diminished together with fucose and galactose moieties. Gene expression levels of sialyltransferases responsible for the formation of α-2,3-linked sialic acids were monitored (data not shown). The results showed that 3′sialyllactose treated—as compared with mock-treated 7 days cultured cells—reduced expression levels of *ST3Gal-I* (2.5×), *-II* (2×), and *–IV* (5×). *ST3Gal-III*, *-V*,

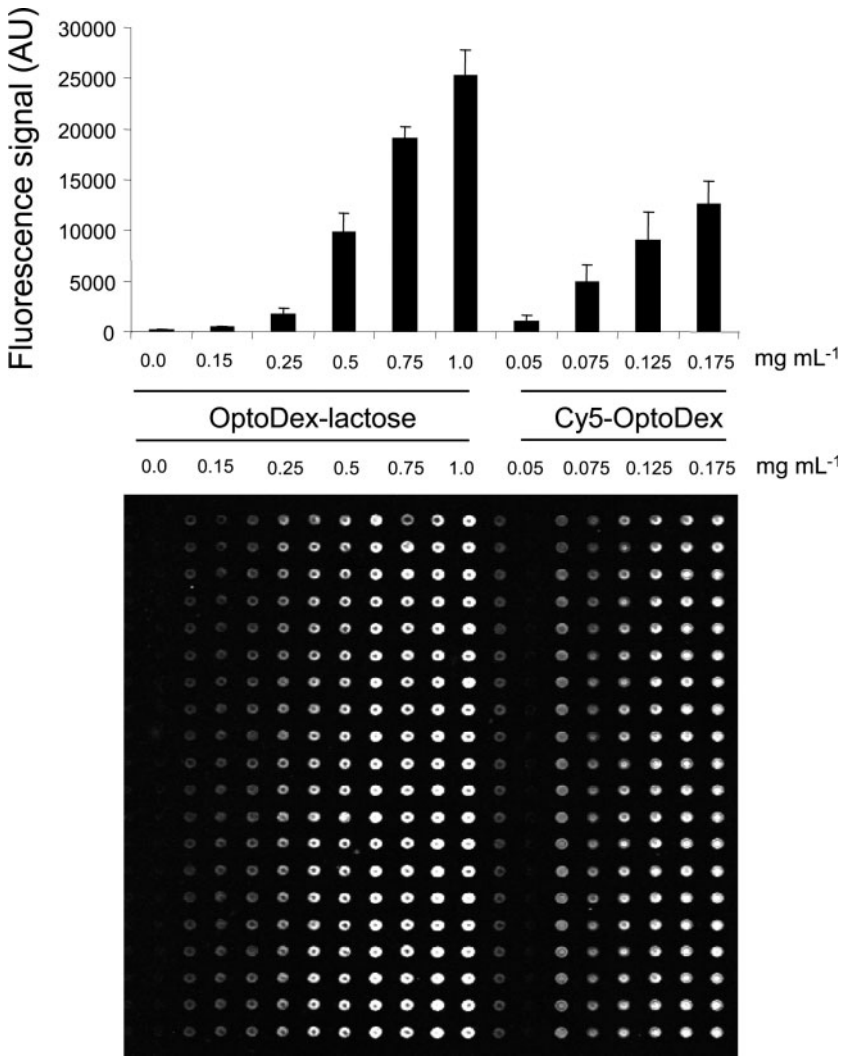

Fig. 4. Dilution series of OptoDex-lactose detected by fluorescent lectin PNA (Cy5-PNA) together with fluorescent OptoDex (Cy5-OptoDex). Indicated print solution concentrations are in mg/ml. The lower half shows the actual recognized spots of the array with decreasing signal intensities. Mean signals and SDs are shown.

and *-VI* were not affected. Thus reduction of MAA-detected glycans was reflected by a reduction in respective gene expression levels.

Additionally, cell extracts were spotted manually onto nitrocellulose membranes for lectin staining and signal quantification (data not shown). The results confirmed the observation made using the microarray platform: Cell glycosylation phenotypes changed with increasing culture time and responded to oligosaccharide addition to the growth medium. This conclusion is further supported by the following finding. Lactosamine-containing glycans recognized by DSA showed lower expression levels in 7 days cultured cells on treatment with 3′sialyllactose as compared to mock-treated cells, whereas levels were equally high in 7 days and 21 days control cells and 21 days 3′sialyllactose-treated cells (Figure 6F). Seven days cultured Caco-2 cells apparently sensed the presence of 3′sialyllactose in the growth medium and responded by changing glycan expression profiles, and 21 days cultured cells did not respond to the oligosaccharide treatment.

Bacterial challenge

To evaluate the significance of the observed glycan changes in the context of bacterial–epithelial cell cross-talk, 3′sialyllactose-, lactose-, and mock-treated cells were challenged with an enteropathogenic *Escherichia coli* (EPEC). Total bacterial adherence to such pretreated cells was monitored (Figure 7). About 10 times more bacteria attached to 7 days cultured cell (~40% of added bacteria) as compared to 21 days cultured cells (~3% of added bacteria). On pretreatment of 7 days cultured cells with 3′sialyllactose and subsequent washing of the cells, EPEC adherence was reduced to about 50% as compared with the lactose- or the mock-treated control cells (Figure 7). This was seen in an EPEC adherence reduction from 35% to 40% of added bacteria to about 20%. EPEC adherence to 21 days cultured cells either

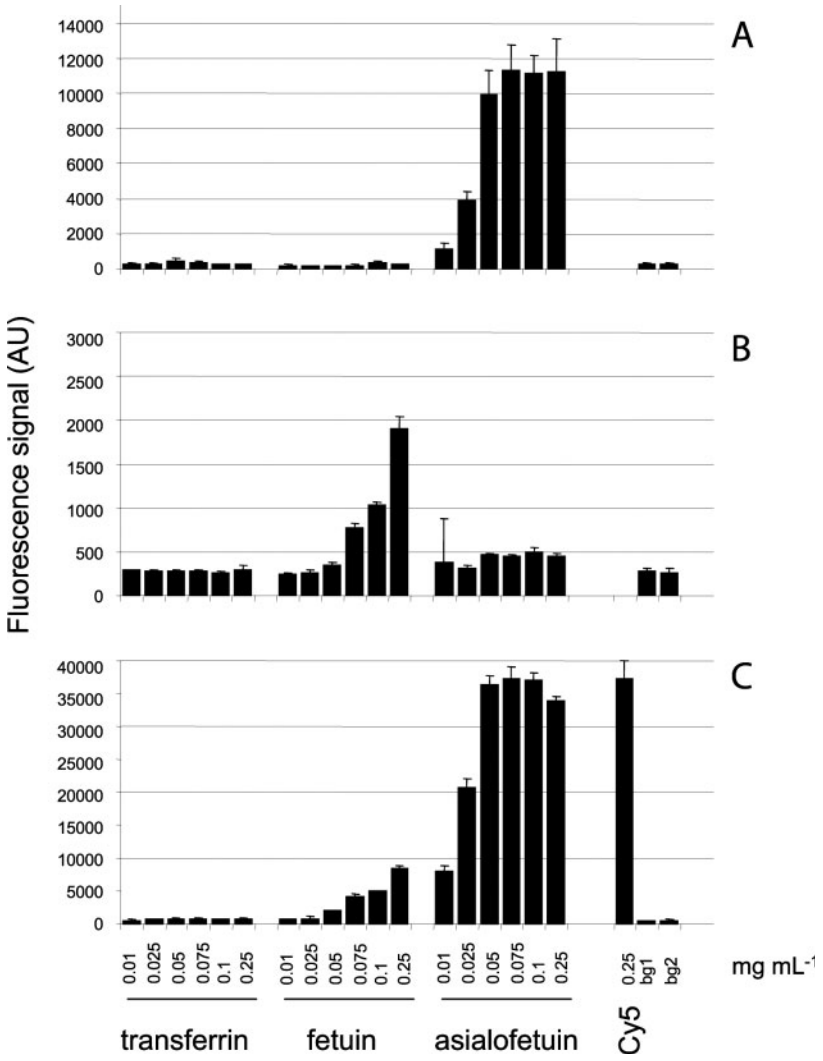

Fig. 5. Dilution series of glycoproteins detected by fluorescent lectins PNA (**A**), MAA (**B**), and DSA (**C**). Cy5-OptoDex (Cy5, **C**) was printed as an internal reference (0.25 mg/ml). Amounts indicated are in mg/ml. bg1 indicates background inside the array grid, bg2 indicates background outside the array grid (indicating the efficiency of unspecific binding suppression). Mean signals and SDs are shown.

mock-, lactose-, or 3′sialyllactose-pretreated was indifferent and, as mentioned, reduced by about 90% as compared to 7 days cultured mock-treated control cells. The reduced EPEC adherence to 21 days as compared to 7 days cultured cells coincided with lower glycan expression levels as determined by lectin-based glycan profiling described.

Lectin microarray

To further explore the application range of the OptoDex-based platforms for microarraying, in particular the Opto-Dex-biotin platform, four plant lectins were immobilized through a biotin-neuravidin-biotin bridge and probed with fluorophore-labeled glycoconjugates (Figure 8). Differential glycan profiles were detected with the immobilized lectins. OptoDex-lactose bound primarily to immobilized PNA, asialofetuin to DSA and to a lesser extent to PNA, fetuin to SNA, lactosamine-BSA to DSA, 2′fucosyllactose-BSA to UEA, and transferrin to SNA (Figure 8).

Signal-to-noise ratios were comparable to and as good as for the glycoprotein arrays. Homogeneous lectin binding to individual spots was achieved on addition of glycerol or a mock protein, such as BSA, to the print solution.

The lectin array technology complements antibody arrays, previously shown to be functional on immobilization on PhotoChips and diverse functional platforms (Gao *et al.*, 2003). Hybrid lectin/antibody arrays may serve as profiling tools, not only for glycan expression but also for the detection of specific immunoreactive markers.

Discussion

The insight that glycan structures are of key importance for numerous biological processes in health and disease lead to the recent need for the development of high-throughput platforms for analysis of glycan information content. Thus the past few years have seen increasing numbers of studies

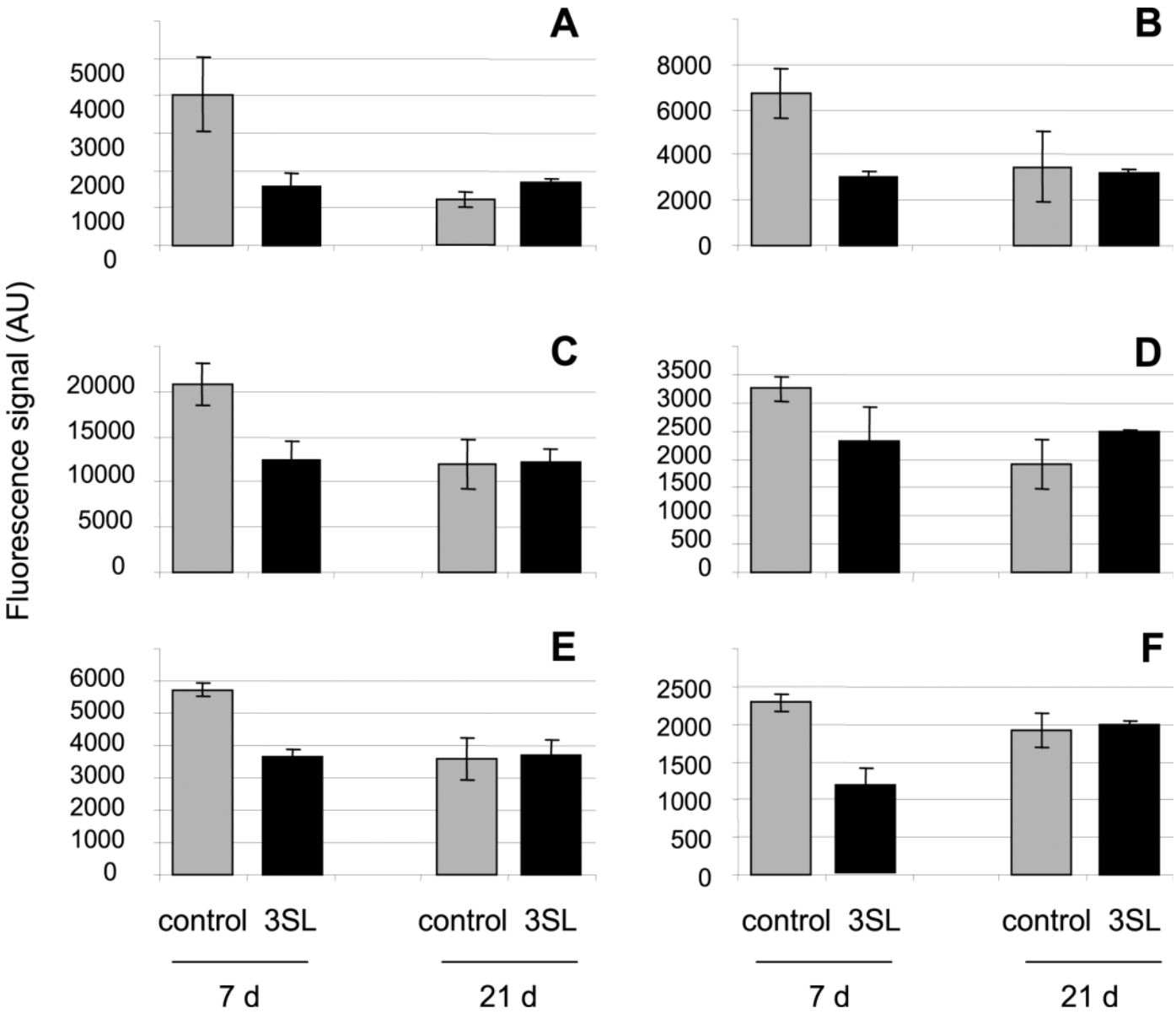

Fig. 6. Glycan display profiles of mock treated control and 3′sialyllactose (3SL)-treated cells at 7 days and 21 days in culture. (**A**) Sialic acid–linked α-2,3 detected by MAA; (**B**) sialic acid linked α-2,6 detected by SNA; (**C**) fucose linked α-1,2 detected by UEA; (**D**) fucose detected by TPA; (**E**) galactose detected by PNA; (**F**) lactosamine detected by DSA. Error bars indicate SDs ($n = 2$).

reporting the development and use of microarray-based glycan display platforms (Feizi *et al*., 2003). Mono- and oligosaccharides were covalently linked to gold or modified glass and plastic surfaces and thus became accessible for functional carbohydrate–protein interaction measurements (Houseman and Mrksich, 2002; Park and Shin, 2002; Schwarz *et al*., 2003). Furthermore nitrocellulose- and polystyrene-coated surfaces were reported as suitable platforms for microarray format display of biomolecules, such as neoglycolipids (Fukui *et al*., 2002), glycoproteins and glycans (Willats *et al*., 2002). However, as for microtiter plate-based display of biomolecules, such as glycoproteins, these nitrocellulose- and polystyrene-based platforms rely on noncovalent adsorptive immobilization.

Here we described the use of PhotoChips and OptoDex-biotin platforms for covalent and ligand-mediated immobilization of biomolecules on microarray formats. Procedures related to PhotoChips enabled single-step covalent immobilization of (1) bacterial glycans, (2) reference glycoproteins and neoglycoconjugates, and (3) cell extracts. Furthermore, OptoDex-biotin platforms allowed for bridging biotinylated lectins through neutravidin. All mentioned immobilized biomolecules remained biologically active and allowed on-array functional protein–carbohydrate interaction measurements. Differential recognition profiles were in accordance with the available structural and compositional information on the profiled reference compounds.

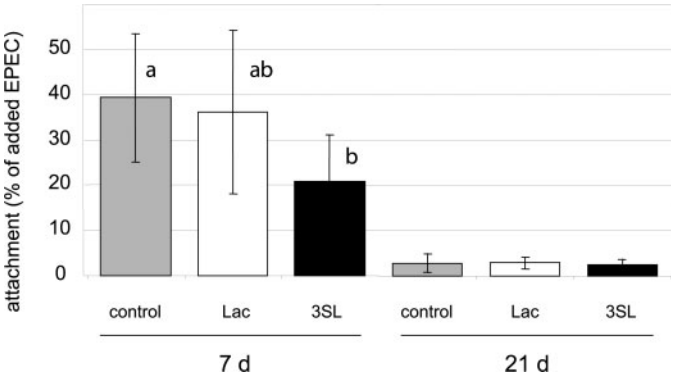

Fig. 7. EPEC challenge of Caco2 cells grown 7 days and 21 days, which were either mock-pretreated (control), lactose (lac)-, or 3′sialyllactose (3SL)-pretreated and washed before bacterial challenge. Error bars indicate SDs ($n = 6$). Statistical analysis was done using unpaired *t*-test, *p*-value 0.0265.

The microarray investigations presented here extend the versatility of the nitrocellulose and polystyrene surfaces for glycoconjugate and glycan display. PhotoChips allow for direct covalent biomolecule immobilization through reactive carbenes, and OptoDex-biotin platforms enable neutravidin mediated display of biotinylated biomolecules (Barie *et al.*, 1998; Gao *et al.*, 1994, 2003).

It is worth noting that all dextran-based platforms are hydrophilic and suppress nonspecific binding. The dextran network provides a stabilizing environment for biomolecules and increases the surface for biomolecule display. All features are essential for reliable detection of biological interactions. The one-step covalent immobilization of unmodified biomolecules on PhotoChips is a most direct immobilization approach. It is highly valuable for glycan immobilization, the binding of high-molecular-weight neoglycoconjugates and glycoproteins. Although this study focuses on the immobilization and arraying of glycoproteins and bacterial EPS, it is equally foreseeable to covalently crosslink via the photoactivatable dextran other glycan preparations, such as those derived from plant, fungal, and bacterial cell walls. Direct covalent immobilization of lipid- or polyacrylamide-linked neoglycoconjugates (Bovin, 2002; Feizi and Childs, 1994) has been demonstrated with nitrocellulose coated surfaces (Feizi *et al.*, 2003; Fukui *et al.*, 2002).

Oligosaccharide immobilization presented here, required precoupling to proteins, for example, 2′fucosyllactose-BSA, or to dextran, for example, OptoDex-lactose. Alternatively, oriented immobilization via biotin/neutravidin allowed for functional on-array protein–carbohydrate recognition. To this end, dual-functionalized dextran was chosen having photoactivatable groups in combination with biotin.

With increasing numbers of immobilization techniques, it should be interesting and important to perform comparative glycan–protein interaction studies using differently immobilized glycans to assess the influence of the immobilization protocol and the platforms on the interaction analysis (Feizi *et al.*, 2003; Leteux *et al.*, 1999).

For lectins, direct cross-linking through the photoactivatable carbenes was attempted, and lectins could be bound to the surface. However, carbohydrate binding activity was lost in this approach. This was likely due to structural rearrangements of the oligomeric lectins. On the other hand, biotin-neutravidin-biotin bridging yielded functional lectin arrays. In the future, these lectin microarrays might be combined with array of antibodies (Gao *et al.*, 1994, 2003) to have at hand microarrays specifically designed to assess the physiological status of cells or tissues.

To apply the dextran platform for microarray-based glycan quantification, cell extracts of intestinal model cells were printed and covalently linked on PhotoChips. Displayed glycans were profiled and quantified with plant lectins. As known for intestinal surface glycosylation occurring during development (Biol-N′garagba and Louisot, 2003), cell lines changed glycosylation phenotypes depending on the time in culture or differentiation status, respectively. Most prominently, α-2,3-linked sialic acid epitopes diminished from 7 days to 21 days in culture, which reflects a similar pattern as observed *in vivo* during development. Interestingly, treatment of 7 days cultured cells with the oligosaccharide 3′sialyllactose led to decreased glycosylation as compared to mock-treated cells of the same time in culture. For α-2,3-linked sialic acids, this reduction correlated with a reduced gene expression level for respective genes *ST3Gal-I*, *-II*, and *-IV*, whereas the other *ST3Gal* genes were not affected.

In summary, such 3′sialyllactose-treated 7 days cultured cells showed glycosylation patterns similar to 21 days cultured control cells. The only exceptions were lactosamine epitopes, which were reduced on oligosaccharide treatment of 7 days cultured cells but remained unaltered over time in mock-treated controls. Pretreatment of 7 days cultured cells with 3′sialyllactose led to about 50% reduction of EPEC adhesion as compared with lactose- or mock-pretreated cells. The reduced adherence correlated with reduced sialic acid and lactosamine epitopes. Both sialic acid and lactosamine epitopes were previously proposed to play a key role in EPEC adherence to Chinese hamster ovary cells (Vanmaele *et al.*, 1995). Furthermore, lactosamine moieties were also shown to alter EPEC localized adherence phenotype (Cravioto *et al.*, 1991; Vanmaele *et al.*, 1999). Considering a scenario with lactosamine as key player might be valid for the 7 days cultured cells. However, another mechanism should come into play to explain the overall reduced EPEC adherence to 21 days as compared to 7 days cultured cells, because (1) lactosamine levels were comparable between the mock-treated 7 days and 21 days cultured cells, and (2) total EPEC adherence was much lower to 21 days cultured cells as compared with 7 days cultured and 3′sialyllactose-pretreated cells.

Future studies will elaborate the connection between the expressed sialylated and lactosamine-displaying glycans on the cells and the adherence of EPEC. To this end, it might be possible to perform on-array adhesion analysis using bacteria or potentially viral particles. For mammalian cells, however, the ~150 μm spot diameter of the herein presented microarray platform might be limiting as compared to another recently presented glycan array that was exploited for cell adhesion studies (Nimrichter *et al.*, 2004).

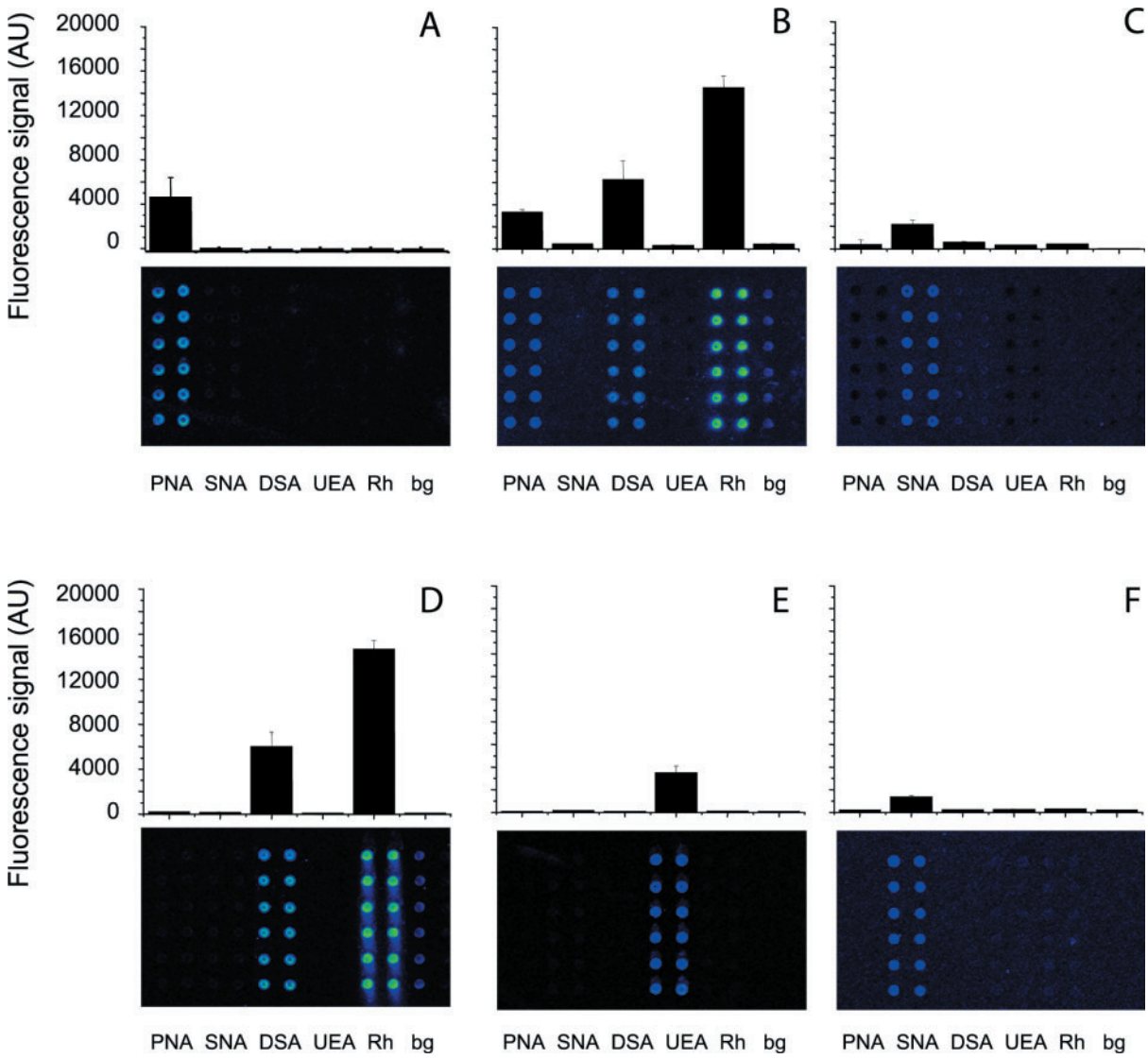

Fig. 8. Lectin microarray probed with Cy5-OptoDex-lactose (**A**), Cy3-asialofetuin (**B**), Cy5-fetuin (**C**), Cy3-lactosamine-BSA (**D**), Cy5-2′fucosyllactose-BSA (**E**), and Cy5-transferrin (**F**). Rh: rhodamine and biotin double-labeled dextran as internal standard (detectable using the Cy3 scanning set-up), bg: background. Mean signals and SDs are shown.

Materials and methods

Biomolecule preparation

Lectins for Cy3 or Cy5 labeling were purchased from Sigma (St. Louis, MO): PNA, UEA, MAA, SNA, DSA, and TPA. Biotinylated lectins were purchased from Vector Laboratories (Burlingame, CA): biotinylated PNA, biotinylated SNA, biotinylated DSA, biotinylated UEA. Rhodamine and biotin double-labeled dextran was a product of Molecular Probes (Leiden, Netherlands). PhotoChips and OptoDex-Biotin platforms were obtained from CSEM (Switzerland).

Control glycoproteins were either purchased from Sigma (transferrin, fetuin, asialofetuin, BSA) or from Dextra Laboratories (Reading, UK; 2′fucosyllactose-BSA, lactosamine-BSA). OptoDex-lactose was prepared by derivatizing amino-group functionalized OptoDex with mono(lactosylamido)mono(succinimidyl)suberate (Pierce, Rockford, IL). The reaction product was purified by gel filtration.

Caco-2 cells were seeded at a concentration of 100,000 cells/ml and grown at 37°C (10% CO_2) in Dulbecco's modified Eagle medium containing 3.7 g/L $NaHCO_3$; 1 g/L D-glucose, fetal bovine serum (FBS) (#2-01F00-1, 10% for Hep-2 and 20% for Caco-2 cells), 200 mM glutamine (Gibco BRL #25030-024, Rockville, MD) and penicillin/streptomycin (10,000 IU/ml–10,000 UG/ml, Gibco BRL #15140-114). Cells were grown for up to 21 days before harvest.

Cell cultures were treated with medium without FBS or with the same medium supplemented either with lactose or 3′sialyllactose at concentrations of 2 mg/ml. Cells were treated for 2 days prior to harvesting, and treatments were renewed after 1 day.

For extraction, cells were washed in phosphate buffered saline (PBS), scraped from the culture wells, and collected in microcentrifuge tubes. Cells from one well of a six-well plate were resuspended in 0.5 ml PBS supplemented with protease inhibitors (Complete, Roche, Switzerland). Cell extracts were homogenized by successive passing through a 25-gauge syringe needle. Homogenized extracts were frozen on dry ice and kept at −80°C until printing. Just before printing, samples were homogenized again as before.

Bacterial EPSs were isolated and prepared according to a standard protocol (Stingele *et al.*, 1996). Briefly, proteins were removed from spent culture supernatants by trichloracetic acid precipitation (20% v/v final trichloracetic acid). After protein removal by centrifugation, EPSs were precipitated on addition of acetone (final 50% v/v). Precipitated EPSs were recovered in water, and the solution was adjusted to pH 7 before dialysis against water for 24 h. After removal of insoluble material, EPSs were freeze-dried. Before printing, EPSs were dissolved in PBS at a concentration of 0.5 mg/ml.

Biomolecule immobilization

PhotoChips and OptoDex-biotin platforms in slide formats were used as analytical platforms. On printing as detailed later, the arrayed platforms were illuminated for 4 min with high-power ultraviolet lamp (1000 W) at 365 nm (11 mW/cm^2). After rinsing, OptoDex-biotin platforms were incubated with neutravidin (0.05 mg/ml) in PBS for 30 min at ambient temperature.

Biomolecules were arrayed with a pin-and-ring printer (Affymetrix Arrayer 417), applying ~50 pl per spot, which gave an average spot diameter of ~150 μm. Print automation was supported by the instrument specific Affymetrix 417 Arrayer Software.

PhotoChips with printed biomolecules were irradiated as described to effect covalent probe molecule immobilization. After photobonding, the surfaces were rinsed with PBS containing 1% BSA (once), PBS/Tween-20 (3 times), PBS (3 times), and deionized water (3 times).

Biotinylated lectins were prepared in HEPES buffer supplemented with divalent ions (Mg^{2+}, Mn^{2+}, Ca^{2+}, 1 mM each) and BSA (0.5 mg/ml) for printing. Printed slides were incubated for 10 min before rinsing them with Tris-buffered saline (TBS) complemented by Tween-20 (0.05%; TBS-Tween) and free biotin (0.5 mg/ml) for 10 min. Thereafter slides were rinsed once each with TBS-Tween, TBS, and deionized water.

All processes related to platform handling, printing, and photoimmobilization were carried out in a dedicated clean room. Prepared microarrays were vacuum-sealed and stored at 4°C (lectin arrays) or −20°C until used.

Array probing

Cyanine reagents (Cy5 or Cy3), either monofunctional or bifunctional, were used for fluorescence labeling of target biomolecules according to manufacturer's instructions (Amersham Biosciences, Little Chalfont, UK). For lectin labeling, free dye was removed by size exclusion chromatography on prepacked PD10 columns preequilibrated with TBS buffer at pH 7.2 supplemented with divalent ions (Mg^{2+}, Mn^{2+}, Ca^{2+}, 1 mM each, TBS+) at room temperature. Labeled samples were stored at 4°C in the dark.

Refrigerated platforms with arrayed biomolecules were allowed to warm to room temperature for at least 30 min before rehydration. To this end, slides were sequentially rinsed for 5 min each in PBS-BSA (1% w/v), PBS-Tween 3 times, and deionized water. Thereafter, a frame was applied to the slides (Gene Frame, ABgene, 65 μl) to generate an incubation chamber around the printed spots.

Fluorescence-labeled lectins were diluted to a final concentration of 0.02 mg/ml in TBS+ buffer complemented by blocking reagent (final 10% v/v, Roche). Each slide was incubated with 65 μl lectin medium for 30 min at room temperature in the dark. Thereafter slides were rinsed for 5 min each with PBS-BSA (1% w/v), PBS-Tween 3 times, PBS 3 times, and deionized water. Slides were air-dried and kept in the dark before fluorescence reading.

For lectin arrays on OptoDex-biotin platforms, all incubations were carried out in TBS+ buffer. Incubation and rinsing steps were as described, however, slides were not allowed to dry before the final wash.

Microarrays were read using a gene array scanner (Affymetrix 428Array Reader) and signals were quantified using the ImaGene software package (Biodiscovery, El Segundo, CA).

Cell culture and bacterial challenge

Caco-2 cells were seeded and treated as described. Before bacterial challenge, Caco-2 cells were washed twice with PBS. EPEC strain E2348/69 was grown overnight at 37°C without shaking in BHI supplemented with 10 μCI/ml ^{3}H-thymidine (Pharmacia). Bacteria were washed in PBS and adjusted to an OD_{600} of 0.1 in PBS. Fifty microliter of bacterial solution were added to 1 ml Dulbecco's modified Eagle medium without FBS and then applied onto pretreated and washed Caco2 cells in 12-well plates. Two hours later, cells were washed twice with PBS. Cells were lysed with 0.5 ml 0.5 N NaOH. Thereafter scintillation cocktail was added and radioactivity counted in a scintillation counter. An aliquot of labeled bacteria was kept to determine total counts added to the cells in order to calculate the % of adherent bacteria. Mean bacterial attachment ($n = 6$) and SD are presented.

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Abbreviations

BSA, bovine serum albumin; DSA, *Datura stramonium* agglutinin; EPEC, enteropathogenic *Escherichia coli*; EPS, exopolysaccharide; FBS, fetal bovine serum; MAA,

Maackia amurensis agglutinin; PBS, phosphate buffered saline; PNA, peanut agglutinin; SNA, *Sambucus nigra* agglutinin; TBS, Tris-buffered saline; TPA, *Tetragonobolus* lectin; UEA, *Ulex europaeus* agglutinin.

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