Glycan Arrays

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MALDI-TOF Mass Spectrometric Analysis of Enzyme Activity and Lectin Trapping on an Array of N-Glycans**

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Glycan microarrays are an established platform for the high-throughput screening of substrate specificities of carbohydrate-binding proteins and processing enzymes.[1] The most common detection method, which is based on fluorescently tagged lectins, can only give a measure of binding specificity, as quantification is often compromised by the specific lectin affinity.[1a,b] Therefore, a label-free technique that could overcome these analytical problems is needed for the analysis of array spot compositions.

MALDI-TOF-based assays on surface-bound carbohydrates have been reported. Mrksich and co-workers have shown that this technique can be used to study enzyme activity or to trap affinity ligands on biofunctionalized self-assembled monolayers (SAMs) of oligoethylene glycols on gold surfaces.[2] Unfortunately, the covalent attachment of glycans to the monolayer requires high ligand concentrations that are not suitable when working with complex oligosaccharides.[3,4] More recently, Wong, Siuzdak, and co-workers studied 2,3-sialyltransferase and β-galactosidase activity on fluorous-tagged lactose immobilized on a perfluorinated surface.[5] The difficult preparation of the nanostructured surface and the multistep tagging procedure, however, render this approach less appealing for routine and high-throughput use in on-chip mass spectrometric analysis of enzyme activity.

Inspired by the flexible and mobile organization of glycolipids in lipid bilayers,[6] we present herein a novel strategy for the surface-based MALDI-TOF analysis of glycan arrays that is conceptually a fusion of the approaches of Wong, Siuzdak, Mrksich, and their respective co-workers.[2a,5] Glycans are functionalized with a lipid tag (Scheme 1) and noncovalently immobilized on the MALDI plate by insertion into a self-assembled alkylthiolate monolayer. This setup was tested in a series of glycomics applications.

The facile preparation of both a hydrophobic MALDI plate and tagged ligands, as well as the general applicability for large oligosaccharides make this method stand out from other surface-based MALDI-TOF approaches. Hydrophobic surfaces have been applied to MALDI-TOF-based proteomics for sample desalting,[7] but, to the best of our knowledge, these surfaces have not been applied to the oriented immobilization of lipid-tagged biomolecules for surface MALDI-TOF analysis.

We chose a commercially available gold-coated MALDI sample plate as surface to prepare a hydrophobic self-assembling monolayer of 1-undecanethiol by following a published procedure.[8] Analysis of the monolayer under standard MALDI-TOF conditions showed only peaks corresponding to known matrix ions,[9] while no disulfide or thiolate ions were detected (Figure 1). The hydrophobically tagged carbohydrate ligands **1**–**10** used in this study were prepared from synthetic N-glycan structures[10] (**2**–**8**) by conjugation with stearic acid or from commercial reducing sugars by reductive amination (**1**, **9**, and **10**). Glycan arrays (see the Supporting Information) were made by spotting the conjugates **2**–**10** onto individual wells of the hydrophobic sample plate, drying, and rinsing the plates with water to remove unbound material. MALDI-TOF analysis using 2,4,6-trihydroxy-acetyophenone (THAP) as matrix showed strong ion signal intensities with signal-to-noise values of typically 100 or higher for all compounds; these values are comparable to those reported by Siuzdak and co-workers.[5] Ions were detected as sodium adducts with a detection limit of around 5 picomol. The maximum surface capacity for glycan immobilization was determined by deposition of increasing amounts of conjugate **2**, washing, and measurement of the signal for **2** normalized to an internal standard. Surface saturation was reached after deposition of around 2 nmol of conjugate **2** per well, which translates to a surface concentration of 0.2 mmol mm^{-2}.

To avoid the unnecessary waste of valuable analytes, glycans were spotted at half the saturation concentration without compromising signal intensity. The stability of the immobilized glycans to repeated washing with aqueous buffers, water, or organic solvents was then determined. Tagged glycans typically resisted 3–5 wash cycles of 1 minute duration without significant reduction of signal intensity, and the intensity of the model glycan **2** was essentially unchanged after 60 seconds of continuous sonication (Figure 1d). However, the glycans were completely removed from the surface

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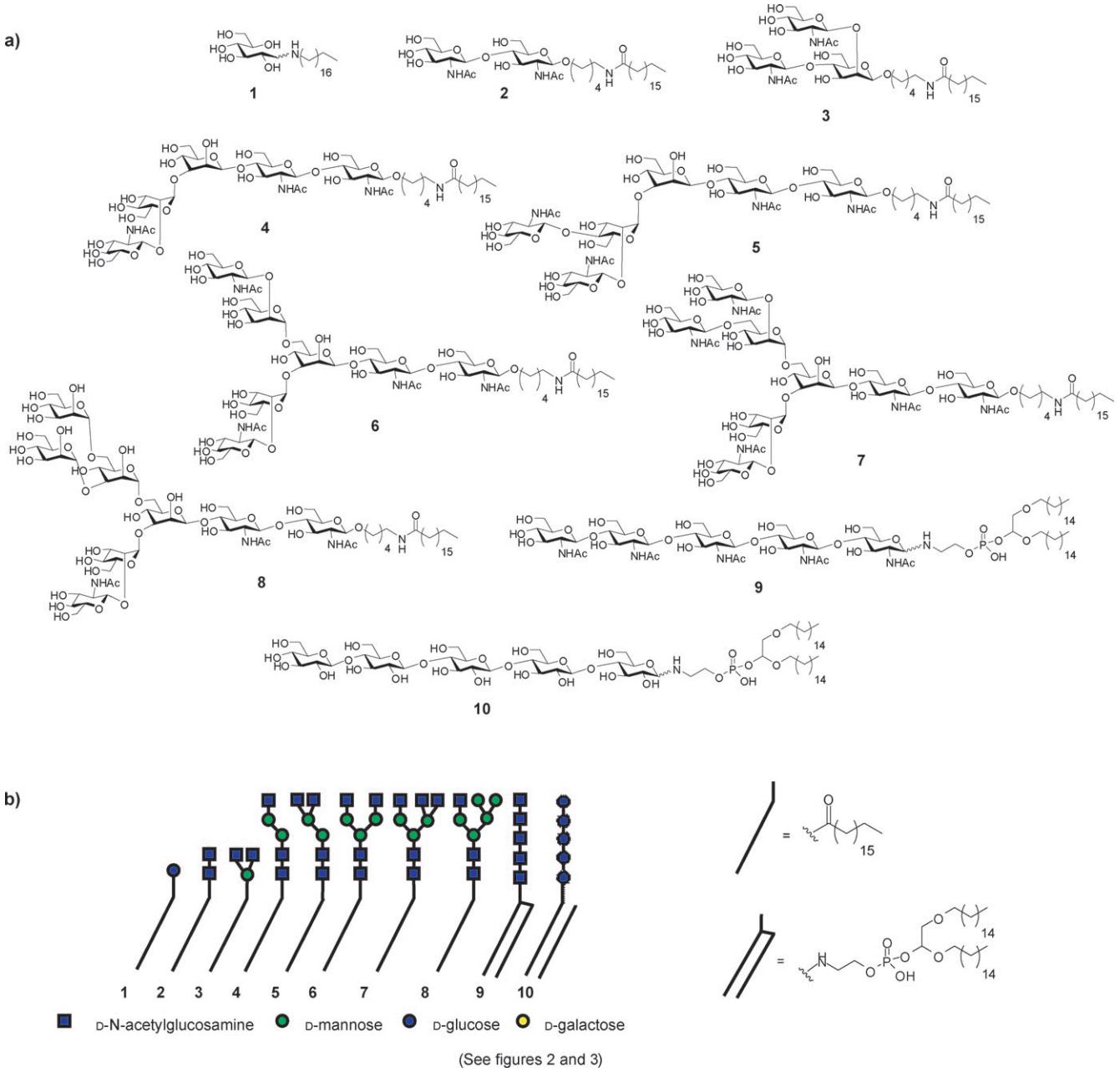


Scheme 1. a) Tagged glycan structures employed in this study. b) Pictogram representation of the glycans.

after washing with organic solvents. Since enzymatic reactions are usually carried out in buffered aqueous solutions, the immobilization method seemed suitable for our purposes. Thus an array of immobilized glycans was tested for three glycomics applications: substrate screening of glycosyltransferases, measurement of glycosidase activities,[2a,5] and lectin identification.

The first trials for the enzymatic galactosylation of the immobilized glycans **2**–**8** with bovine β-1,4-galactosyltransferase (GalT) and UDP-galactose (UDP-Gal) with addition of 30% glycerol to avoid evaporation showed incomplete conversion even after prolonged reaction times. When the blocking sugar **1** and a sample glycan were spotted in a ratio of 10:1, however, complete conversion of all glycans was observed. We assume that the major function of the blocking sugar is to cover the exposed hydrophobic patches and to dilute the ligand surface density, thereby improving enzyme accessibility. We were able to reduce the blocking sugar/glycan ratio to 2:1 to increase the signal strength while maintaining complete conversion in the enzyme reaction.

Enzyme activity could be detected as soon as 10 minutes after incubation, but full conversion of multiantennary glycans, which is a requisite for on-chip enzymatic preparation of glycan arrays,[10] took up to 60 hours. The resulting array of fully galactosylated glycans was then used to follow the action of a β-1,4-galactosidase from *Aspergillus oryzae* (Figure 2). After 1.5 hours, almost complete hydrolysis of galactose residues was observed for most of the glycans with

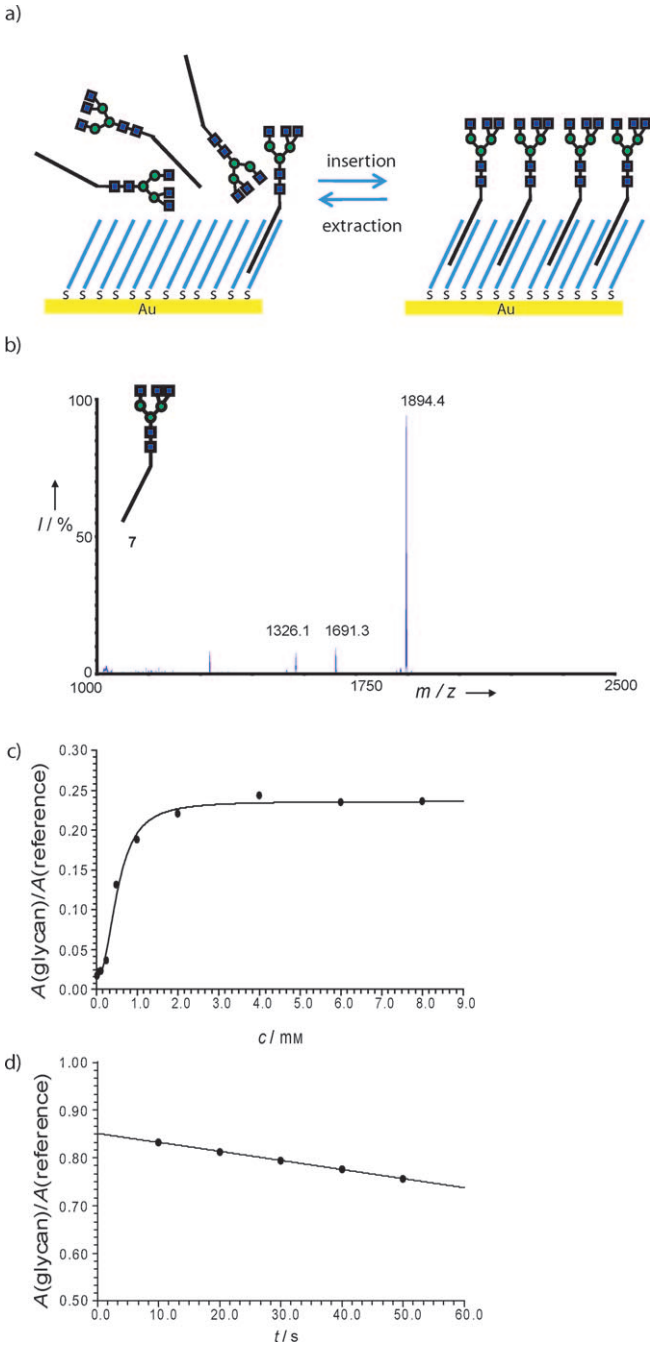
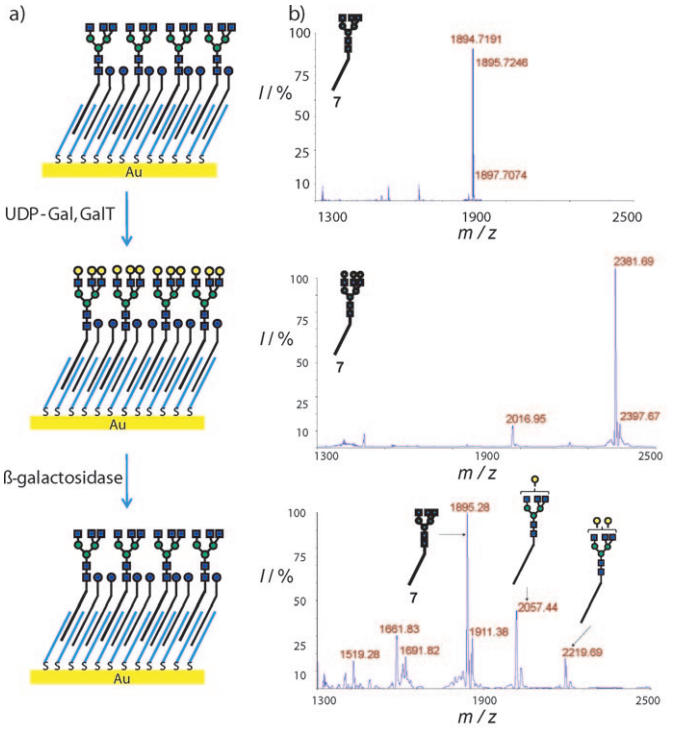


Figure 1. a) Formation of glycan-functionalized surfaces for MALDI-TOF MS analysis. b) MALDI-TOF spectrum of immobilized triantennary glycan **7** after washing. c) Saturation graph of the MALDI plate for chitobiose. d) Desorption graph of chitobiose adsorbed on the MALDI plate. *A* = peak area.



c)

Glycan	Observed *m/z* [M+Na$^+$]	Observed *m/z* [M+Na$^+$], (no. Gal residues)	Observed *m/z*, [M+Na$^+$], (rel. signal intensity, no. Gal residues)
2	799.9	962.3 (1 Gal)	799.9 (100%, 0 Gal)
3	962.2	1285.0 (2 Gal)	1285.0 (73%, 2 Gal), 1122.9 (27%, 1Gal)
4	1327.6	1488.2 (1 Gal)	1488.2 (21%, 1 Gal), 1326.1 (79%, 0 Gal)
5	1531.1	1854.2 (2 Gal)	1530.2 (100%, 0 Gal)
6	1693.2 * 45 minutes reaction	2015.2 (2 Gal)	2014.8 (4%, 2 Gal), 1853.8 (1%, 1 Gal), 1690.8 (95%, 0 Gal)
7	1894.7	2381. (3 Gal)	2219.7 (5%, 2 Gal), 2057.4 (19%, 1 Gal), 1895.3 (74%, 0 Gal) *
8	1814.7	1974.6 (1 Gal)	1812.2 (100%, 0 Gal)

Figure 2. a) Scheme of enzymatic processing on surface-bound glycans. b) Transformations performed on glycan **7**. c) Observed *m/z* values of C_{18}-tagged glycans after treatment with β-1,4-galctosyltransferase (60 h, 37 °C) and β-galactosidase (1.5 h, 30 °C).

the exception of glycans **3** and **7**, which showed only partial hydrolysis of galactose residues (Figure 2b).

To explore the utility of this platform for lectin identification, we incubated sample wells functionalized with different tagged glycans (**2**, galactosylated **8**, **9**, and **10** as control) and investigated binding of three purified lectins, a mixture with different specificities, and the selective trapping of a lectin from a cell lysate (Figure 3). A well of the sample plate functionalized with tagged glycan **9** was incubated with wheat germ agglutinin (WGA) for 40 minutes at room temperature, rinsed with water to remove nonspecifically bound lectin, and analyzed. MALDI-TOF analysis of **9** bound in the well showed peaks for the single- and double-charged lectin at 17160 Da and 8705 Da, respectively (Figure 3c), while no signal for the lectin was observed from a control well functionalized with maltopentaose derivative **10**. This interaction could be detected down to a WGA concentration of 130 nM.

As a second lectin, we tested *Phytolacca americana* lectin (PAL), which specifically recognizes the N-acetylglucosamine trimer. Immobilized glycoconjugate **9** was incubated with the lectin, washed, and analyzed by MALDI-TOF MS. The observed signals at 13795 Da, 9343 Da, and 9130 Da corre-

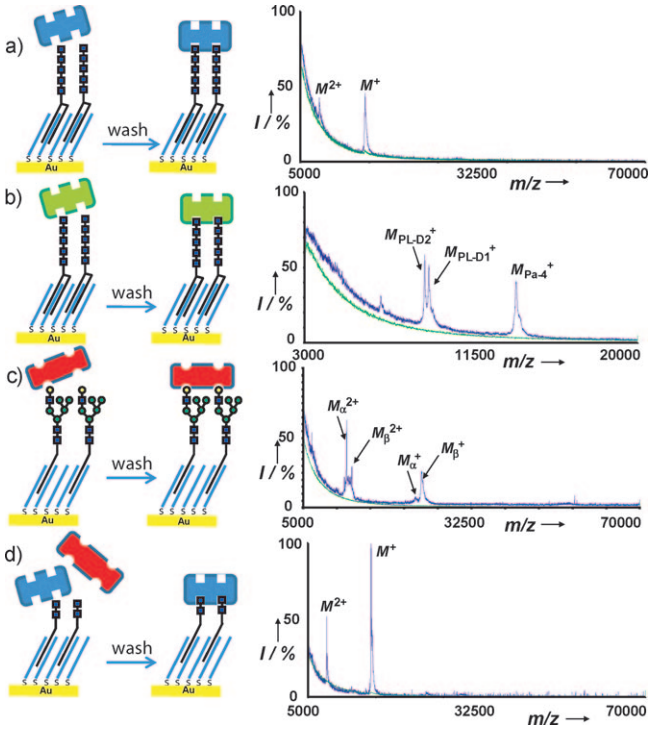


Figure 3. a) WGA binding to a well functionalized with chitopentaose **9** (blue trace) and control on surface functionalized with **10** (green trace). b) PAL binding to **9** (blue) and control with **10** (green). c) ECA lectin binding fully galactosylated glycan **8**. (blue) and control with glycan **8** (green). d) Specific trapping of WGA lectin in presence of a fourfold excess of ECA. (blue) and control for ECA binding on well containing glycan **2** (green).

spond to the subunits of the Pa-4/PL-C and PL-D1/PL-D2 isoforms.

When a galactose-binding lectin from *Erythrina cristagalli* (ECA) was studied on immobilized fully galactosylated glycan **8**, broad peaks for the single-charged (27825 Da) and double-charged (14401 Da and 13443 Da) α and β subunits were observed. However, no peaks corresponding to ECA were observed in a control experiment with maltopentaose **10**.

To assess the ability of our platform to specifically trap and identify a lectin from a mixture of proteins, wells with immobilized chitobiose **12** were incubated with a 4:1 ECA/WGA mixture to compensate for the differences in ionization efficiency. MALDI-TOF analysis showed strong peaks for single- and double-charged WGA at 17160 kDa and 8705 kDa, respectively. Immobilized maltopentaose **10** showed no binding to the lectin mix, thus demonstrating the specificity of the interaction. In a related experiment, immobilized chitobiose **2** was incubated with a lysate from an *E. coli* culture spiked with WGA at 580 nM, washed, and analyzed. Again, WGA was detected with good signal intensity only on the chitobiose-functionalized wells.

In summary, we have developed a simple and efficient platform for the immobilization and analysis of surface-bound complex oligosaccharides. With this system, it was possible to follow the action of a glycosyltransferase and a glycosidase on surface-bound multiantennary N-glycans. This technique will allow optimization of reaction conditions for the enzymatic on-chip elongation of surface-bound glycans to increase structural diversity on glycan microarrays.[10] In addition, it has been shown that this method is sensitive enough to observe the specific but weak interaction between immobilized glycans and lectins in various cases.

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