Proteomics

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Analysis of Microarrays by MALDI-TOF MS**

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Mass analyzers are universal detectors with an exceptional sensitivity, dynamic range, and resolution. Their combination with microarrays could create a valuable tool for functional genomics, proteomics, and glycomics, to help with gene annotation, discovery of new enzyme functions or assign receptor specificities.[1]

Here we report on the development of a new microarray platform that allows analysis by fluorescence spectroscopy, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) and optical microscopy without a change of the format. Ligand libraries are printed without previous tagging[2] onto a noncovalent sandwich composed of activated bidentate lipids embedded in a hydrophobic layer conjugated to a indium–tin oxide (ITO) surface (Figure 1 a). These functionalized surfaces are transparent, conductive, resistant to repeated aqueous washing and provide the signal intensity required for the MS readout of micrometer-sized spots.

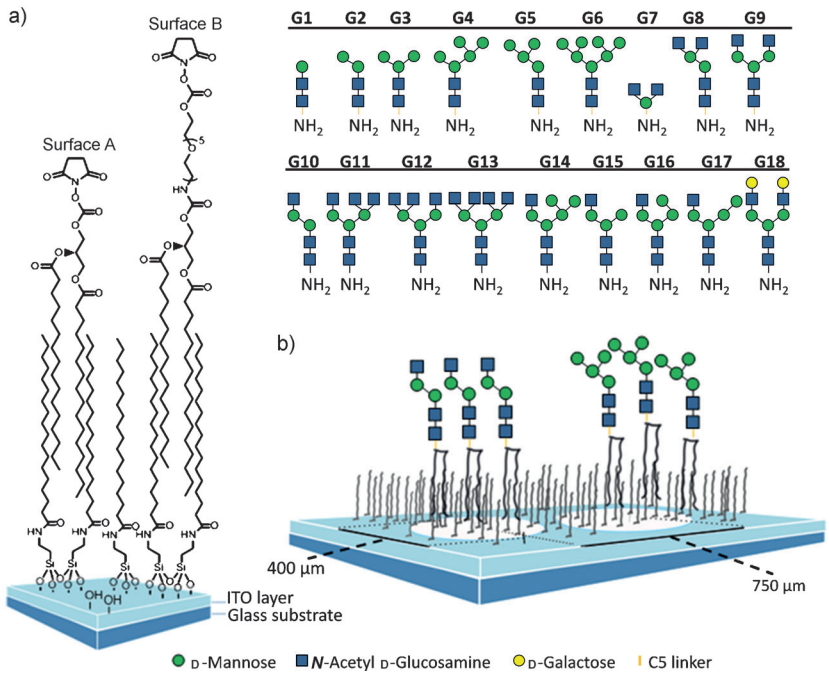

Figure 1. Preparation of a *N*-glycan array on an activated ITO platform. a) An activated bidentate linker is stabilized on an ITO surface through hydrophobic interactions. b) Amino-functionalized glycans **G1**–**G18** (box) were printed on surfaces A and B.

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Supporting information for this article, including details for the surface functionalization and all described applications, is available on the WWW under http://dx.doi.org/10.1002/anie.201302455.

To demonstrate its scope this platform has been applied to 1) study the action of eight recombinant glycosyltransferases on *N*-glycan acceptors, 2) assign the specificity of a fucosyltransferase by on-chip product fragmentation, 3) profile the proteins bound to a lectin array by MS, and 4) identify a lectin bound to a glycan array by on-chip tryptic digestion and in situ sequencing of peptide fragments.

Several approaches for studying interactions and chemical[3] or enzymatic[4] transformations on surface-bound substrates by MALDI-TOF MS have been reported. Köster et al. developed a DNA-sequencing method based on the detection of hybridized DNA on a silicon wafer DNA array[5] while Becker et al. employed MS for the detection of Ras-protein–receptor interactions on a protein–oligonucleotide conjugate attached to a silicon wafer through DNA-directed immobilization.[6]

A linkage to the surface, which is cleavable under laser irradiation, facilitates the observation of mass changes directly on the surface-bound substrate. Mrksich and co-workers measured enzyme activity on carbohydrate and peptide substrates tethered by oligoethylene thiolates to a gold sample chip[4b,7] and Flitsch and co-workers employed this platform to study acyl amine exchange on a peptide

array.[8] Siuzdak, Wong and ourselves developed glycan-array-based enzyme assays with substrates attached to porous aluminium oxide or gold by fluorous[2b,4a] and hydrophobic interactions,[4a] respectively. Although very promising, in situ cleavage of photolinker bound substrates in MALDI-Tof MS has been complicated by low yields.[9] These studies show that assay sensitivity, resolution, and array stability are major challenges for the development of MS-compatible microarrays that requires a careful match of surface materials, conjugation chemistry, and instrumentation. Most efforts have been limited to arrays on larger well plate formats or slides with manually deposited ligands.[2b,9b]

In the search for a material transparent and conductive as required for optical and MS chip analysis, we chose commercial ITO-coated glass slides. Silanization with 3-aminopropyltriethoxysilane (APTES) and subsequent coupling of NHS-activated stearic acid created a hydrophobic support layer for the immobilization of lipid-tagged biomolecules via hydrophobic interactions.[4a] This stepwise process resulted in higher surface coverage and hydrophobicity than ITO surfaces directly functionalized with stearic acid or alkylthiolates. 1,2-*sn*-Dipalmitoyl glycerol, activated as a carbonate, was then immobilized onto the hydrophobic layer forming a reactive novel surface (Figure 1, surface A). Likewise, immobilization of the lipid linker extended with a hexaethylene glycol spacer produced a surface likely to reduce nonspecific interactions and improve enzyme accessibility (Figure 1, surface B). Slides thus functionalized could be stored under vacuum at room temperature for weeks without losing activity. Robotic printing of aminopentyl glycans **G1** to **G18**[4a,10] (Figure 1) onto this surface produced an array with excellent spot morphology, reproducibility, and assay sensitivity (Figure 2).

Imaging the array by MS produced clean peaks absent of unreacted glycans or cross-contamination from adjacent spots (Figure 2e). The spot-wise analysis of the 54-spot array was completed in less than 3 minutes. With a detection limit of 30 femtomoles (S/N of 40) for glycan **G10**, this is one of the most sensitive methods for the analysis of immobilized glycans by MS.[2b,11] A ligand leakage of less than 20% after a rigorous 5×5-minute wash program, which included sonication, demonstrated the stability of the surface.

As a first application, we incubated arrays of putative *N*-glycan acceptors with eight recombinant glycosyltransferases in the presence of the nucleotide sugar donors. In a high-throughput screening of putative glycosyltransferases, Mrksich and co-workers employed self-assembled monolayers for matrix assisted laser desorption ionization mass spectrometry (SAMDI-MS) to the analysis of an array of simple sugar acceptor substrates.[11] Table S1 (see the Supporting Information) resumes the MS analysis of our array (surfaces A and B) after incubation with bovine β-1,4-galactosyltransferase (GalT),[12] a human α-2,6-sialytransferase (SialT),[13] a β-1,4-*N*-acetylgalactosaminyltransferase (GalNAcT)[14] from *C. elegans*, two core α-1,6-fucosyltransferases from *C. elegans* (CeFUT8)[15] and *A. gambiae* (AgFUT6),[15] two core α-1,3-fucosyltransferases (CeFUT1,[16] AtFutA[17]) from *C. elegans* and *A. thaliana,* respectively, and a *C. elegans* fucosyltransferase (CeFUT6)[18] of reported Lewis-type specificity. Enzymatic conversions were calculated as the ratio of the peak areas for starting sugar and reaction product[4b] with a limit for detecting

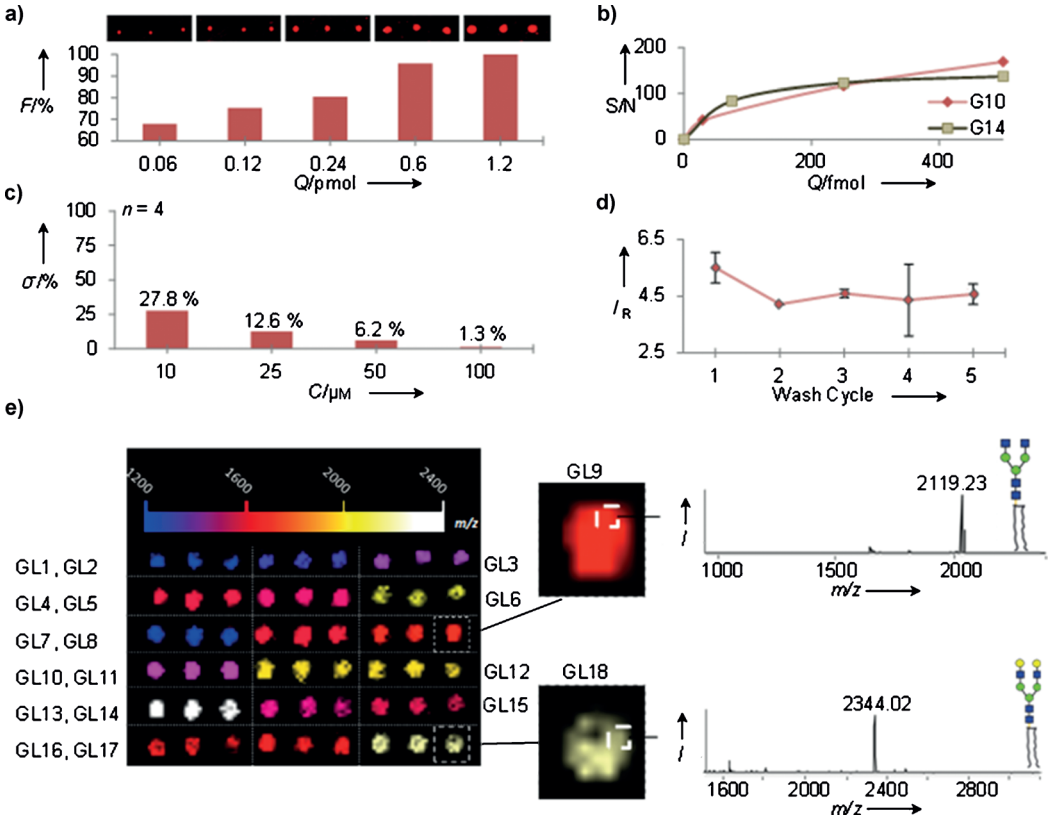

Figure 2. Characterization of ITO–glycan slides by a) fluorescence and b–e) MALDI-TOF MS. a) Evaluation of spot size and fluorescence intensity with WGA-Cy5. b) Assay sensitivity measured at a signal-to-noise ratio (S/N) for 2 glycans. c) S/N reproducibility at different spotting concentrations. d) Surface stability during successive wash cycles. e) MALDI image of glycan array with mass-dependent color coding. Inset: mass spectra from individual spots.

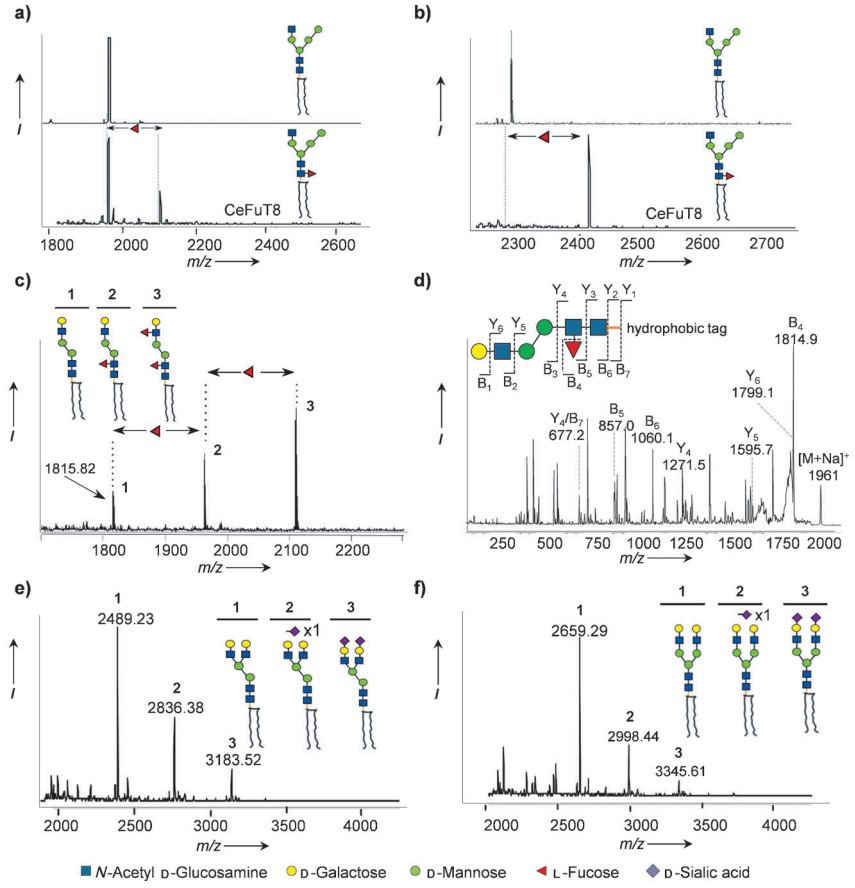

Figure 3. a) Core fucosylation with low conversion on surface A. b) Improved conversion on surface B. c) MS spectra of GL10 after incubation with GalT and CeFUT6. d) Assignment of fucosylation sites through diagnostic fragment ions generated by on-chip fragmentation. e and f) Detection of multiple sialylated products after on-chip derivatization.

unreacted glycan close to 5%. The glycosylation of the panel of *N*-glycan acceptors followed closely reported enzyme specificities[10b,c,19] and showed a generally large degree of conversion after a single incubation cycle underlining the viability of on-chip enzymatic derivatization. The hexaethylene glycol spacer on surface B dramatically improved the enzymatic conversion for core-fucosyltransferases operating close to the surface (Figure 3a and b). Consequently the AtFutA and AgFUT6 activities were measured only on surface B. The lability of sialic acid moieties often compromises the analysis of sialylated glycans by MALDI-TOF MS.[20] After on-chip derivatization[21] with acetohydrazide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)[22] however, we detected the formation of multiple sialylated *N*-glycans next to the starting sugars, a requirement for calculating the conversion by MS (Figure 3e and f). Enzyme activity is conveniently measured by MS but to assign the regio-specificity of a glycosyltransferase requires the generation of diagnostic fragment ions.[23] Incubation with CeFUT6, reported to perform Lewis-type fucosylation,[18] produced a bisfucosylated product for GL10, suggesting an additional activity of the enzyme (Figure 3c). On-chip fragmentation of the monofucosylated product ion **2** (Figure 3c) produced the diagnostic fragment ions Y_5 ($m/z = 1595$), Y_4 ($m/z = 1271$), and B_5 ($m/z = 857$; Figure 3d) which allowed the localization of the additional fucose at the second GlcNAc residue.

As a further application we incubated a small lectin array comprising *Concanavalin A* (ConA), *Galantus nivalis* agglutinin (GNA) and wheat germ agglutinin (WGA) with fluorescently tagged glycoprotein RNAse B. The scanometric analysis showed binding to mannose-binding lectins ConA and GNA only (inset, Figure 4a) and MALDI-TOF analysis of selected spots produced ions related to RNAse B only on GNA and ConA spots (Figure 4a). Five different RNAse B glycoforms decorated with high-mannose-type glycans M5–M9 differing in a single mannose unit (±162 Da) could be easily distinguished[24](Figure 4).

Next we attempted to profile low-weight urinary glycoproteins (e.g. microtubulins). Some of these are clinical markers of (tubular) proteinuria[25] and disease-related glycosylation changes might be of prognostic value in renal pathologies. After

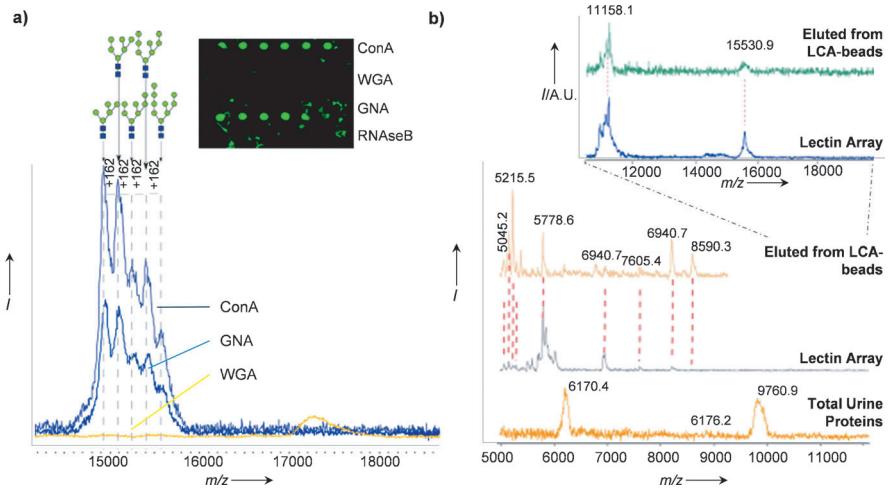

Figure 4. Dual readout of a lectin array. a) MS spectra showing RNAse B high mannose-glycoforms bound to ConA and GNA on the lectin array. (WGA, negative control, inset: fluorescence readout). b) MS spectra of total urinary proteins and fractions (5–12 KDa) and 11–22.5 KDa (amplified region) bound to a lectin array or LCA-beads.

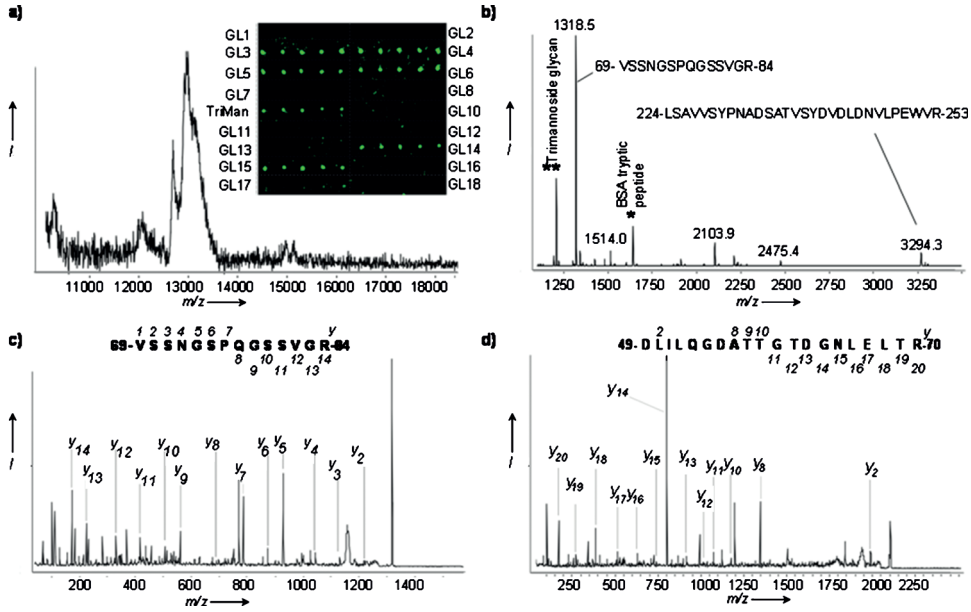

Figure 5. Identification of binding partners after on-chip tryptic digestion. a) MALDI-MS of ConA bound to α-mannoside-containing glycan array (inset: fluorescence image). b) On-chip tryptic digest of ConA. c) On-chip sequencing of 50–69 aa (*m*/*z* 1318), and d) the 70–83 aa peptides (*m*/*z* 2103).

incubation with a urinary protein extract, the lectin array analysis showed mainly binding to fucose-binding lectins, in particular *Lens culinaris* lectin (LCA). As control, the incubation was repeated in a suspension assay with LCA-functionalized beads. Figure 4 shows the similar profiles captured by both surface and suspension trap and strong signals for several LCA-binding glycoproteins with masses between 3.5–20 kDa. The LCA included in the lectin array concentrated fucose presenting proteins[19] which were barely visible in the MS-profile of the original protein mixture with a similar efficiency than a bead-based suspension assay (Figure 4).

We also studied on-chip tryptic digestion for the identification of proteins. In a proof-of-principle experiment we incubated a glycan array with ConA (Figure 5). MALDI-Tof analysis of selected spots showed a strong signal for the doubly charged Con A monomer at 13 KDa. (Figure 5) Trypsin printed over individual ConA-positive spots at varying concentrations (Figure S12) produced intense peptide peaks between 1–3.3 kDa with higher intensity for smaller peptide fragments than for the solution phase digest. A database search for tryptic peptides returned ConA as the only hit with over 30% sequence coverage. With a view on future protein identification within more complex mixtures, we subjected the two most abundant peaks of the ConA tryptic digest to on-chip peptide sequencing by tandem mass spectrometry (MS/MS; Figure 5). The assignment of fragment ions allowed us the in situ sequencing of the 50–69 aa (*m*/*z* 1318) and the 70–83 aa (*m*/*z* 2103) Con A tryptic peptides (Figure 5).

In conclusion, we have developed a new platform for microarray analysis by MS and traditional optical readout methods with unparalleled sensitivity. The direct printing of amino-functionalized glycans, glycoproteins, and lectins onto the activated slide produced arrays with strongly improved spot morphology and homogeneity avoiding solubility problems, ligand tagging,[2,9b] and strict incubation requirements associated with printing glycolipids or fluorous-tagged glycans.[26] Work in progress is directed towards exploiting this new platform for the discovery, characterization, and identification of lectins and carbohydrate processing enzymes and as a general functional proteomics tool.

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