

GLYCOARRAYS ON GOLD SURFACES

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ABSTRACT

Self-assembled monolayers (SAMs) on gold have become widely used as a platform for studying chemical and biochemical reactions, for studying biomolecular interactions and for the development of nanoscale devices. We have used this platform to study the solid-supported synthesis of carbohydrates and glycopeptides using both chemical and enzymatic methods. An attractive feature of the technology is the opportunity for miniaturisation and *in situ* analysis using mass spectrometry, SPR and fluorescence spectroscopy. Applications for the synthesis of complex oligosaccharides and glycopeptides to generate glycoarrays and their application in biology and medicine are discussed.

INTRODUCTION

The sequences of oligo- and polysaccharides in cells and tissues are not directly encoded in their genomes, but are determined by the expression and substrate specificity of a large set of ‘glycoenzymes’, which catalyse the formation or hydrolysis of glycosidic bonds (Figure 1). These enzymes are involved in the biosynthesis of glycan structures by controlling regio- and stereoselectivity of glycosylation, a process which is highly dynamic. The understanding of the activity and substrate specificity of these glycoenzymes is a key to determining and understanding the ‘glycome’, the set of carbohydrate structures in a biological system.

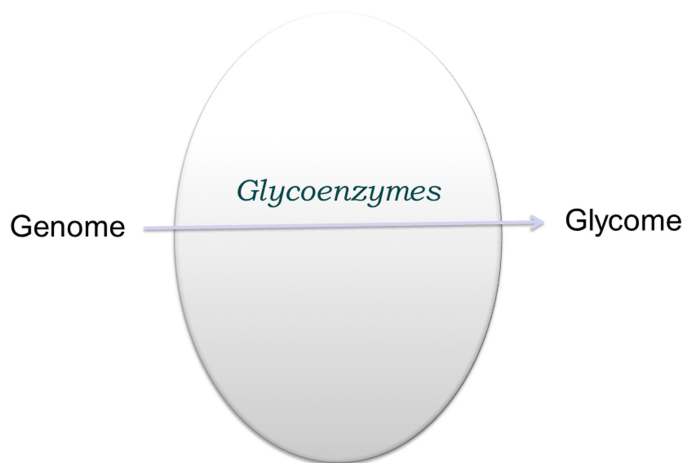


Figure 1. The Glycome presents the set of carbohydrate sequences in a cell or organism. The sequences are determined by glycoenzymes such as glycosyltransferases and glycosidases which control formation and hydrolysis of specific glycosidic linkages.

Glycoenzymes can often be identified in genomes for their characteristic conserved polypeptide sequence motifs and over 100,000 members are currently catalogued in the CAZy database (www.cazy.org/). Most of these glycoenzymes are highly specific for generating or cleaving a defined saccharide sequence, including selectivity for monosaccharide units as well as regio- and stereochemistry of linkage. This precise substrate specificity needs to be determined for each enzyme by biochemical studies, since computational methods are currently not able to make accurate predictions on substrate recognition. Thus, there is a major drive in the glycosciences to find biochemical methods that can allow us to go beyond sequence determination to obtain structural and functional information on glycoenzymes.

One of the emerging technologies in the glycosciences involves microarrays containing diverse carbohydrate probes (Glycan arrays) which have been used to study carbohydrate-protein interactions. Such microarrays have been generated from either natural or synthetic carbohydrate samples (Figure 2) and have been interrogated by fluorescently labelled protein for binding. Over the past five years, our laboratory and others have applied this glycan array technology to the study of glycoenzymes [1]

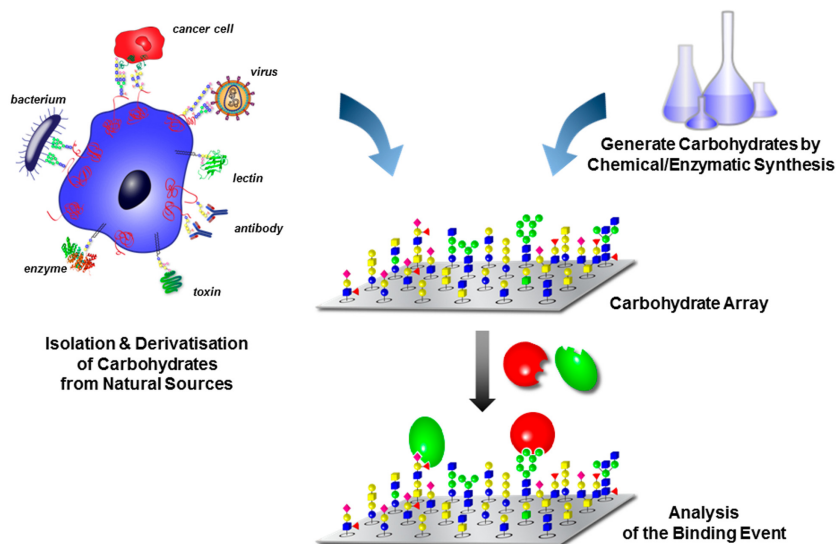


Figure 2. Carbohydrate arrays have become important tools for the investigation of protein/carbohydrate interactions in a high-throughput manner.

METHODS AND RESULTS

Biochemical reactions are generally studied in solution, but for microarray studies, such reactions need to be transferred to solid phase. Although immobilised enzymes have been used frequently in biotechnological applications, the study of enzymatic modifications of immobilised substrates (Figure 3) has been less well explored [2]. Extensive fundamental studies of enzyme-catalysed reactions on diverse solid supports have shown that issues of substrate accessibility [3–13], reaction equilibria [14–17] and stereospecificity [18–21] need to be considered when choosing solid supports. Porous supports such as copolymers of polystyrene or polyacrylamide with polyethylene glycol are generally not suitable as supports for efficient enzyme catalysis because of poor yields. Such polymer supports also offer little opportunity for *in situ* molecular analysis of reactions.

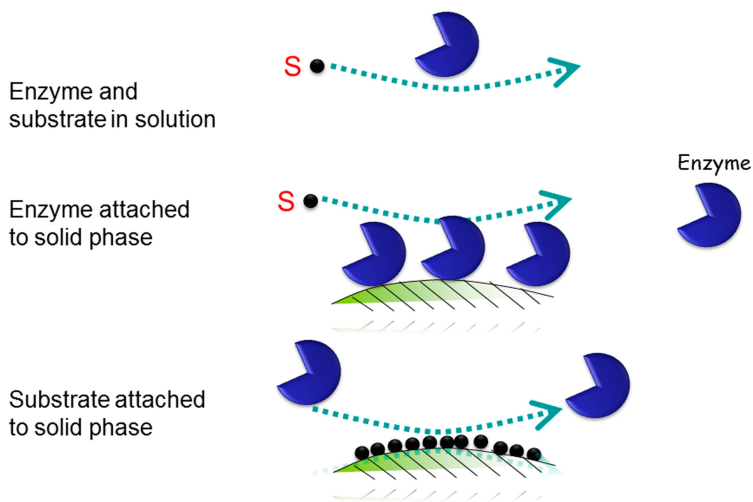


Figure 3. Classically, enzymes have been in used solution (upper panel) or immobilised on solid phase (middle panel). Applications on microarrays require the substrate to be immobilised during the reaction (lower panel).

Given these considerations, a solid support consisting of self-assembled monolayers (SAMs) on gold (subsequently referred to as ‘gold platform’) [22, 23] was chosen as a more suitable system to study glycoenzymes on arrays (Figure 4). Such arrays can easily be derivatized with structures containing an amine functionality such as peptides, glycopeptides or glycosyl ethanolamines. The SAM surfaces can be used with both organic solvents and aqueous buffers and have been optimised for biocompatibility [22]. Peptides can be coupled directly through their amino-termini or amino side chains, whereas glycosyl aminoethanols are easily prepared by short synthetic routes [23].

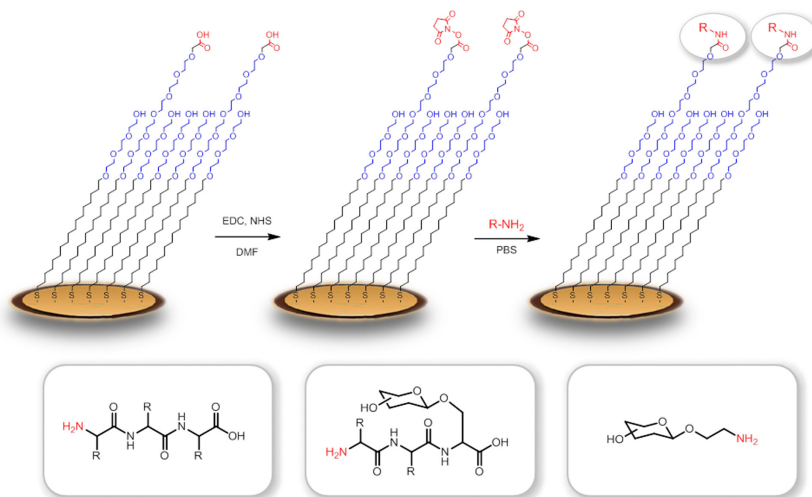


Figure 4. The ‘gold platform’ presented in this paper consists of self-assembled monolayers linked *via* thiols to gold surfaces.

A particular advantage of the gold platform is the possibility of *in situ* analysis of surfaces through MALDI-TOF mass spectrometry, a technology termed SAMDI [24, 25] (Figure 5). Thus, any chemical or enzymatic transformation can be monitored directly on the surface without the need for cleavage. The surface can also be re-used for further chemical or enzymatic reactions since only small fractions of the analyte are sampled by MALDI-TOF MS analysis. Figure 5 shows as an example the mass spectra taken from array spots before and after glycosylation using a β -1,4-galactosyltransferase. The glycan probe shows several peaks due to thiol dimerization to generate homo- and hetero-disulfides, but all peaks can be assigned unambiguously to either starting material or product. The MALDI-TOF mass spectrum of the product illustrates that the glycosylation can be high-yielding – in this case no starting material was observed in the spectrum, suggestion quantitative conversion. We have investigated a diverse range of glycosyltransferases on the gold platform such as GlucNAc-, GalNAc-, Neu5Ac- and Fuc-transferases (Figure 6, unpublished results) and have found that this platform provides accessible acceptor substrates to all of these enzymes [26–28]. A further advantage of the gold platform is compatibility with other spectroscopic methods, In particular surface plasmon resonance spectroscopy [29], which also needs attachment of ligands to conducting surfaces such as gold, in order to measure carbohydrate/protein interactions [29].

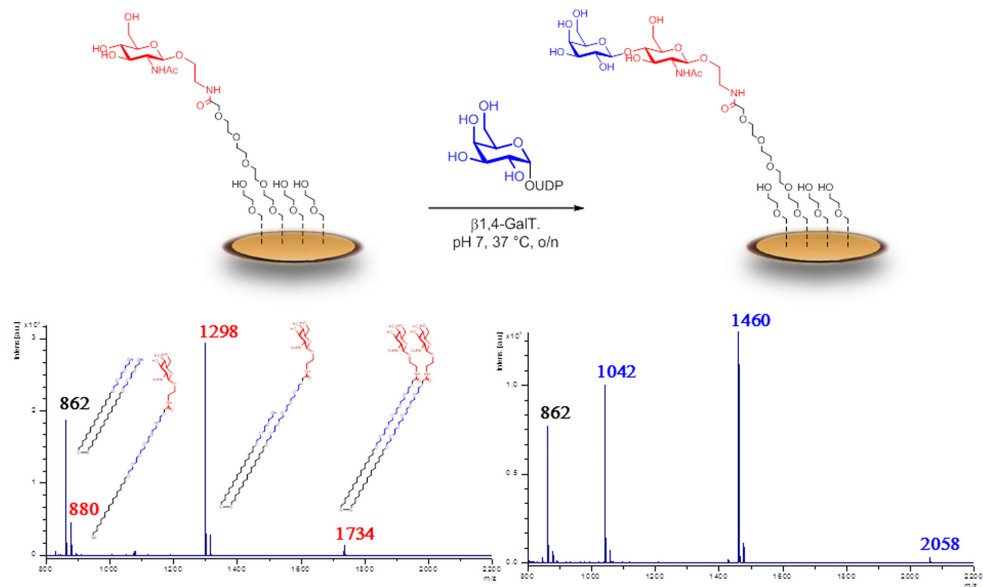


Figure 5. The enzymatic galactosylation of a surface-linked GlcNAc can be monitored using MALDI-TOF mass spectrometry with trihydroxy acetophenone as a matrix. The spectrum shows monomers, homo- and heterodimers of the functionalised alkyl thiols.

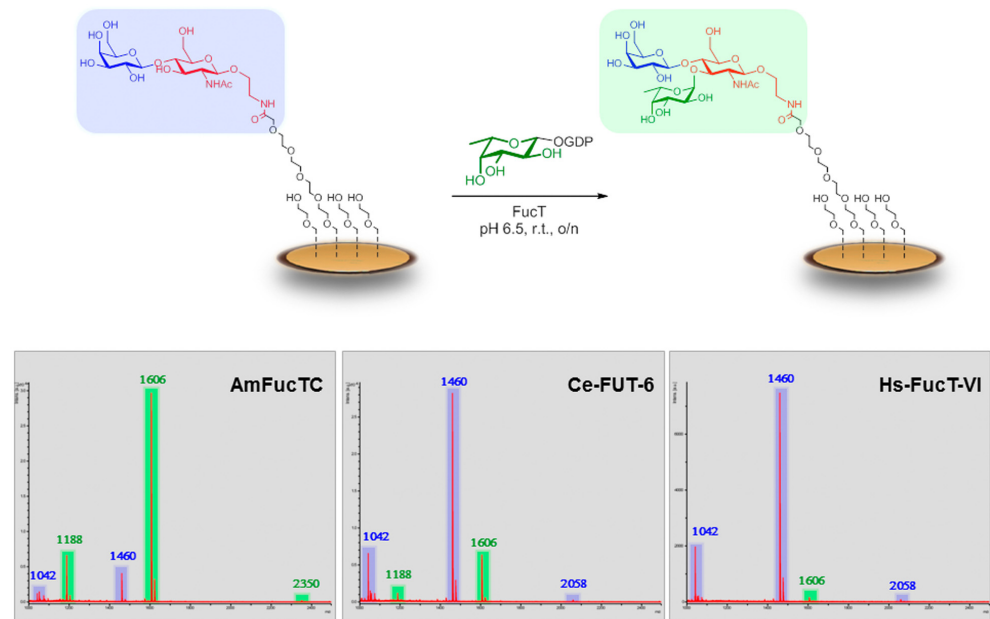


Figure 6. In collaboration with Prof Iain Wilson(BOKU, Austria), we have investigated a number of fucosyltransferases on the array platform. Varying degrees of glycosylation were observed with recombinant enzymes.

One of the most intriguing questions in protein glycosylations concerns the choice of glycosylation site on a polypeptide sequence. Whilst the site of *N*-glycosylation is highly conserved and predictable for tripeptide motifs (Asn-X-Thr/Ser), consensus motifs for *O*-glycosylation are poorly understood and need to be determined by biochemical studies for each glycosyltransferase. Microarrays of peptide acceptor substrates are very useful for this purpose, because of high-throughput and miniaturisation. Particularly attractive for these studies is spot-synthesis of peptides directly on an array, because it allows for the fast and cheap synthesis of tailor-made peptide libraries to investigate specific peptide substrates around a known lead structure.

A common problem with spot synthesis, however, is quality control. Peptide synthesis is not always reliable and on chip synthesis can be difficult to monitor. Given the *in situ* analysis capability of the gold platform, we have developed a methodology that allows us to do spot synthesis with concomitant analysis. Figure 7 shows the individual steps involved, which are fully compatible with Fmoc-protected amino acids used in many automated peptide synthesis systems [30, 31]. Hence, all the building blocks needed are commercially available. We have shown that the *in situ* analysis using MALDI-TOF MS allows for tight quality control after every step. Thus, poor couplings can easily be detected and if necessary be repeated before proceeding with synthesis. MALDI-TOF analysis can also give us information about the purity of the final peptide, and in particular detect any truncated side products.

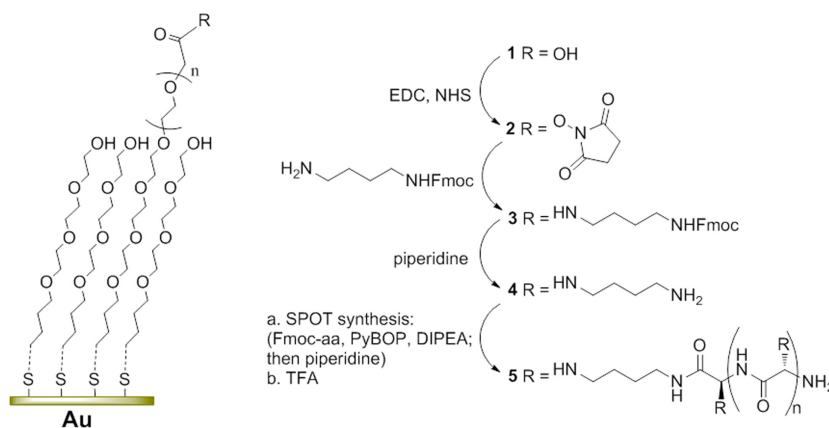


Figure 7. Solid phase peptide synthesis on 'gold array' (SPOT) using Fmoc technology.

This spot synthesis was used to make a glycopeptide array to probe the substrate specificity of ppGalNAc T2, an isoform of a family of over 20 human glycosyltransferases involved in the first steps of mucin glycosylation (Figure 8). A peptide library around a natural polypeptide sequence was generated and it was found that glycosyltransferase activity was

highly dependent on the nature of the two residues next to the threonine glycosylation site [30]. Interestingly, placing a proline on either site dramatically increased the activity of the enzyme and made the peptide a much better substrate than the natural sequence studied.

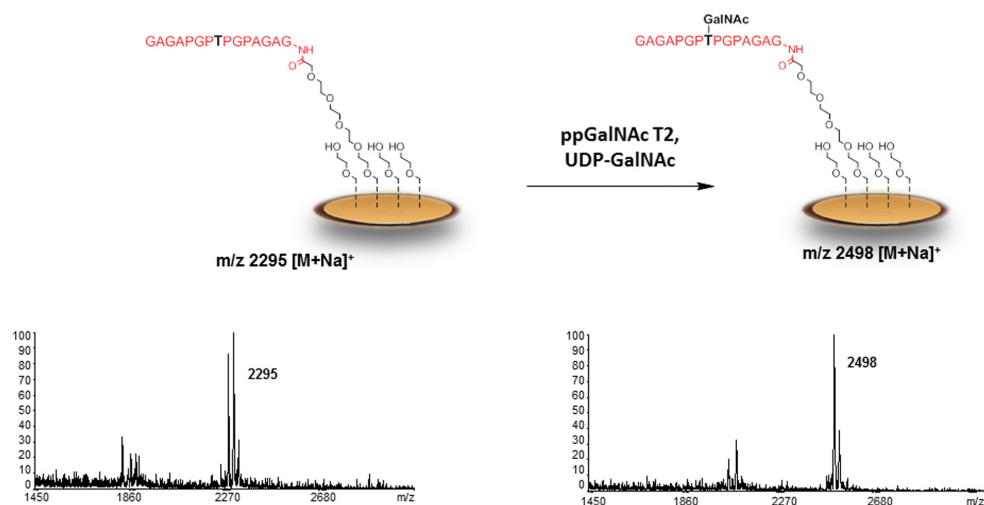


Figure 8. Glycopeptide synthesis appears to proceed to completion on the array surfaces as indicated by MALDI-TOFMS analysis of starting material and product.

Another application of our platform is in the analysis of genetic orders of glycosylation [32, 33]. The glycosyltransferase POMGnT catalyses the glycosylation of an unusual class of *O*-mannosyl glycans (Figure 9), which have been isolated as a major component of mouse brain, but so far have only been identified on a single protein, α -dystroglycan (α -DG), which has been found in muscle and brain tissue. Defective glycosylation of α -DG leads to congenital muscular dystrophies and a number of patients have been diagnosed with mutations in the POMGnT gene. Our technology has allowed us to study clinical mutants of POMGnT for activity against peptides derived from α -DG. Interestingly, one of these clinical mutations did not seem to affect enzyme kinetics, whereas other clinical point mutation abolished activity. Some correlation between enzyme activity and severity of disease was found, although this requires further investigation.

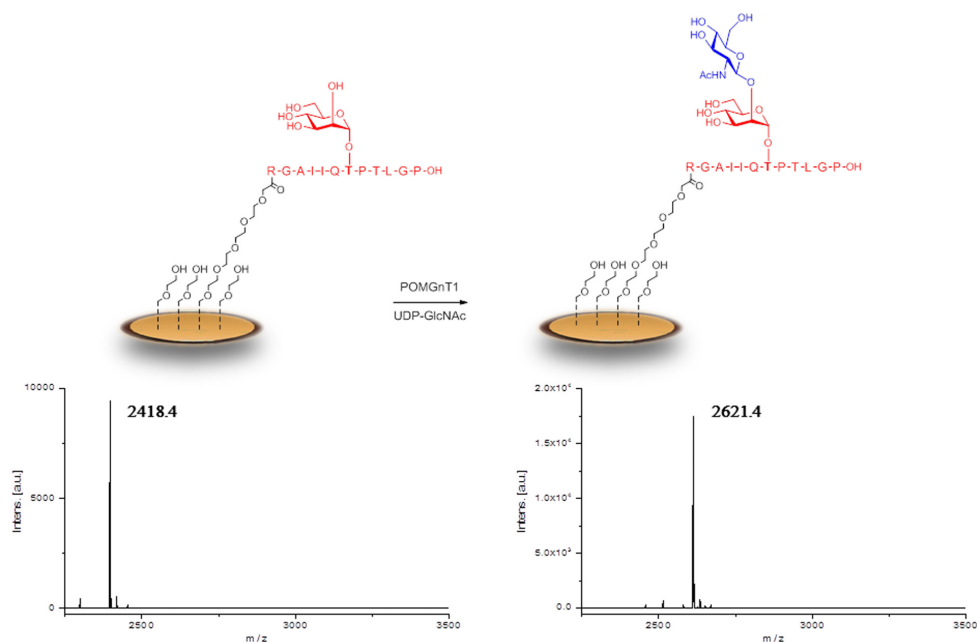


Figure 9. POMGnT1 is involved in the biosynthesis of unusual *O*-mannosyl glycans found in the brain. Mutations in the gene encoding for POMGnT1 can lead to congenital disorders of glycosylations, in particular muscular dystrophies.

It is interesting to note that the glycosylation of peptides using this enzymatic approach appears to proceed with excellent yields. Although quantification of reaction yield using mass spectrometry is difficult, the analysis of the gold surface by MALDI-TOF MS as shown in figures 8 and 9 gives a very clean spectrum in which only the product peak is visible, suggesting near quantitative yields of reaction. Thus, the solid-phase enzymatic synthesis of glycopeptides is possible. Such enzymatic routes are particularly interesting for the generation of linkages that are chemically more difficult to achieve, such as α -GlcNAc and α -GalNAc linkages as shown in figures 8 and 9 respectively.

The results shown so far demonstrate that mass spectrometric readout on gold arrays is very useful for following chemical and enzymatic reactions in a label-free manner. Because of the high resolution of MALDI-TOF MS even at high molecular weights, it should be possible to follow multiple reactions on the surface in parallel (multiplex analysis), provided that there is a difference in molecular weights between individual reaction sets. For proof of principle studies, we have immobilised mixtures of three Muc1 derived peptides on gold arrays (Figure 10, unpublished results). In previous studies we had shown that peptides Muc1fr1 is a good substrate for ppGalNAc T2 and complete glycosylation is observed when incubated with enzyme and UDP-GalNAc. Under the same reaction conditions, Muc1fr2 is only partially glycosylated and for Muc1fr3 we could not observe any glycosylation. The same

results were obtained in peptide mixtures, suggesting that the methodology allows us to investigate several potential substrates in single spots. These investigations are currently extended to more complex mixtures.

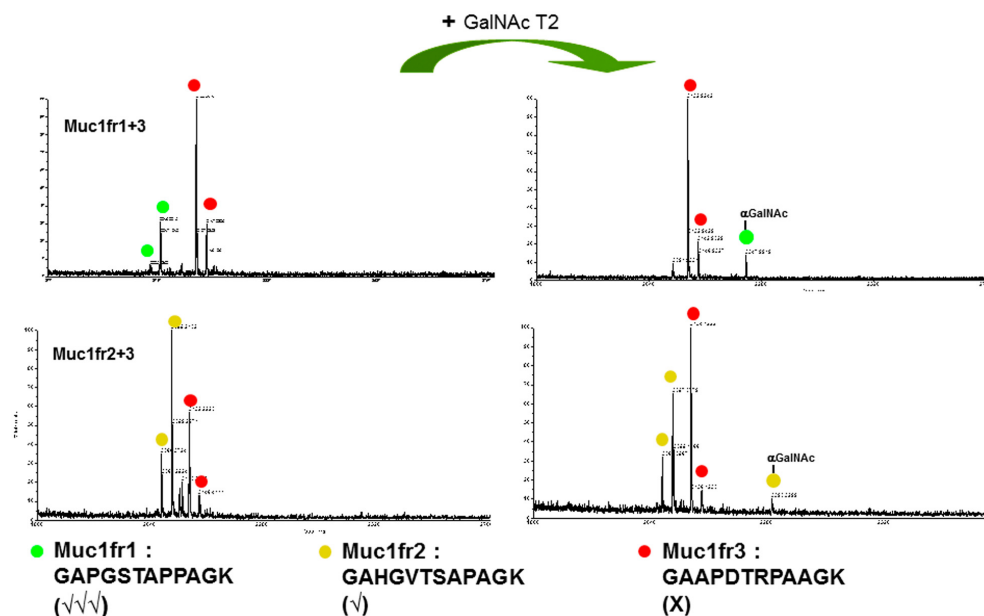


Figure 10. The resolution of the platform readout by mass spectrometry allows for the investigation of multiple substrates in one spot as shown here for pairs of peptides derived from the Muc1 repeat sequence of mucins, which are interrogated as substrates for the mucin transferase ppGalNAc T2.

Some of the most important terminal monosaccharides on cell surfaces of humans and higher organisms are the sialic acids. A number of glycosyltransferases which generate sialosides are available and it was of interest to study these enzymes on gold surfaces. However, the mass spectrometric analysis of sialic acids can present problems, since sialosides are often not stable under MALDI-TOF conditions, and hydrolysis products are observed. This can be overcome by methylation of sialic acids prior to analysis; in particular formation of the methyl ester significantly increases the signal intensity. For this purpose, we tested a number of methylation protocols for suitability on gold surfaces and found that these significantly improved the signal and allowed us to demonstrate that sialylation had indeed taken place. For example, the transsialidase from *Trypanosoma cruzi* was able to transfer sialic acids in an $\alpha 2,3$ manner from the glycoprotein fetuin onto the gold surfaces containing lactose acceptors to generate $\alpha 2,3$ sialyllactose. In collaboration with the group of Paul Crocker at the University of Dundee we could show that CHO cells, engineered to display sialoadhesin on their surfaces, selectively recognised gold surfaces displaying $\alpha 2,3$ sialyllactose over lactose (Figure 11 [34]).

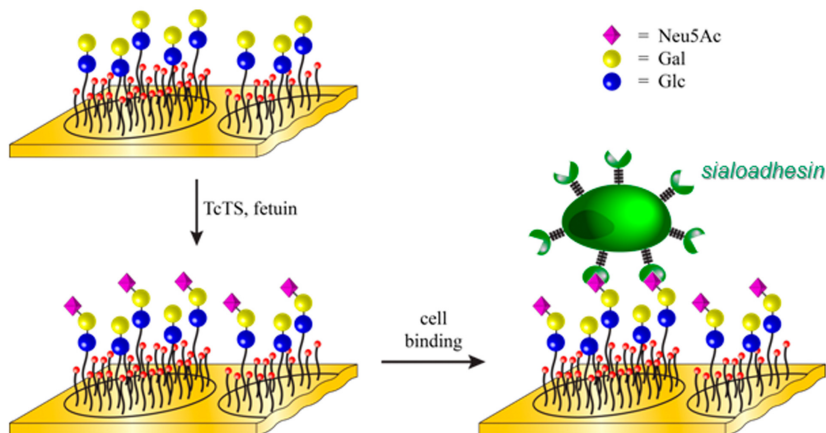


Figure 11: Sialic acids (Neu5Ac) are important components of cell surface oligosaccharides. The transsialidase TcTS from *Trypanosoma cruzi* can transfer sialic acids from glycoproteins such as fetuin to immobilised lactosides. The resulting sialolactosides are recognised by cell surface sialoadhesin receptors (collaboration with Prof Paul Crocker, University of Dundee)

CONCLUSIONS

An array platform consisting of gold surfaces covered with self-assembled functionalised monolayers has proven to be chemically and biologically robust and versatile. Many chemical and biochemical reactions can be studied on this surface and can proceed with very high efficiency, as measured by *in situ* mass spectrometry. In the past few years we have explored the application of this technology to glycosylation reactions and have found that it is suitable for many applications, in particular exploring enzyme specificity and activity. We believe that this technology is ideally suited to address the challenges of glycomics – high-throughput investigation of glycoenzyme activity and investigation of carbohydrate-protein interactions. The platform has the additional advantage that it is suited to a number of analytical techniques such as mass spectrometry and surface plasmon resonance spectroscopy, which makes it suitable for the development of diagnostic devices.

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REFERENCES

- [1] Laurent, N., Voglmeir, J., Flitsch S.L. (2008) *Chem. Commun.* **37**:4371 – 4384.
doi: 10.1039/B814463J.
 - [2] Halling, P.J., Ulijn, R.V., Flitsch, S.L. (2005) *Current Opinion in Biotechnology* **16**:385 – 392.
doi: <http://dx.doi.org/10.1016/j.copbio.2005.06.006>.
 - [3] Ulijn, R.V., Brazendale, I., Margetts, G., Flitsch, S.L., McConnell, G., Girkin, J., Halling, P.J. (2003) *J. Combi. Chem.* **5**:215 – 217.
doi: <http://dx.doi.org/10.1021/cc030024c>.
 - [4] Ulijn, R.V., Bisek, N., Flitsch, S.L. (2003) *Org. Biomol. Chem.* **1**:621 – 622.
doi: <http://dx.doi.org/10.1039/b211887d>.
 - [5] Ulijn, R.V., Bisek, N., Halling, P. J., Flitsch, S.L. (2003) *Org. Biomol. Chem.* **1**:1277 – 1281.
doi: <http://dx.doi.org/10.1039/b211890d>.
 - [6] Basso, A., De Martin, L., Gardossi, L., Margetts, G., Brazendale, I., Bosma, A.Y., Ulijn, R.V., Flitsch, S.L. (2003) *Chem. Commun.* **11**:1296 – 1297.
doi: 10.1039/B301680C.
 - [7] Basso, A., De Martin, L., Ebert, C., Linda, P., Gardossi, L., Ulijn, R.V., Flitsch, S.L. (2003) *Tetrahedron Lett.* **44**:6083 – 6085.
doi: [http://dx.doi.org/10.1016/S0040-4039\(03\)01464-3](http://dx.doi.org/10.1016/S0040-4039(03)01464-3).
 - [8] Bosma, A.Y., Ulijn, R.V., McConnell, G., Girkin, J., Halling, P.J., Flitsch, S.L. (2003) *Chem. Commun.* **22**:2790 – 2791.
doi: <http://dx.doi.org/10.1039/b308078a>.
 - [9] Basso, A., Ulijn, R.V., Flitsch, S.L., Margetts, G., Brazendale, I., Ebert, C., De Martin, L., Linda, P., Verdelli, S., Gardossi, L. (2004) *Tetrahedron* **60**(3):589 – 594.
doi: <http://dx.doi.org/10.1016/j.tet.2003.10.125>.
 - [10] Daines, A.M., Maltman, B.A., Flitsch, S.L. (2004) *Current Opinion in Chemical Biology* **8**:106 – 113.
doi: <http://dx.doi.org/10.1016/j.cbpa.2004.02.003>.
 - [11] Basso, A., Maltman, B.A., Flitsch, S.L., Margetts, G., Brazendale, I., Ebert, C., Linda, P., Verdellia, S., Gardossia, L. (2004) *Tetrahedron* **61**:971 – 976.
doi: <http://dx.doi.org/10.1016/j.tet.2004.11.015>.
 - [12] Bejugam, M., Maltman, B.A., Flitsch, S.L. (2005) *Tetrahedron: Asymmetry* **16**:21 – 24.
doi: <http://dx.doi.org/10.1016/j.tetasy.2004.11.031>.
-

-
- [13] Maltman, B.A., Bejugam, M., Flitsch, S.L. (2005) *Org. Biomol. Chem.* **3**:2505 – 2507.
doi: <http://dx.doi.org/10.1039/b506154g>.
- [14] Ulijn, R.V., Baragana, B., Halling, P.J., Flitsch, S.L. (2002) *J. Am. Chem. Soc.* **124**:10988 – 9.
doi: <http://dx.doi.org/10.1021/ja026912d>.
- [15] Basso, A., Maltman, B.A., Flitsch, S.L., Margetts, G., Brazendale, I., Ebert, C., Linda, P., Verdellia, S., Gardossia, L. (2004) *Tetrahedron* **61**:971 – 976.
doi: <http://dx.doi.org/10.1016/j.tet.2004.11.015>.
- [16] Deere, J., McConnell, G., Lalaoui, A., Maltman, B.A., Flitsch, S.L., Halling, P.J. (2007) *Advanced Synthesis & Catalysis* **349**:1321 – 1326.
doi: <http://dx.doi.org/10.1002/adsc.200700044>.
- [17] Deere, J., Halling, P., Flitsch, S.L., De Oliveira, R., Tomaszewski, B., Millar, S., Lalaoui, A., Solares, L. (2008) *Langmuir* **24**:11762 – 11769.
doi: <http://dx.doi.org/10.1021/la801932f>.
- [18] Humphrey, C.E., Turner, N.J., Easson, M.A.M., Flitsch, S.L., Ulijn, R.V. (2003) *J. Am. Chem. Soc.* **125**:13952 – 3.
doi: <http://dx.doi.org/10.1021/ja037922x>.
- [19] Doeze, R.H.P., Egan, C.L., Ulijn, R.V., Flitsch, S.L. (2004) *Angewandte Chemie* **43**:3138 – 3141.
doi: <http://dx.doi.org/10.1002/anie.200353367>.
- [20] Bejugam, M., Maltman, B.A., Flitsch, S.L. (2005) *Tetrahedron: Asymmetry* **16**:21 – 24.
doi: <http://dx.doi.org/10.1016/j.tetasy.2004.11.031>.
- [21] Maltman, B.A., Bejugam, M., Flitsch, S.L. (2005) *Org. Biomol. Chem.* **3**:2505 – 2507.
doi: <http://dx.doi.org/10.1039/b506154g>.
- [22] Šardžik, R., Noble, G.T., Weissenborn, M.J., Martin, A., Webb, S.J., Flitsch, S.L. (2010) *Beilstein J. Org. Chem.* **6**:699 – 703.
doi: <http://dx.doi.org/10.3762/bjoc.6.81>.
- [23] Ostuni, E., Chapman, R.G., Liang, M.N., Meluleni, G., Pier, G., Ingber, D., Whitesides, G.M. (2001) *Langmuir* **17**:5605 – 5620.
doi: <http://dx.doi.org/10.1021/la010384m>.
- [24] Su, J., Mrksich, M. (2002) *Angew. Chem. Int. Ed.* **41**:4715 – 4718.
doi: <http://dx.doi.org/10.1002/anie.200290026>.
-

- [25] Gurard-Levin, Z.A., Scholle, M., Eisenberg, A.H., Mrksich, M. (2011) *ACS Comb. Sci.* **13**(4):347 – 50.
doi: <http://dx.doi.org/10.1021/co2000373>.
- [26] Laurent, N., Voglmeir, J., Wright, A., Blackburn, J., Pham, N.T., Wong, S.C.C., Gaskell, S.J., Flitsch, S.L. (2008) *ChemBioChem.* **9**:883 – 887.
doi: <http://dx.doi.org/10.1002/cbic.200700692>.
- [27] Zhi, Z., Laurent, N., Powell, A.K., Karamanska, R., Fais, M., Voglmeir, J., Wright, A., Blackburn, J.M., Crocker, P.R., Russell, D.A., Flitsch, S.L., Field, R.A., Turnbull, J.E. (2008) *ChemBioChem.* **9**:1568 – 1575.
doi: <http://dx.doi.org/10.1002/cbic.200700788>.
- [28] Laurent, N., Haddoub, R., Flitsch, S.L. (2008) *Trends in Biotechnology* **26**:328 – 337.
doi: <http://dx.doi.org/10.1016/j.tibtech.2008.03.003>.
- [29] Karamanska, R., Clarke, J., Blixt, O., MacRae, J.I., Zhang, J.Q., Crocker, P.R., Laurent, N., Wright, A., Flitsch, S.L., Russell, D.A., Field, R.A. (2008) *Glycoconjugate Journal* **25**:69 – 74.
doi: <http://dx.doi.org/10.1007/s10719-007-9047-y>.
- [30] Laurent, N., Haddoub, R., Voglmeir, J., Wong, S.C.C., Gaskell, S.J., Flitsch, S.L. (2008) *ChemBioChem* **9**:2592 – 2596.
doi: <http://dx.doi.org/10.1002/cbic.200800481>.
- [31] Haddoub, R., Dauner, M., Stefanowicz, F.A., Barattini, V., Laurent, N., Flitsch, S.L. (2009) *Org. Biomol. Chem.* **7**:665 – 670.
doi: <http://dx.doi.org/10.1039/b816847d>.
- [32] Voglmeir, J., Šardžik, R., Weissenborn M.J., Flitsch, S.L. (2010) *OMICS J.* **14**:437 – 444.
doi: <http://dx.doi.org/10.1089/omi.2010.0035>.
- [33] Voglmeir, J., Kaloo, S., Laurent, N., Meloni, M.M., Bohlmann, L., Wilson, I.B., Flitsch, S.L. (2011) *Biochem. J.* **436**:447 – 55.
doi: <http://dx.doi.org/10.1042/BJ20101059>.
- [34] Šardžik, R., Sharma, R., Kaloo, S., Voglmeir, J., Crocker, P.R., Flitsch, S.L. (2011) *Chem. Commun.* **47**:5425 – 7.
doi: <http://dx.doi.org/10.1039/c1cc10745c>.
-