The mAb-Glyco Chip Kit – a Workflow Solution for Rapid and Fully Automated Characterisation of N-linked Glycans from Monoclonal Antibodies

*Lukas Trojer[1(2)], Kirill Gromadski[1(2)], Tom van de Goor[1] and Stephan Buckenmaier[1]**
**Corresponding author.*
(1) Agilent Technologies R&D and Marketing GmbH & Co. KG, Hewlett-Packard-Str. 8, 76337 Waldbronn, Germany
(2) New adress: Sandoz GmbH, Biochemiestr. 10, 6250 Kundl, Austria

Monoclonal antibodies (mAbs) are an important class in the group of new biological entities with about 30 antibody drugs licensed for treatment of various diseases [1]. These glycoproteins bear complex oligosaccharide moieties within their structure, whose presence, absence, and profile can have significant impact on therapeutic efficacy, pharmacokinetics, and immunogenicity. Glycosylation is influenced by many factors such as the cell line in which the mAb is produced as well as specific production parameters including pH or temperature. Thus, characterisation of glycan profiles is of vital importance throughout the various phases of drug development.

The challenge for the analytical chemist is that conventional methods utilised are complex and time consuming. For instance, a typical workflow could involve multiple sample preparation steps: Enzymatic deglycosylation of mAbs, hydrolysis of glycosylamines to free reducing end glycans, and (dependent on the detection method) the labelling of glycans with fluorescent tags. Glycan analyses apply capillary electrophoresis or HPLC with fluorescence and/or MS detection, or MALDI-TOF-MS. Together with data acquisition and processing the chemist is looking at one half to several days to complete the analysis [2]. In addition, if sample preparation is done manually, each step exhibits a potential source of error.

With this in mind the mAb-Glyco Chip Kit was developed. It is a complete workflow solution designed to characterise N-linked glycans on mAbs. Major development goals were to fully automate the workflow, to reduce the complexity of sample preparation, data acquisition and data processing, and to substantially increase sample throughput. This article provides a technical description of this solution and explains how it can aid in removing a major bottleneck during the development phase of these biological drugs.

mAb-Glyco Chip Kit

The Kit contains mAb-Glyco Chip, reagent pack and a content disk. mAb-Glyco Chip and its function will be described below. The reagent pack provides chemicals needed for ready chip operation: System Conditioning Reagent for flow path coating and carry over minimisation, Glycan Standards for chromatographic checkout and method development, Antibody Standard (IgG type mAb spiked with stable internal standard, a free reducing end glycan) for functional checkout of the chip, and Deglycosylation Buffer for dilution of standards, samples and for loading the mAb samples onto the chip's PNGase F enzyme reactor (see below). The content disk has optimised methods for glycan profiling analysis and data processing, including efficiency tools such as glycan accurate mass and structure database, and reporting templates. More details in [3].

Instrumental setup

All analyses were run on the Agilent 1260 Infinity HPLC-Chip/MS system comprising micro autosampler with thermostat (set to 4°C), capillary and nanoflow pump with micro degasser, and the Chip-Cube that interfaces LC modules and the MS instrument [4-6]. HPLC-grade H_2O [5mM formic acid (FA)] and

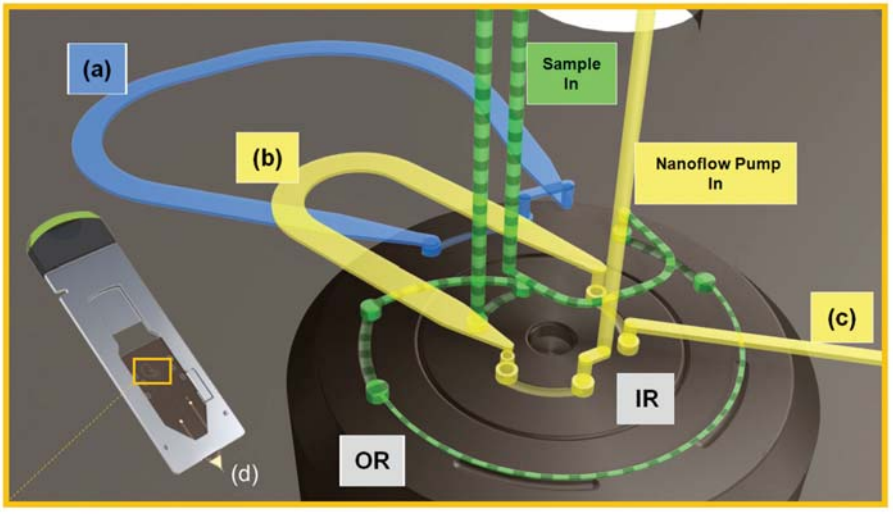

Figure 1: Rotor-in-rotor valve and chip design of the mAb-Glyco Chip incorporating (a) enzyme reactor, (b) glycan enrichment column, (c) glycan separation column, and (d) nano electrospray tip. OR = outer rotor, IR = inner rotor

Inner Rota (IR)	Outer Rotor (OR)	Columns - Nanoflow Pump	Columns - Capillary Pump
Enrichment	Inline	PGC-SC	ER / PGC-EC
Enrichment	Bypass	PGC-SC	PGC-EC
Analysis	Inline	PGC-EC / PGC-SC	ER
Analysis	Bypass	PGC-EC / PGC-SC	None

Table 1: All possible rotor valve switching positions of the mAb-Glyco Chip

ACN [5 mM FA] were used as nanoflow pump mobile phases A and B, respectively. ACN was from Merck (Germany), water from a Milli-Q water purification system, FA from Sigma (USA). A detailed description of the analysis method is given in Table 2.
Mass detection occurred with an Agilent 6520 Q-TOF operated in positive ion mode with Vcap = 1850V, drying gas flow = 3.5L/min at T = 360°C, and a fragmentor voltage of 160V. Data were acquired at 2GHz in MS only mode, range 450-3,000 m/z at a rate of 3 spectra/s. Internal mass calibration used m/z 922.0098. The MassHunter Workstation was used for data acquisition and processing.

mAb-Glyco Chip layout and operation

Figure 1 illustrates the architecture of the mAb-Glyco Chip, which is made from inert, biocompatible polyimide. It integrates: (a) a 310nL enzyme reactor (ER), packed with immobilised PNGase F beads (Peptide-N4-(acetyl-ß-glucosaminyl)-asparagine amidase N-Glycosidase F), (b) a 160nL porous graphitised carbon enrichment column (PGC-EC), (c) a 43mm x 75µm ID PGC separation column (PGC-SC), that directly connects to the metalised nano electrospray tip (Figure 1 (d)). PGC particle size = 5µm. The Chip-Cube interface automatically positions the chip orthogonal to the MS inlet and makes the necessary electrical and hydraulic connections to the chip [4-6]. The mAb-Glyco Chip uses the concentric rotor-in-rotor valve design of the Chip-Cube. The stator-chip-rotor sandwich creates an outer 10-port valve (OR) and an inner 6-port valve (IR). This unique design allows for switching ER and PGC-EC independently into or out of the sample loading flow path. Table 1 summarises all possible chip valve positions. Initial designs used a 6-port valve setup with the enzyme reactor in flow through mode, i.e. ER directly in-line with PGC-EC and thus residence time of the mAb on the enzyme reactor (which determines the deglycosylation efficiency) was a function of flow used to load the mAb onto the chip. Data have shown that a period of 6 seconds obtained at 1µL/min loading flow could efficiently deglycosylate the majority of many mAbs studied on the chip [e.g. 7]. However, some did not react quantitatively and required further lowering the flow rate. This was detrimental for analysis speed.

Description of the on-chip workflow

1) Data acquisition

Figure 2 illustrates the on-chip workflow comprising five automated steps:

1. Sample injection: Outer rotor is set to bypass (enzyme reactor bypass), inner rotor to analysis. A volume of antibody sample is injected and loaded onto the chip using deglycosylation buffer.
2. Enzyme reactor fill: Outer rotor switches enzyme reactor to inline position, which cuts a piece from the heart of the injected sample plug.
3. Deglycosylation: Outer rotor switches enzyme reactor back to bypass. The PNGase F reacts with the mAb and cleaves off the N-linked glycans while the capillary pump flushes the system.
4. Glycan transfer: Both, inner and outer rotors turn simultaneously switching both, enzyme reactor and enrichment column into the loading pump flow path. N-glycans become trapped on the PGC-EC.
5. Glycan separation/detection: Inner rotor turns into analysis position. Both, PGC-EC and PGC-SC are in the nanopump flow path. A reversed phase gradient is used to chromatographically separate glycans prior to TOF-detection. During analysis the outer rotor keeps the inline position for cleaning and re-equilibration of the ER with deglycosylation buffer.

Figure 3 shows results obtained from the analysis of the Kit's Antibody Standard using the HPLC-Chip method outlined in Table 2. Figure 3 shows time segments of the workflow and the nanoflow pump gradient. At a 3µL/min capillary pump flow rate, sample injection requires 1 minute and ER fill 6 seconds. Reaction time was 4 minutes, which was determined to result in complete deglycosylation of antibodies (this time may be shortened and the method optimised for a particular mAb of interest). The transfer of cleaved N-glycans from the ER to the PGC-EC takes 1 min and the separation of the enriched glycans on the PGC-SC including a column flushing step and re-equilibration, takes 6 min. This makes a total of 12 minutes for the entire on-chip workflow including

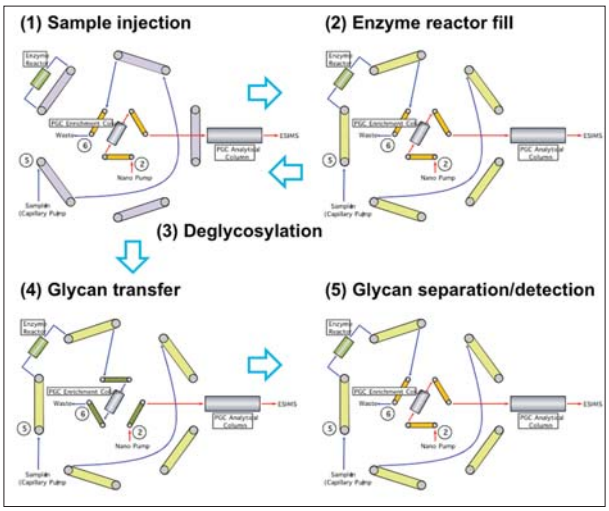

Figure 2: Valve switching scheme for automated on-chip deglycosylation of mAbs, enrichment, separation and MS-detection of N-glycans.

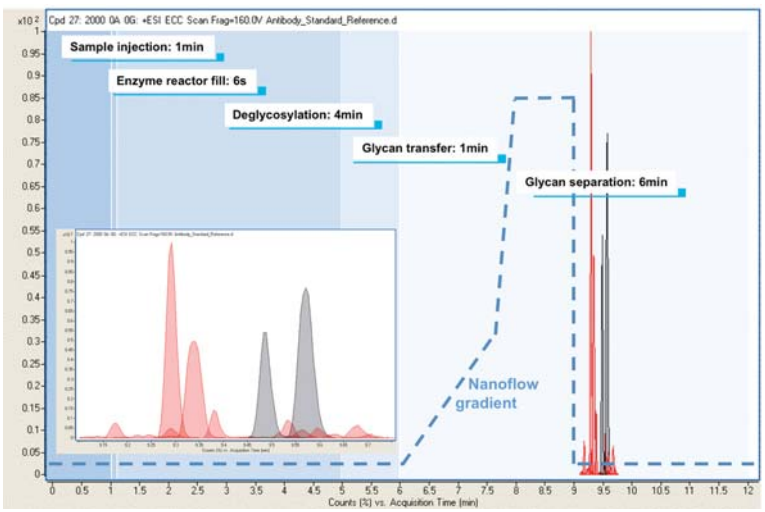

Figure 3: Analysis speed. The whole workflow time is 12 min. Sample: Antibody Standard, 75ng on-column.

		Chip Cube		Capillary Pump		Nanoflow Pump	
Description	Time [min]	IR	OR	Flow [µl/min]	Solvent B [%]	Flow [µl/min]	Solvent B [%]
Sample Injection	0.00	Analysis	Bypass	3	0	0.5	2
Enzyme Reactor Fill	1.00		Inline				
Deglycosylation	1.10		Bypass				
Glycan Transfer	5.00	Enrichment	Inline				
Glycan Analysis and Detection	6.00	Analysis					32
	7.50						85
	8.00						85
	9.00						2
	9.01						
	12.00						

Table 2: Optimised acquisition method.

sample preparation and analysis.
Figure 3 also shows the corresponding chromatogram (inset = zoom out). The red peaks are glycans that result from mAb deglycosylation and the black peaks are from the internal standard that is spiked into the Antibody Standard. The internal standard is a free reducing end glycan that serves for functional checkout of the HPLC-Chip/MS system. For instance, on an intact chip the ratio between the antibody glycans and the internal standard should be similar to that shown in Figure 3.

Taking a closer look at the glycan chromatogram of the antibody standard, which is shown in Figure 4 including structure assignments*: Why is the same glycan structure assigned to three distinct peaks? Figure 4 illustrates that during enzymatic deglycosylation by PNGase F, glycosylamines are released from the polypeptide backbone of the antibody. These are reactive intermediates that hydrolyse under acidic conditions to free reducing end glycans [8]. Due to the anomeric equilibrium at the carbon on the reducing end, each glycosylamine has two corresponding free glycan structures. The chromatogram on the right shows that these diastereomeric species can be resolved on the PGC separation column so that each cleaved N-glycan results in three peaks. Since the workflow with the mAb-Glyco Chip occurs within a short time frame, the predominately detected species are glycosylamines. Yet, exposure to the acidic nanoflow pump gradient converts some amount of glycoslyamine to free reducing end glycans (between 5% and 10%).
*The peaks for the internal standard were removed for reasons of simplicity.

2) Data processing

Data obtained from the analysis of N-glycans cleaved off mAbs can be complex. Therefore an automated data analysis and reporting procedure was developed to facilitate interpretation. Figure 5 provides an overview of the steps that convert a TOF raw data file (TIC) to a report of identified glycans with information on structure, retention time, mass error, abundance (volume) and relative abundance.

Extraction and identification

The (Q)-TOF data file is processed using the Molecular Feature Extractor (MFE) that is part of MassHunter. MFE utilises an algorithm taking into account LC retention time, accurate mass, charge states, adducts and the generation of oligomers (up to trimers, which might typically occur in the ESI process, in particular when the concentration increases) to extract unique compounds from the TIC data set. Furthermore, comparisons are made between the compound masses detected and those stored in the accurate mass mAb-Glyco database. The search of the

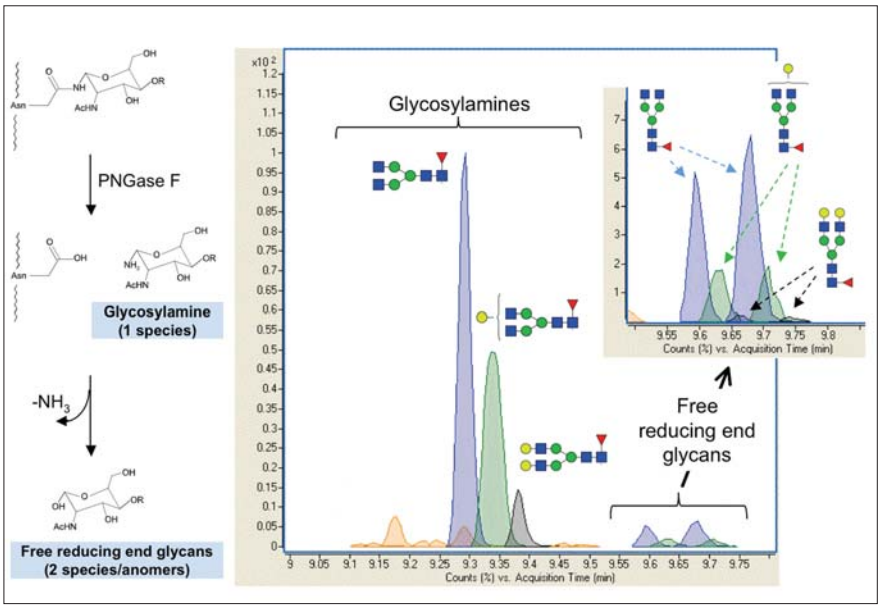

Figure 4: Left: Chemistry of the enzymatic PNGase F cleavage of N-linked glycans from a mAb. Glycosylamine intermediate hydrolyses to two anomeric free reducing end glycans. Right: mAb-Glyco Chip predominately detects glycoslyamines. Sample: Antibody Standard, 75ng on-column.

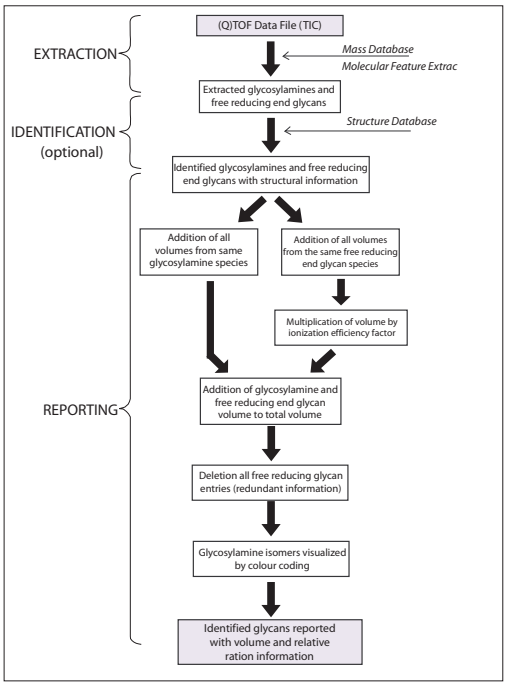

Figure 5: Schematic of the automated glycan extraction, identification, clustering and reporting procedure.

Compound Label	RT	Mass	Name	Formula	Tgt Mass	Diff (ppm)	Isomer(s) Present	Total Volume	Total Vol%
Cpd 28: 2121 0A 1G N	9.589	2092.7583	2121 0A 1G N	C79H132N6O58	2092.7564	0.93		8906	0.3
Cpd 23: 1100 0A 0G N	9.373	1258.4803	1100 0A 0G N	C48H82N4O34	1258.481	-0.58	1258.481	40030	1.5
Cpd 20: 2120 0A 0G N	9.349	1785.6693	2120 0A 0G N	C68H115N5O49	1785.6661	1.83		11972	0.5
Cpd 19: 3100 0A 0G N	9.317	1664.6406	3100 0A 0G N	C64H108N6O44	1664.6398	0.46		10792	0.4
Cpd 17: 2110 0A 0G N	9.305	1623.6155	2110 0A 0G N	C62H105N5O44	1623.6132	1.39		284929	10.8
Cpd 16: Man5 N	9.262	1233.4477	Man5 N	C46H79N3O35	1233.4494	-1.36	1233.4494	134626	5.1
Cpd 15: 2100 0A 0G N	9.25	1461.5628	2100 0A 0G N	C56H95N5O39	1461.5604	1.66		2044974	77.4
Cpd 13: 1100 0A 0G N	9.23	1258.4825	1100 0A 0G N	C48H82N4O34	1258.481	1.15	1258.481	40030	1.5
Cpd 11: 2010 0A 0G N	9.203	1477.5546	2010 0A 0G N	C56H95N5O40	1477.5553	-0.5		5076	0.2
Cpd 10: 1110 0A 0G N	9.198	1420.5339	1110 0A 0G N	C54H92N4O39	1420.5339	0.03		864	0.0
Cpd 9: Man4 N	9.17	1071.3928	Man4 N	C40H69N3O30	1071.3966	-3.55	1071.3966	6937	0.3
Cpd 5: 2000 0A 0G N	9.133	1315.5029	2000 0A 0G N	C50H85N5O35	1315.5025	0.3		59267	2.2
Cpd 4: 1000 0A 0G N	9.103	1112.4235	1000 0A 0G N	C42H72N4O30	1112.4231	0.31		34631	1.3
Cpd 3: Man4 N	9.082	1071.3956	Man4 N	C40H69N3O30	1071.3966	-0.9	1071.3966	6937	0.3
Cpd 1: Man5 N	9.068	1233.4475	Man5 N	C46H79N3O35	1233.4494	-1.52	1233.4494	134626	5.1

Table 3: Extract of a compound report.

extracted glycan signals against the database attaches corresponding structures to the hits. Figure 6 shows results obtained from the Antibody Standard. The identified hits are listed in the Data Navigator pane and corresponding peaks in Chromatogram Results (here, superposition of Extracted Compound Chromatograms). The Structure Viewer illustrates the glycan structure of the selected peak. m/z values, charge states and isotopes of the selected glycan hit are displayed in MS Spectrum Results and MS Spectrum Peak List.

Reporting: Volumes of glycosylamines with identical mass are merged. The same occurs for the volumes of the free reducing end glycan isomers. Latter are multiplied with an ionisation factor that compensates for the difference in ionisation efficiency between glycosylamines and free reducing end glycans. Corresponding glycosylamines and free glycans are clustered and the total volume is reported. Volumes of free reducing end glycan entries are deleted as redundant information. Finally, glycosylamine isomers are visualised by colour coding as shown in Table 3, which shows the summary table of a compound report that usually also includes chromatograms and mass spectra.

Data processing including reporting (all performed by a single mouse click) typically needs less than 5 minutes per sample. This makes a total of about 20 minutes for an assay that traditionally could take one half to several days.

Chip stability, reproducibility and lifetime

Table 4 summarises characteristics of the mAb-Glyco Chip. Retention time stability over 200 injections was in the range 0.3 to 0.5% RSD (4 chips evaluated), reproducibility of the relative abundance was typically better

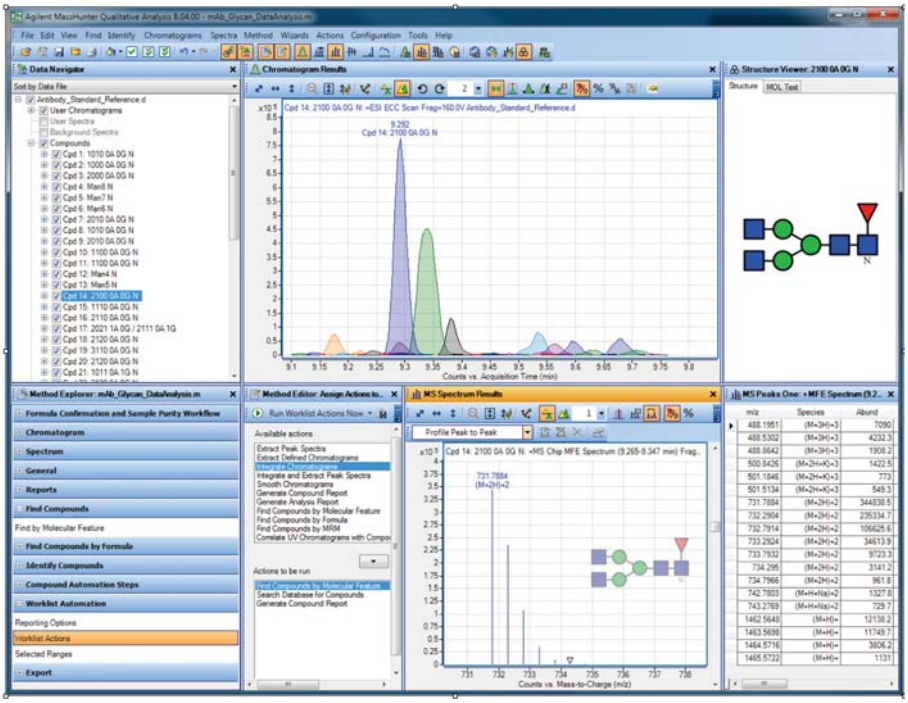

Figure 6: Typical results obtained from the extraction and identification procedure using MFE and glycan database search, respectively. Sample: Antibody Standard, 75ng on-column.

Investigated Item	# of Chips tested	Results
Retention time stability over 200 injections	4	0.3-0.5% RSD
Intra-day reproducibility of rel. glycan ratios	2	5% RSD on average (glycans > 1% relative ration)
Inter-day reproducibility of rel. glycan ratios	2	7% RSD on average (glycans > 1% relative ration)
Spray stability	1	> 200 hours
Chip lifetime	10	200-300 injections (75ng IgG from bovine serum (Sigma) on-column each injection)
PNGase F activity // long-term storage	1	90% remaining activity after 3 months of storage at -20°C
PNGase F activity // freeze/thaw cycles	2	83% remaining activity after 74 freeze/thaw cycles

Table 4: Performance characteristics of the mAb-Glyco Chip.

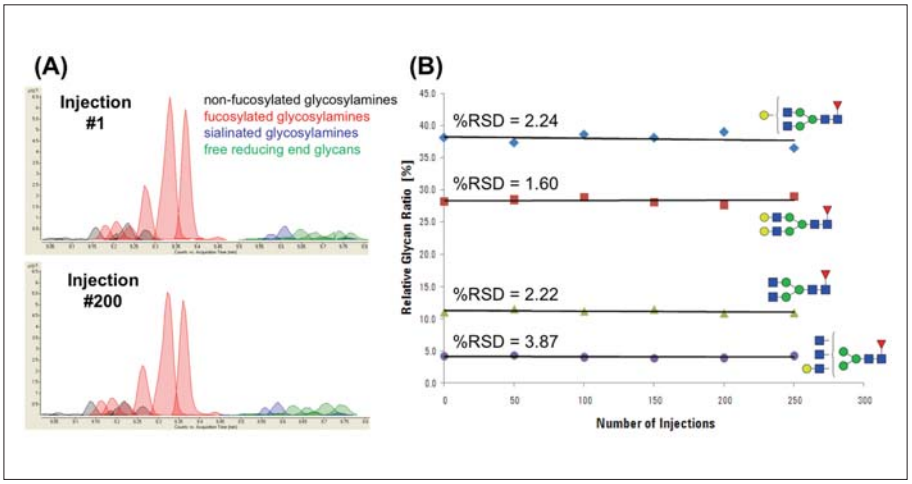

Figure 7: Long-term stability and robustness of the mAb-Glyco Chip: (A) Extracted glycan pattern of the analysed antibody at injection number 1 and 200. (B) Relative glycan ratio as function of the number of injections (4 most intense N-glycans considered). Sample: IgG from bovine serum (Sigma), 75ng on-column.

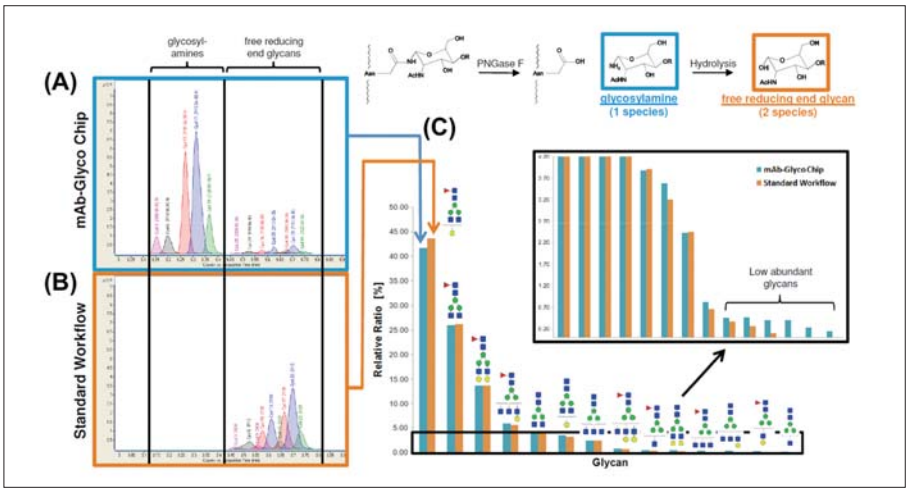

Figure 8: Chromatograms obtained (A) from analysis with mAb-Glyco Chip and (B) in-solution workflow. (C) Identified N-glycans together with calculated ratios, relative to glycan-total. Sample: IgG1 Kappa from human myeloma plasma (Sigma), 75ng and 100ng on-column for mAb-Glyco Chip and standard workflow, respectively.

	mAb-Glyco Chip	In-Solution Deglycosylation
Amount of antibody per sample	1µg	20µg
Deglycosylation time	4 min	3 hours
Protein (deglycosylated mAb and PNGase F) removal	not required (immobilized PNGase F)	10 min
Glycosylamine hydrolysis	not required (analysis on glycosylamine level)	2 hour
Glycan seperation and detection	6 min	6 min
Total workflow time	12 min	5-6 hours

Table 5: Summary of experimental details: mAb-Glyco Chip and standard in-solution deglycosylation workflow.

5% RSD for glycans > 1% relative ratio. Spray stability was maintained for more than 200 hours. Typical lifetime was in the range of 200-300 injections (10 chips evaluated). Under appropriate storing conditions (-20 °C, wet ER), the immobilised PNGase F conserved 90% of its original activity after a period of three months. Multiple freeze/thaw cycles do not severely affect PNGase F activity after immobilisation.

Figure 7 (A): Glycan chromatograms obtained from injection 1 and 200. Retention time, relative glycan distribution and absolute signal intensities of the identified glycan pattern remain well comparable demonstrating full catalytic activity of the PNGase F reactor over the course of 200 injections. Figure 7 (B): %RSD values for relative glycan ratios in the range 1.6 to 3.9 % show robustness and the stability of the on-chip deglycosylation workflow.

Comparison: On-chip versus standard in-solution deglycosylation experiment

Figure 8 shows a comparison of mAb-Glyco Chip based N-glycan analysis to results obtained from an in-solution PNGase F deglycosylation workflow*. Table 5 summarises experimental details.

In this current case 5 to 6 hours, as required with standard analyses, were reduced to 12 min. The most substantial time saving was due to the reduction of the deglycosylation time from 180 in standard analyses to 4 min with the mAb-Glyco Chip. In standard analyses long exposure times of the mAb to the PNGase F enzyme are needed in order to cleave all the glycans quantitatively. This is crucial since quantification occurs relative to the total amount of glycans. The strong reduction in deglycosylation time with the mAb-Glyco Chip is due to the large amount of PNGase F enzyme that is immobilised on a highly macroporous, large surface support material, leading to a high enzyme to substrate ratio and thus to a fast deglycosylation rate.

Additional time is saved because the chip based approach does not require conversion of glycosylamines to free glycans. The chromatogram in Figure 8 (A) shows that the mAb-Glyco Chip predominately detects glycosylamines, whereas the standard workflow, the later eluting free reducing end glycans (Figure 8 (B)). The graphic in Figure 8 (C) demonstrates that relative ratios of all glycans are well comparable between the two analyses. Moreover, the mAb-Glyco Chip analysis typically results in a higher number of identified low abundant glycans, which could be attributed to: 1.) Higher ionisation efficiency of glycosylamines, 2.) Conversion of one glycosylamine into two, free reducing end glycans, which could lower the limit of detection in the in-solution workflow, and 3.) potential losses of low abundant glycans in the multi-step in-solution procedure in contrast to the integrated on-chip workflow.

*In-solution deglycosylation experiments were done using a PNGase F kit from New England Biolabs. 20µg of the protein was combined with 4µl of G7 Reaction Buffer, 10µl of PNGase F and water to make a total reaction volume of 40µL. This mixture was incubated for 3h at 37°C. Deglycoslyated antibody was removed by centrifugation using Vivaspin 4 vials with a molecular cut-off

at 10,000Da. The remaining glycans were then converted to free reducing end glycans by dilution of the sample with 1% FA to obtain an antibody concentration of 0.1mg/ml; incubation for 2h at 37°C.

Conclusion:

This article provides a technical description of the mAb-Glyco Chip Kit that was designed for fast and automated characterisation of N-glycans from monoclonal antibodies (mAb). It was demonstrated that the complete workflow including on-chip deglycosylation of the mAb as well as chromatographic separation, and Q-TOF detection of the cleaved glycans, and data processing can be completed within a 20 min time period.

Data have verified chip stability, reproducibility and lifetime as well as excellent comparability of results obtained from chip analysis to those obtained from a typical standard workflow (that in this case took 5-6 hours to complete). It can be concluded that the mAb-Glyco Chip Kit provides a robust workflow solution that helps to remove a major bottleneck during the development phase of mAb-based biological drugs allowing the analyst to quickly provide answers.

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