Improved and semi-automated reductive β-elimination workflow for higher throughput protein *O*-glycosylation analysis

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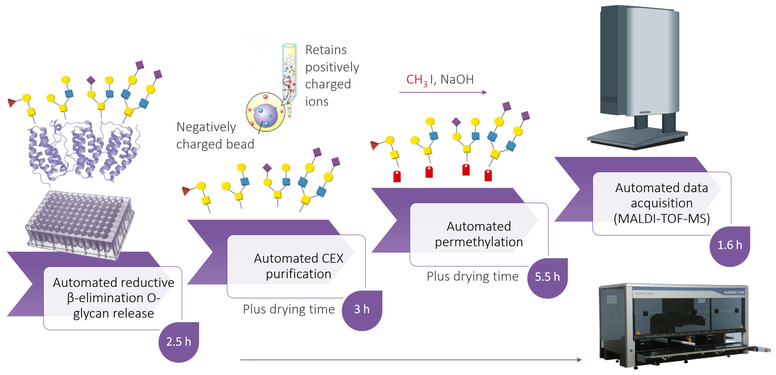
Introduction

Protein *O*-glycosylation has shown to be critical for a wide range of biological processes, resulting in an increased interest in studying the alterations in *O*-glycosylation patterns of biological samples as disease biomarkers as well as for patient stratification and personalized medicine.[1,2] Given the complexity of *O*-glycans, often a large number of samples have to be analysed in order to obtain conclusive results.[4] However, most of the *O*-glycan analysis work done so far has been performed using glycoanalytical technologies that would not be suitable for the analysis of large sample sets, mainly due to limitations in sample throughput and affordability of the methods.[4] Here we report a largely automated system for *O*-glycan analysis. We adapted reductive β-elimination release of *O*-glycans[5] to a 96-well plate system and transferred the protocol onto a liquid handling robot. The workflow includes *O*-glycan release, purification and derivatization through permethylation followed by MALDI-TOF-MS. The method has been validated according to the ICH Q2 (R1) guidelines for the validation of analytical procedures.[6] The semi-automated reductive β-elimination system enabled for the characterization and relative quantitation of *O*-glycans from commercially available standards. Results of the semi-automated method were in good agreement with the conventional manual in-solution method[5] while even outperforming it in terms of repeatability. Release of *O*-glycans for 96 samples was achieved within 2.5 hours, and the automated data acquisition on MALDI-TOF-MS took less than 1 minute per sample. This largely automated workflow for *O*-glycosylation analysis showed to produce rapid, accurate and reliable data, and has the potential to be applied for *O*-glycan characterization of biological samples, biopharmaceuticals as well as for biomarker discovery.

Method

Preparation: Sample preparation for *O*-glycan release was adapted to a 96-well plate system to allow for its use on the liquid handling robot with the exception of few steps such as off-deck plate sealing, incubation and centrifugal evaporation. *O*-glycan release: O-glycans were released by reductive β-elimination, using 1M potassium borohydride (KBH_4) solution in 0.1M potassium hydroxide (KOH) prior to incubation in an ultrasonic bath at 60°C for 2 hours. Clean-up: Released *O*-glycans were cleaned up using cation exchange (CEX) resin-packed VersaPlate tubes (CEX cartridges) and methanol (MeOH). Derivatization and analysis: Released and purified *O*-glycans were permethylated using the LudgerTag™ permethylation microplate kit (LT-PERMET-96)[3] prior to to MALDI-TOF-MS analysis in positive ion mode.



Experimental data

The methods for *O*-glycan release, purification, and permethylation were validated according to the ICH Q2 (R1) guidelines for the validation of analytical procedures. The validation characteristics accuracy, repeatability (intraday variation), intermediate precision (interday variation), linearity and working range are addressed in the following section.

Accuracy

In this study, the in-solution reductive β-elimination method was used as reference method for the development of the semi-automated method. We analysed bovine submaxillary mucin (BSM) type I-S using the semi-automated reductive β-elimination method and compared the data to those obtained from the manual in-solution protocol (Fig 1). The relative areas (RAs), standard deviations (SDs), and coefficients of variation (CVs) from 10 major *O*-glycan peaks were calculated from sample analysis using MALDI-TOF-MS. For MALDI-TOF-MS signals with RAs above 3 %, the CVs were < 6.4 % compared to CVs of 20.3 % for the manual in-solution method.

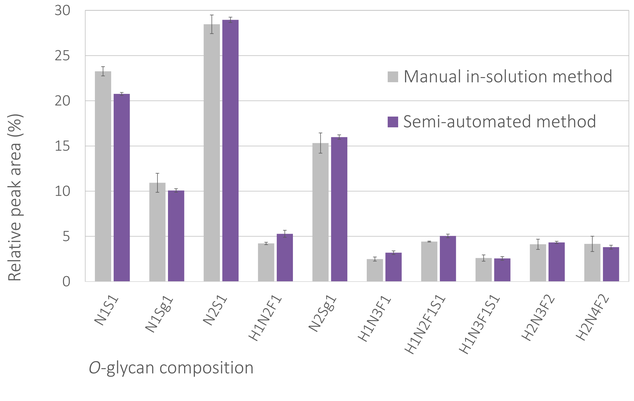


Fig 1. Comparison of the relative peak areas of 10 major *O*-glycans from 50 µg of BSM type I-S glycoprotein analysed after sample preparation using the manual in-solution method and the liquid handling robot.

Repeatability (intraday variation)

In order to show the precision of the procedure under the same operating conditions, glycans from 8 independent BSM type I-S samples (50 µg) were released, purified, permethylated and analysed by spotting these 8 samples singly on the MALDI-target. The areas under each peak corresponding to glycans from these spectra were integrated, normalized and the RAs, SDs, and CVs were calculated for the 10 major *O*-glycan peaks. The CVs for RAs were < 11.2 % for O-glycans with RAs ≥ 2.6 % (Table 1).

Peak no.	Glycan composition	Avg. RA (%)	SD	CV
1	N1S1	23.02	0.70	3.06
2	N1Sg1	11.34	0.74	6.56
3	N2S1	28.25	0.98	3.48
4	H1N2F1	4.39	0.24	5.40
5	N2Sg1	15.71	0.83	5.27
6	H1N3F1	2.64	0.20	7.65
7	H1N2F1S1	4.45	0.37	8.42
8	H1N3F1S1	2.49	0.24	9.69
9	H2N3F2	4.00	0.39	9.83
10	H2N4F2	3.72	0.42	11.22

Table 1. Glycan composition, average relative areas (Avg. RAs), standard deviations (SDs) and coefficients of variation (CVs) for 10 major *O*-glycan structures calculated after triplicate analysis.

Intermediate precision (interday variation)

For this test, the same experimental set up used for the intraday variation experiment was applied to 8 BSM type I-S samples (50 µg) on two separate days. Glycan areas from the spectra were integrated, normalized and the RAs, SDs and CVs were calculated for the 10 major *O*-glycan peaks. A linear regression plot of the relative values from two different days gave an R^2 value of 0.97, indicating a high level of correlation between the two data sets (Fig 2).

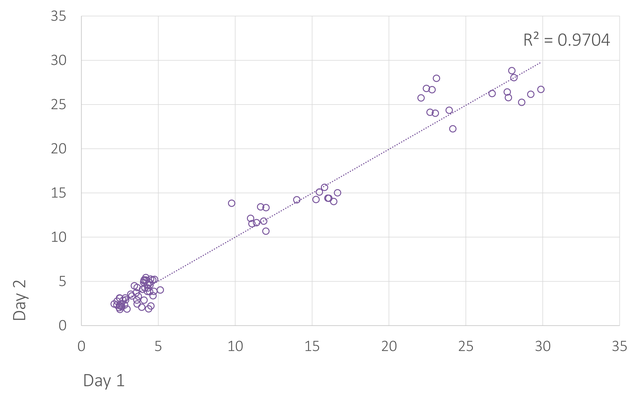


Fig 2. Linear regression plot comparing RA values of 10 major *O*-glycan structures. Released glycans from 8 independent BSM type I-S samples were purified, permethylated and analysed by MALDI-TOF-MS in two separate days (8 samples on day 1 versus 8 samples on day 2).

Linearity

In order to evaluate the ability of the analytical procedure (within a given range) to obtain test results which are directly proportional to the amount of analyte in the sample, glycans from 200 µg, 100 µg, 50 µg, 10 µg and 5 µg of starting material of BSM type I-S glycoprotein were released, purified, permethylated and analysed in triplicate. Each concentration was plotted for four major BSM type I-S *O*-glycan peaks against their relative peak intensities (RIs). The R^2 values from the linear regression plot for the four major BSM type I-S *O*-glycan peaks were all above 0.93 (Fig 3). We believe that the negative slope shown in the concentration-signal intensity relationship of the *O*-glycan structure N_2S_1 and N_2Sg_1 is due to the stepwise degradation of these polysaccharides starting at the reducing end and removing one sugar residue at a time (peeling).[1]

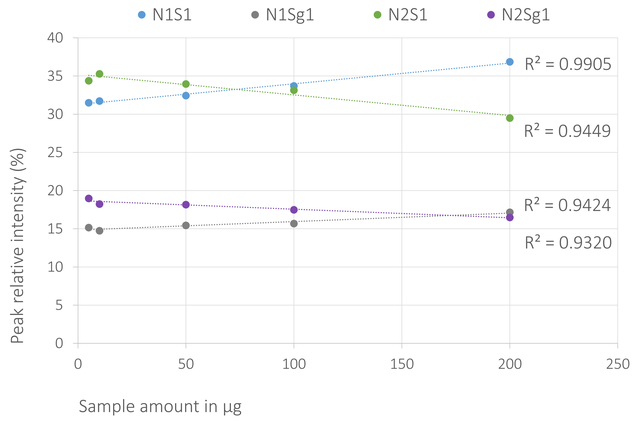


Fig 3. Triplicate samples of BSM type I-S glycoprotein from different starting material amounts (200 µg, 100 µg, 50 µg, 10 µg and 5 µg) were released, purified and permethylated using the liquid handling robot.

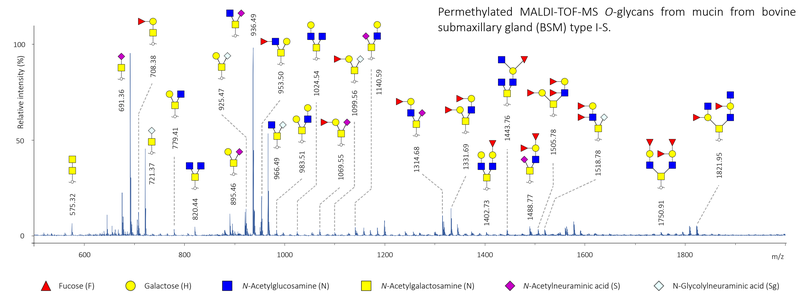
Working range

The working range was derived from linearity studies, and is the interval between the lower and upper concentration (amount) of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. From these data, it was determined that the working range was between 5 µg to 200 µg.

Amount (µg)	Glycan composition	Avg. RA (%)	SD	CV
5	N1S1	26.71	0.16	0.59
	N1Sg1	13.17	0.48	3.64
	N2S1	38.39	0.29	0.76
	N2Sg1	21.73	0.17	0.76
10	N1S1	26.58	2.48	9.31
	N1Sg1	12.66	1.18	9.33
	N2S1	39.69	1.46	3.69
	N2Sg1	21.07	2.73	12.93
50	N1S1	27.40	0.11	0.40
	N1Sg1	13.30	0.24	1.78
	N2S1	38.21	0.44	1.16
	N2Sg1	21.09	0.28	1.31
100	N1S1	28.57	1.30	4.54
	N1Sg1	13.61	0.58	4.29
	N2S1	37.47	1.28	3.41
	N2Sg1	20.35	0.60	2.97
200	N1S1	31.38	0.28	0.90
	N1Sg1	15.05	1.64	10.87
	N2S1	33.99	1.54	4.52
	N2Sg1	19.58	0.33	1.67

Table 2. Glycan composition, average relative areas (Avg. RAs), standard deviations (SDs) and coefficients of variation (CVs) of the 4 major *O*-glycan structures, calculated after triplicate analysis, for the different amounts of glycoprotein used.

Conclusions and future perspectives



We have presented a largely automated *O*-glycosylation analysis workflow which was used for characterization and relative quantitation of *O*-glycans from commercially available standards. This work showed that the data for the identification and quantitation of *O*-glycans by MALDI-TOF-MS obtained from the semi-automated procedure were comparable to those of the conventional manual in-solution method. Release of *O*-glycans for 96 samples can be performed within 2.5 h, significantly reducing the procedure timeline of the reference method (overnight incubation), without compromising the quality and integrity of the generated data. While providing advantages such as taking out time-consuming and labor-intensive steps, this system has showed to even outperform the current manual in-solution reductive β-elimination method in terms of repeatability, being able to produce lower CVs than previously described. The automated data acquisition using the MALDI-TOF-MS takes less than 1 minute per sample, greatly reducing the time required to measure glycan samples. In conclusion, the semi-automated reductive β-elimination system described in this article produces rapid, accurate and reliable data. We believe that this technique has the potential to be utilized for *O*-glycan characterization of biological samples, biopharmaceuticals as well as biomarker discovery.

Acknowledgements and References

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