Development of a high throughput method for O-glycosylation analysis

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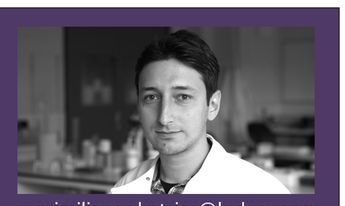

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Introduction

Colorectal cancer (CRC) is the second most common cancer in Europe, and is one of the most curable cancers when detected in its early stages. However, the disease remains undiagnosed due to its aspecific symptoms. Although population-based screenings are effective for early detection and prevention, the current CRC screening methods either lack sensitivity and/or specificity, or cause discomfort and pain due to their invasive character. There is an urgent need for discovering disease biomarkers and personalised treatment. With the exception of CEA (carcinoembryonic antigen) levels in plasma, which have shown limited value, there are many other proposed CRC plasma biomarkers at the DNA, protein and carbohydrate level that have not yet reached clinical application.

Glycans are promising candidates as diagnostic and prognostic CRC biomarkers, since various studies have shown CRC-associated changes in glycosylation profiles. Much of the previous glycan biomarker work has been performed on N-glycans. However, the study of protein O-glycosylation is receiving increased attention in biological, medical and biopharmaceutical research.

In order to study O-glycans (serine- or threonine-linked oligosaccharides), chemical release is currently the only effective method for obtaining the full range of these compounds. Improved high throughput techniques are required to allow reliable, reproducible and quantitative analysis of O-glycans.

At Ludger Ltd we are developing technologies that can help researchers to understand the structure-function relationship of O-glycosylation in CRC. Here we report our progress in method optimisation and development of a high throughput system for O-glycan analysis. We describe how we have adapted reductive β-elimination release of O-glycans to a 96-well plate system to allow for its use with a liquid handling robot, making this technology a good candidate for high throughput analysis of cancer related samples.

Method

O-glycan analysis:

Glycan release. In order to assay the functionality of our high throughput method for O-glycosylation analysis, bovine fetuin **(FET)** and bovine submaxillary mucin **(BSM)** O-glycans were released by reductive β-elimination, using 1M potassium borohydride (KBH_4) solution in 0.1M potassium hydroxide (KOH). Samples were incubated in an ultrasonic bath at 60°C for 2 hours.

Cleanup. Purification was performed using cation exchange resin cartridges (*LC-CEX, Ludger Ltd, UK*) and an additional methanol (MeOH) evaporation step.

Derivatisation and analysis. Released O-glycans were permethylated (*LT-PERMET-96, Ludger Ltd, UK*) prior to MALDI-TOF-MS analysis in positive ion mode.

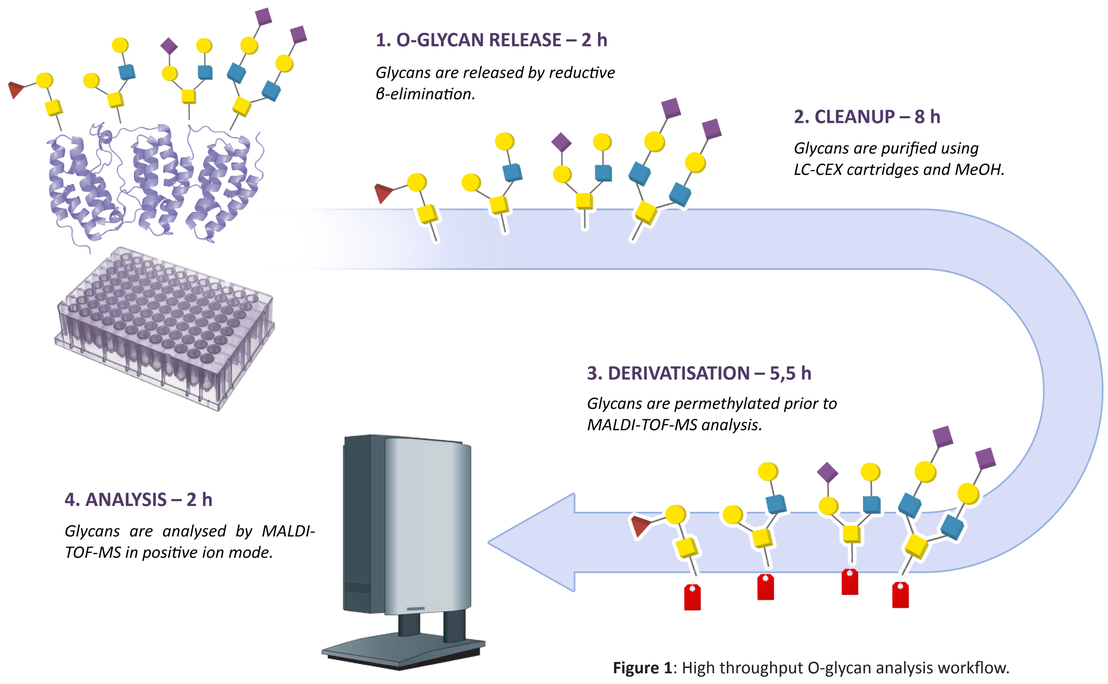

Figure 1: High throughput O-glycan analysis workflow.

Experimental Data

Regarding the development of a high throughput method for O-glycan analysis, we tested the efficiency of our new technology **(figure 2A, 3A)** when compared to the manual procedure **(figure 2B, 3B)**. A comparison of the O-glycan data generated from BSM **(figure 2)** and FET **(figure 3)** can be observed below. The data generated from both procedures has been shown to be comparable.

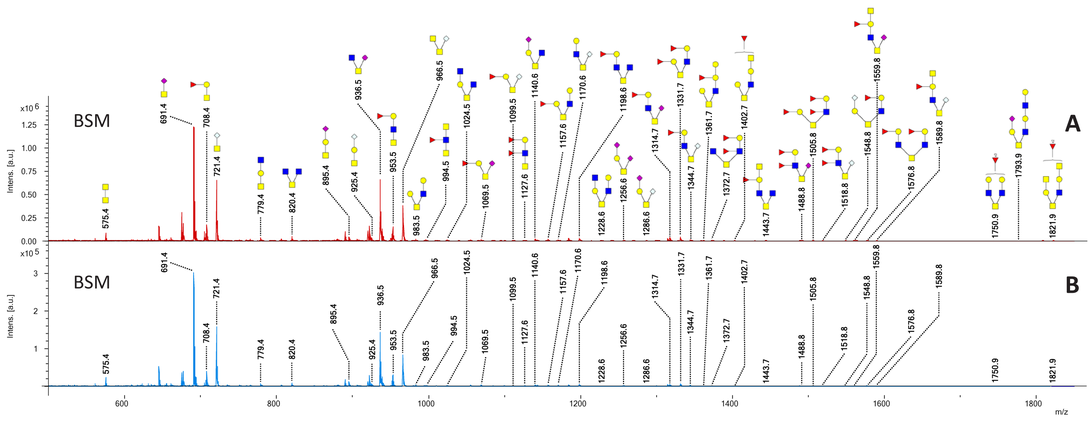

Figure 2. BSM MALDI-TOF-MS O-glycome spectra. *Major glycan peaks are annotated and represent compositions.* 50 µg of glycoprotein were incubated in an ultrasonic bath at 60°C for 2 hours using 1M potassium borohydride (KBH_4) solution in 0.1M potassium hydroxide (KOH). A comparison between high throughput **(A)** and manual **(B)** procedure is shown.

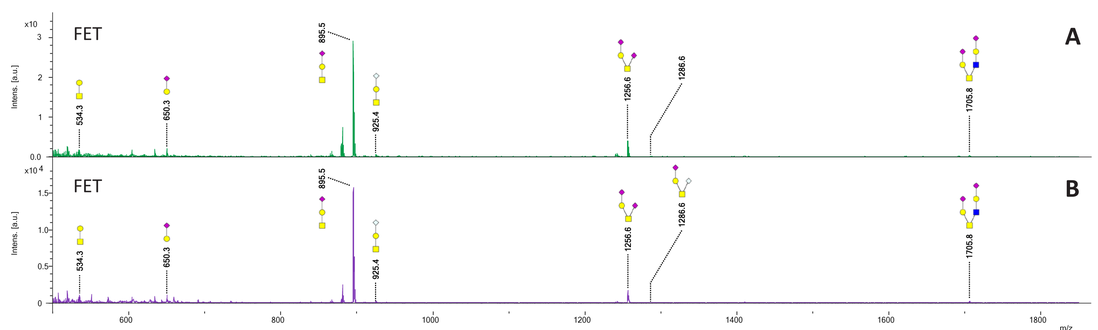

Figure 3. FET MALDI-TOF-MS O-glycome spectra. *Major glycan peaks are annotated and represent compositions.* 50 µg of glycoprotein were incubated in an ultrasonic bath at 60°C for 2 hours using 1M potassium borohydride (KBH_4) solution in 0.1M potassium hydroxide (KOH). A comparison between high throughput **(A)** and manual **(B)** procedure is shown.

By optimizing many of the different method parameters, we have greatly reduced the procedure timeline. Reaction conditions were initially optimized for 21 hours incubation time **(figure 4A)**. In order to reduce it, samples were incubated in an ultrasonic bath at various temperatures and times **(figures 4B, 4C, 4D, 4E)** and the MS signal obtained from each condition was point compared. Signal intensity and the areas of the separated O-glycan species from BSM were used for the comparisons. Following these experiments, the incubation at 60°C for 2 hours in an ultrasonic bath was chosen as the standard operating condition as a compromise between structural coverage and incubation time. A comparison of the data generated from each approach is shown in **figure 4**. The actual method **(figure 4D)** offers shorter timelines, permitting rapid O-glycan release within two hours.

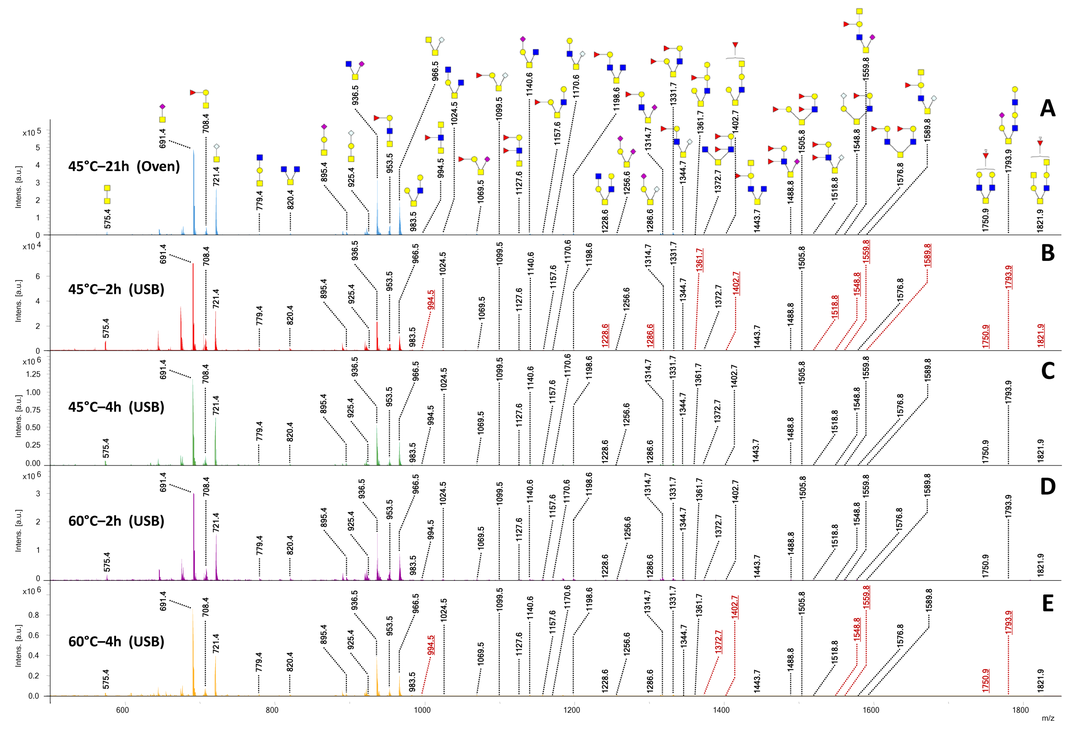

Figure 4. BSM MALDI-TOF-MS O-glycome spectra. 50 µg of glycoprotein underwent several incubation methods using 1M potassium borohydride (KBH_4) solution in 0.1M potassium hydroxide (KOH). **A.** *Oven incubation at 45°C for 21 hours.* **B.** *Ultrasonic bath incubation (USB) at 45°C for 2 hours.* **C.** *Ultrasonic bath incubation at 45°C for 4 hours.* **D.** *Ultrasonic bath incubation at 60°C for 2 hours.* **E.** *Ultrasonic bath incubation at 60°C for 4 hours. Undetected O-glycan structures from each approach are marked in red.*

In order to test the accuracy of the high throughput procedure, O-glycans were released from a large range of starting material (*5-200 µg of BSM*) **(figure 5)**. Although structural coverage and signal intensity of the O-glycan structures detected for each different sample amount is comparable, with a small decrease in signal intensity for the amounts analysed above 100 µg and non detection of a few small structures at amounts analysed below 50 µg, the O-glycan data generated shows that the optimal amount of starting material to use is 50 µg.

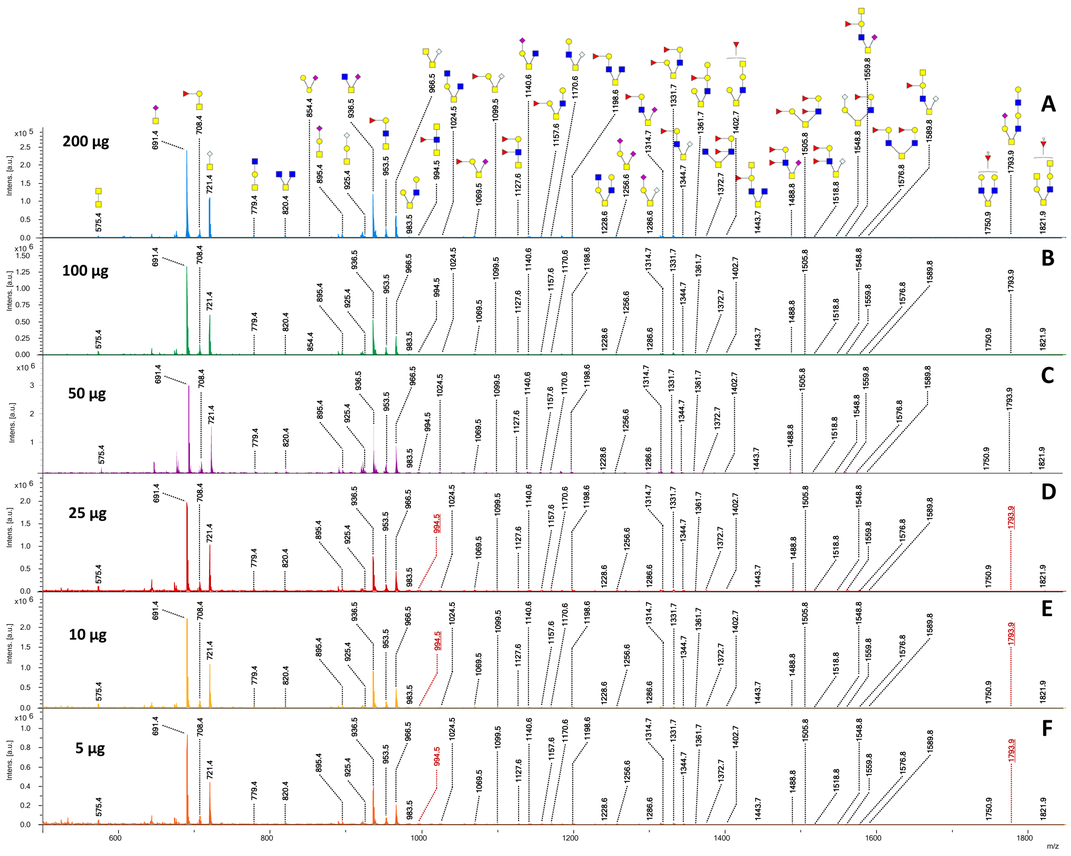

Figure 5. BSM MALDI-TOF-MS O-glycome spectra. 200 **(A)**, 100 **(B)**, 50 **(C)**, 25 **(D)**, 10 **(E)** and 5 **(F)** µg of glycoprotein were incubated in an ultrasonic bath at 60°C for 2 hours using 1M potassium borohydride (KBH_4) solution in 0.1M potassium hydroxide (KOH). After permethylation, all samples were dried down in a centrifugal evaporator and re-constituted in 10 µL of 70% MeOH prior to MALDI-TOF-MS analysis. *Undetected O-glycan structures from each approach are marked in red.*

Conclusions and Future Perspectives

The results shown on this poster prove that rapid, accurate, sensitive and reproducible O-glycan data can be generated from a large range of starting material, and that our method is suitable for high throughput studies. The actual glycan workflow is optimized for O-glycosylation analysis of purified glycoproteins. We aim to further validate our method to enable its use as a technique for the high throughput analysis of colorectal cancer samples.

References

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