Integrative Biology

PAPER

Automated, high-throughput serum glycoprofiling platform†‡

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Complex carbohydrates are rapidly becoming excellent biomarker candidates because of their high sensitivity to pathological changes. However, the discovery of clinical glycobiomarkers has been slow, due to the scarcity of high-throughput glycoanalytical workflows that allow rapid glycoprofiling of large clinical sample sets. To generate high-quality quantitative glycomics data in a high-throughput fashion, we have developed a robotized platform for rapid serum-based *N*-glycan sample preparation. The sample preparation workflow features a fully automated, rapid glycoprotein denaturation followed by sequential enzymatic glycan release, glycan purification on solid-supported hydrazide and fluorescent labelling. This allows accurate glycan quantitation by ultra-high performance liquid chromatography (UPLC). The sample preparation workflow was automated using an eight-channel Hamilton Robotics liquid handling workstation, allowing the preparation of almost 100 samples in 14 hours with excellent reproducibility and thus should greatly facilitate serum-based glyco-biomarker discovery.

Insight, innovation, integration

It is well recognized that glycans affect cellular function in biological system; consequently glycoprofiling can be useful for disease pathophysiology and to identify biomarkers for various diseases. Serum contains a high abundance of glycans and whole serum glycomic approaches have been used in disease diagnosis studies. We have developed a high-throughput robotic platform for whole serum *N*-glycan analysis which allows accurate glycan quantification by ultra-high performance liquid chromatography. The method offers a facile and rapid preparation of almost 100 samples in 14 hours with excellent reproducibility. Diagnostic information about disease progression and biological mechanism can be extracted from the data and should expedite the emergence of novel biomarkers. This technique can also serve as a complimentary approach to proteomic research.

Introduction

The majority of all human proteins are glycosylated, and alterations in glycosylation impact numerous physiological and pathological processes.[1–3] Attached glycans significantly affect protein function and pathophysiology but they are not directly encoded by genes, and the regulation of glycan expression is poorly understood. Recently, large-scale glycoprofiling studies have been combined with human genome-wide association studies (GWAS) to examine the regulation of glycan expression in human populations.[4–6] For instance, the combination of GWAS and high-throughput glycomics analysis of more than 2500 individuals showed that common variants in the Hepatocyte Nuclear Factor α (HNFα) and fucosyl-transferase genes influence *N*-glycan levels in human plasma, thereby revealing a new role for HNFα as a master transcriptional regulator of multiple stages in the fucosylation process.[5] However, future GWAS will require the processing of substantially larger sample sets in a rapid and cost-effective fashion.

N-glycan profiling can be enabled by several analytical approaches, including separation chromatography such as reverse phase chromatography, hydrophilic interaction chromatography and capillary electrophoresis and these techniques been reviewed extensively.[7,8] Matrix-assisted laser desorption/ionization (MALDI) time-of-fight (TOF) mass spectrometry (MS) has also been comprehensively utilised; however problems associated with stability of the sialylated glycan species, which is often overcome by permethylation reactions hinders its high throughput capabilities.[9] One of the latest advances in the field of glycan profiling is the implementation of HILIC UPLC, exhibiting increased separation efficiency compared to HPLC for increased speed and throughput.[10,11]

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We have recently reported the first low-cost, high-throughput automated glycan sample preparation platform, which allows accurate glycan quantification of glycans derived from affinity purified proteins such as Immunoglobulin G (IgG).[12] Although glycoprofiling of individual glycoproteins is extremely useful for understanding disease pathophysiology and biomarker discovery, the serum glycome encodes much more biological information than glycans derived from individual serum glycoproteins. Thus, whole serum glycomics has been used as a valuable complementary tool in glycobiology research. Our laboratory had previously developed a HPLC-based analysis of serum N-glycans on a 96-well plate and breast cancer patients have been identified using UPLC-based analysis.[13] Furthermore, N-glycan abnormalities have been found in children with galactosemia, which yielded a better understanding of the pathophysiology of the disease and paves the way for new treatment strategies.[14] Moreover, glycosylation changes in the serum of inflammatory arthritis patients on anti-TNF therapy were found to be associated with a decrease in inflammatory processes and reflect the effect of anti-TNF on the immune system.[15] Although the 96-well glycan sample preparation platform has been very useful in many glycomics studies, the method is labour-intensive and difficult to automate, which is often an industry expectation. Thus, we adapted our existing robotized glycomics platform for whole serum glycan sample preparation and compared this with the existing 96-well manual method.[12,16]

Experimental

All chemical reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO). Solid-supported hydrazide was obtained from Fisher Scientific (Waltham, MA). Solvinert filter plates were from Merck-Millipore (Billerica, MA) and Acroprep filter plates from Pall (Port Washington, NY). Samples were prepared on a Hamilton Robotics StarLet liquid handling platform (Reno, NV). The instrument is equipped with 8 software-controlled pipettes, a vacuum manifold and an automated heater shaker. Samples were analyzed on a Waters Acquity H-Class UPLC instrument (Milford, MA). The sample preparation workflow was implemented as a robotic program on Hamilton Robotics Venus One software. Glycoprotein denaturation, release and solid phase extraction can be performed on up to 96 samples in parallel.

Native human serum (NHS) samples

A total of 100 adult serum samples from apparently healthy male and female adult blood donors were pooled and used as a source of serum glycoproteins for subsequent glycan analysis (courtesy of the UK Blood Transfusion Service).

Glycoprotein denaturation and glycan release

Denaturation buffer (55 μL per well, 100 mM ammonium bicarbonate, 12 mM dithiothreitol) was dispensed into a 96-well V-bottom PP plate (Greiner Bio-One Cat.No.: 651201) containing serum (5 μL per well), which was placed on a robotic heater shaker and fully covered and insulated with an anti-evaporation lid.

This assembly was incubated at 65 °C with agitation at 700 rpm for 20 min. After cooling to room temperature for 15 min, an iodoacetamide solution (120 mM, 10 μL per well) was added, the multiwell plate was covered with an anti-evaporation lid and incubated at room temperature with agitation at 700 rpm for 30 min. A trypsin solution (10 μL of a 40 000 U mL^{-1} solution per well) was added, the plate was sealed with adhesive aluminium film, placed on the robotic heater shaker and fully covered with an anti-evaporation lid. Incubation was performed at 40 °C with agitation at 700 rpm for 120 min. Next, the temperature was increased to 105 °C and incubation was continued for 10 min. The plate was left to cool to room temperature, briefly spun and the seal removed. PNGase F (Prozyme © Glyco N-Glycanase ©, code GKE-5006D, 10 μL per well, 0.5 mU in 1 M ammonium bicarbonate, pH 8.0) was added and the plate was insulated with an anti-evaporation lid and incubated at 40 °C with agitation at 700 rpm for 120 min.

Hydrazide-mediated glycan clean-up

Hydrazide-assisted glycan clean-up and glycan labelling was performed essentially as previously described:[12] Each well of a 96-well chemically inert filter plate (Millipore Solvinert, hydrophobic polytetrafluoroethylene membrane, 0.45 μm pore size) was washed with 100 μL methanol (MeOH). Ultralink hydrazide resin (40 μL of a suspension in water, ThermoScientific) was dispensed to each well. The resin was sequentially washed with MeOH, H$_2$O and acetonitrile (MeCN) and the plate was placed on a heater (70 °C, 10 min) to seal the membranes. 180 μL MeCN/acetic acid (98 : 2) was added to the resin, followed by 20 μL of the glycan solution. The filter plate was incubated with shaking at 700 rpm at 70 °C for 45 min. 50 μL MeCN/acetic acid (98 : 2) were added and shaking was continued at the same temperature for 10 min to disrupt resin aggregates. The resin was washed sequentially with MeOH, guanidine (2 M), H$_2$O, triethylamine/MeOH (1 : 99) and MeOH. Fresh MeOH (180 μL) and acetic anhydride (20 μL) were added and the plate was incubated for 10 min with agitation at 700 rpm. Excess reagent was removed by filtration and the resins were washed sequentially with MeOH, H$_2$O and MeCN. Acetic acid/MeCN (2 : 98, 180 μL) and H$_2$O (20 μL) were sequentially added and the plate was incubated at 70 °C with agitation at 700 rpm for 60 min. Fluorescent labelling mix (50 μL, 350 mM 2-aminobenzamide, 1 M sodium cyanoborohydride in acetic acid/dimethyl sulfoxide (30 : 70)) was dispensed into each well and the plate was incubated at 70 °C with agitation at 700 rpm for 120 min.

Glycan solid phase extraction

The labelling reaction was quenched by the addition of 200 μL MeCN/H$_2$O (95 : 5). The suspension was transferred to a 2 mL collection plate, the beads were left to settle, 200 μL of the supernatant was aspirated and dispensed back into the filter plate. After extensive mixing the suspension was transferred back into the collection plate. This cycle was repeated once more to ensure a quantitative transfer of the resins.

HyperSep Diol SPE cartridges (Thermo Scientific) were washed with 1 mL MeCN/H$_2$O (95 : 5), 1 mL H$_2$O and 1 mL

MeCN/H$_2$O (95 : 5). Next, the supernatants of the quenched reaction mixtures without the beads were transferred onto the SPE cartridges. 10 min incubation typically led to complete drainage of the solvent by gravity. The SPE cartridges were washed three times with 700 μL MeCN/H$_2$O (95 : 5). A collection plate was placed inside the robotic vacuum manifold and the SPE cartridges were washed twice with 200 μL H$_2$O/MeCN (80 : 20), with an intermittent incubation period of 10 min. The samples were concentrated to dryness in a vacuum evaporator (typically 4–6 hour for 96 samples). The samples were dissolved in 30 μL MeCN/H$_2$O (70 : 30) and filtered (Pall Acroprep GHP membrane, 0.45 μm pore size). A 10 μL aliquot of the filtrate was analyzed by UPLC.

Ultra performance liquid chromatography (UPLC)

2-AB derivatized N-glycans were separated by UPLC with fluorescence detection on a Waters Acquity UPLC H-Class instrument consisting of a binary solvent manager, sample manager and fluorescence detector under the control of Empower 3 chromatography workstation software (Waters, Milford, MA, USA). The HILIC separations were performed using a Waters Ethylene Bridged Hybrid (BEH) Glycan column, 150 × 2.1 mm i.d. 186004742, 1.7 μm BEH particles, with 50 mM ammonium formate, pH 4.4, as solvent A and MeCN as solvent B. The separation was performed using a linear gradient of 70–53% MeCN at 0.56 mL min^{-1} in 30 min. An injection volume of 10 μL sample prepared in 70% v/v MeCN was used throughout. Samples were maintained at 5 °C prior to injection and the separation temperature was 40 °C. The fluorescence detection excitation/emission wavelengths were λ_{ex} = 330 nm and λ_{em} = 420 nm, respectively. The system was calibrated using an external standard of hydrolyzed and 2AB-labelled glucose oligomers to create a dextran ladder, as described previously.[16] A fifth-order polynomial distribution curve was fitted to the dextran ladder to assign glucose unit (GU) values from retention times (using Empower software from Waters).

Results and discussion

The most widely used method for releasing N-glycans from glycoprotein mixtures is in-solution release with PNGaseF.[17,18] To accurately quantify released glycans, they are typically labelled with fluorophores via reductive amination. However, whole serum consists of a complex mixture of proteins, salts and lipids, all of which can interfere with the fluorescent labelling reaction, and the reductive amination in such complex biological matrices typically results in side-products and poor reaction conversion. This, in turn, adversely impacts assay performance, e.g. specificity, accuracy, precision, and limit of quantitation. Although specific guidelines have not yet been defined for qualifying an analytical method for glycan-biomarker discovery, it is important that biomarker assays demonstrate acceptable method performance. To avoid problems arising from the presence of contaminants in a sample, an important step has been the immobilization of glycoproteins

to allow the removal of buffers, matrix components, and the reagents used to denature, reduce, and alkylate the glycoproteins. Protein immobilization has been accomplished by trapping glycoprotein samples in polyacrylamide gel pieces[16] or immobilizing glycoproteins onto PVDF membranes.[19] When using membranes for immobilization, it is important to ensure, especially for mixtures of glycoproteins, that the binding is non-selective because in practice it is difficult to achieve quantitative binding of the analyte. Trapping glycoproteins in polyacrylamide gel pieces, although conventionally a reliable technique is difficult to automate on liquid handling robotic workstations and hence relies on manual sample processing.

Our recently published automated method for antibody glycoprofiling is a fully integrated platform that combines glycoprotein affinity purification, protein denaturation, enzymatic glycan release, hydrazide-mediated glycan enrichment, fluorescent glycan labelling and sample clean-up.[12] To achieve robust glycan fluorescence labelling and reproducible quantification, glycans are captured on solid supports, followed by the removal of contaminants prior to glycan fluorescence labelling. Nishimura et al. pioneered a method to capture released glycans on custom made solid supported hydrazide.[17,20] In the present paper we investigated whether UltraLink hydrazide resin a complex biological matrix, can be used to isolate glycans from native human serum.

This glycomic approach can then assist in differential diagnosis of diseases such as cancers and autoimmune diseases in a high-throughput fashion and can give valuable biological insights into the mechanism of the diseases. The analysis of glycoproteins can be crucial to the pharmaceutical industry; variations of N-glycan structures between glycoforms of erythropoietin (EPO), a glycoprotein hormone, were found to be linked to biological activities of the pharmaceutical agents.[21] Moreover, we created a robust robotic program with automatic error recovery to automate the entire serum glycomics workflow for fast efficient analysis (Fig. 1). First, serum samples are denatured and trypsinized in a 96-well plate. Trypsinization was found to be important to circumvent aggregation of the UltraLink solid supports later in the protocol. Next, glycans are enzymatically released and reacted with solid-supported hydrazides to form hydrazones, capturing glycans covalently on solid supports. Contaminants such as excess reagents and buffer salts are removed by simple filtration. Subsequently, glycans are released by acid catalysis in the presence of water. The reaction solvent evaporates during glycan release and the dry glycans, which are non-covalently bound to the solid supports at this stage, and are fluorescently labelled by reductive amination with 2-aminobenzamide (Fig. 2).

The following protocol was efficiently programmed on a Hamilton StarLet robot (Fig. 3A). The workstation is software-controlled and was equipped with pipette tip racks, plate carriers, reagent reservoirs, a software-controlled vacuum manifold (Fig. 3B), a plate transport tool to move multiwell plates between positions and operate the vacuum manifold (Fig. 3C), eight robotic pipettes with individual liquid level and pressure sensors
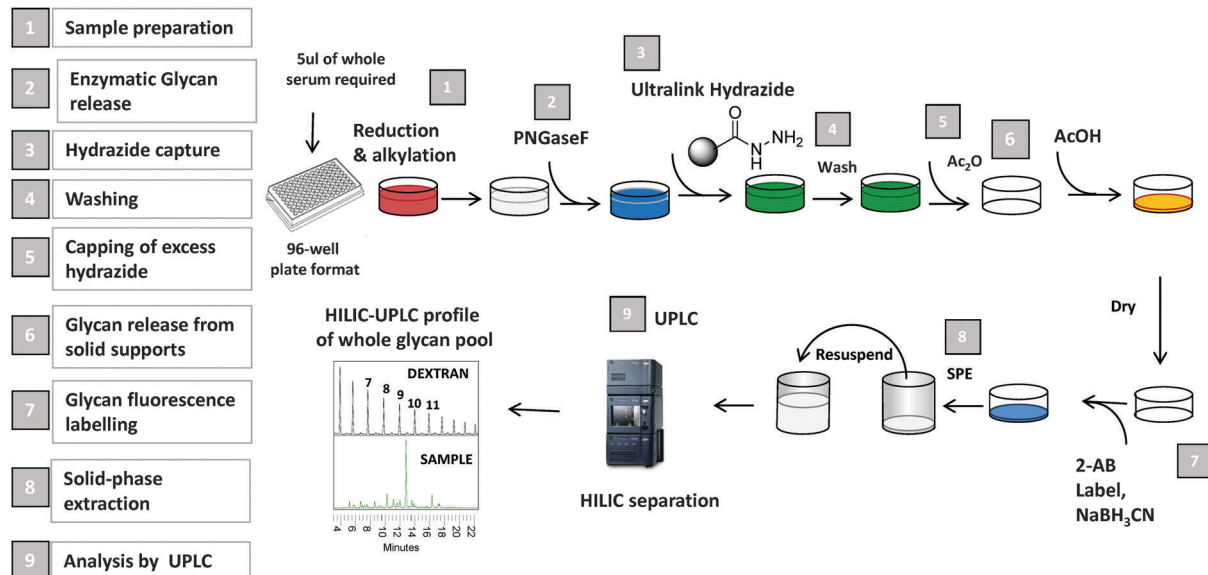
Fig. 1 Sample preparation workflow, consisting of glycoprotein affinity purification, protein denaturation, enzymatic glycan release, glycan immobilization on solid-supports, removal of contaminants, glycan release, labelling, solid-phase extraction and UPLC analysis.
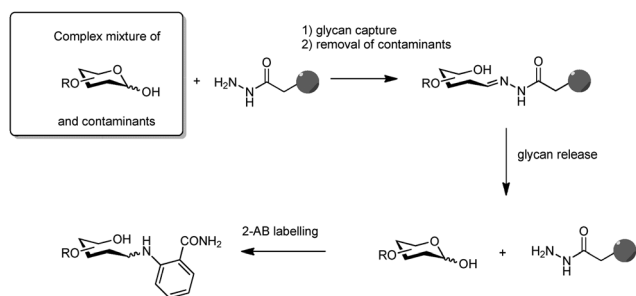

Fig. 2 Solid-supported hydrazide-mediated sample clean-up. Reducing end glycans are reacted with solid supported hydrazides to form hydrazones, capturing glycans on solid supports. Contaminants such as excess reagents and buffer salts are removed by filtration. Next, glycans are released by acid catalysis in the presence of water. Finally, glycans are fluorescently labelled with 2-aminobenzamide (2-AB).
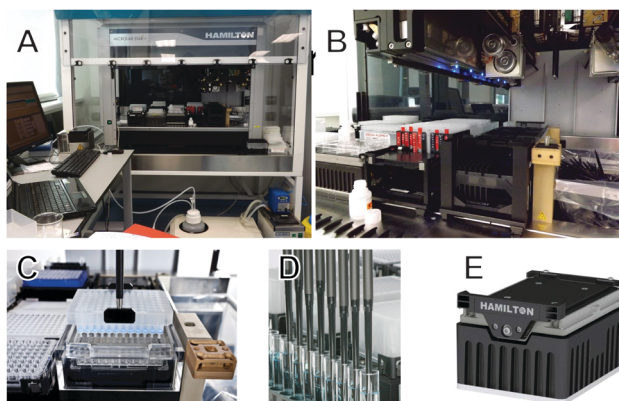

Fig. 3 Hamilton liquid handling workstation and safety cabinet (A) deck view (B), together with its key components: Robotic vacuum manifold and plate transport tool (C), eight independent pipetting channels with liquid-level detection and anti-droplet control (D), temperature-controlled orbital shaker (E).

(Fig. 3D), and a temperature controlled orbital shaker (Fig. 3E). The protocol starts with a one-pot sequence of protein denaturation and tryptic protein digestion in a 96-well plate. After trypsin inactivation, glycans are released from peptides using PNGase F. Starting with 5 μL of serum, the sequence results in *ca.* 50 μL reaction volume with a glycan concentration of *ca.* 70 μM, of which 20 μL (*ca.* 1.4 nmol glycans) are used for the downstream steps. Glycans are first captured on 0.75 μmol hydrazide on UltraLink Biosupport medium under acid catalysis at elevated temperature (Fig. 2). The solid-supported reactions were conducted under vigorous shaking conditions to maintain the solid supports in suspension during reaction thereby improving the reaction kinetics. Next, contaminants were removed by a sequence of washing steps with both organic solvent and aqueous buffers. This was followed by acetylation of unreacted hydrazide and reagent removal by washing. Glycans are then released from the solid supports under acid catalysis at 60 °C. This results in free glycans whose reducing ends can be labelled with fluorescent dyes such as 2-aminobenzamide. The labelling reactions are quenched by the addition of a mixture of acetonitrile and water and the glycans are thoroughly extracted from the solid supports. The supernatants are transferred to solid-phase extraction (SPE) cartridges packed with polymerically bonded diol to capture glycans and to remove excess reagents. To generate reproducible results, it was found to be important not to transfer any solid supports to the SPE cartridges.

Method verification

To verify our protocol, eight serum samples (from pooled native human serum) were prepared on both the Hamilton Star workstation as well as manually using the in-gel-block (IGB)
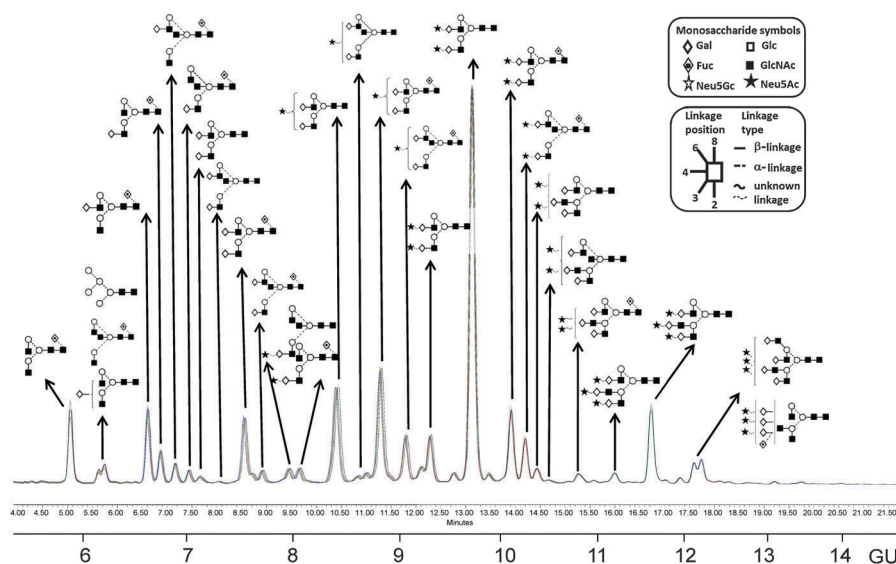
Fig. 4 Reproducibility of the automated high-throughput platform for the human whole serum *N*-glycome. The major glycans are highlighted. Human serum *N*-glycome was analysed in detail in Saldova *et al.*[13] GU: glucose unit.

method. The samples were then analyzed on a UPLC system with fluorescence detection and equipped with a BEH HILIC glycan column. An overlay chromatogram resulting from the automated sample preparation method (eight samples), together with the corresponding peak assignments are shown in Fig. 4.

Whole native human serum was processed on the liquid handling workstation as described in the Experimental section, followed by glycan analysis by UPLC with fluorescence detection. The chromatograms depict the *N*-glycosylation of human whole serum as well as the corresponding peak assignments. The overlay of the profiles is shown and the peaks corresponded to those assigned in Saldova *et al.*[13] The

chromatograms obtained using the robotic method showed 46 integratable peaks, in accordance with previously acquired data.[13] Furthermore, the glycan peak areas were very similar to those of the IGB method, with only a slight bias towards smaller glycans in the robotic method (Fig. 5). The coefficients of variation between the samples prepared with the automated method were below 10% for all major peaks, *i.e.* those peaks with a relative percentage area above 1%. In addition, the coefficients of variation of the automated method were lower than those of the IGB method for most glycan peaks (Fig. 6). Sample cross-over was checked by processing water blanks alongside serum samples. The UPLC profiles indicated that sample cross-over was below the UPLC detection limit.
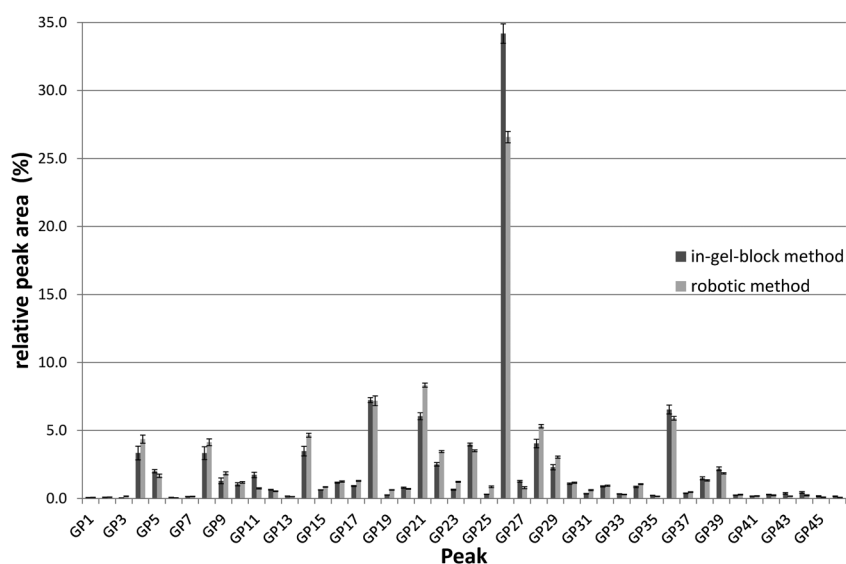

Fig. 5 Comparison of the glycan peak areas from human serum for eight samples between the automated assay and the in-gel-block (IGB) method. Samples were prepared on the robotic platform or using the IGB method followed by UPLC analysis. GP: glycan peak number.
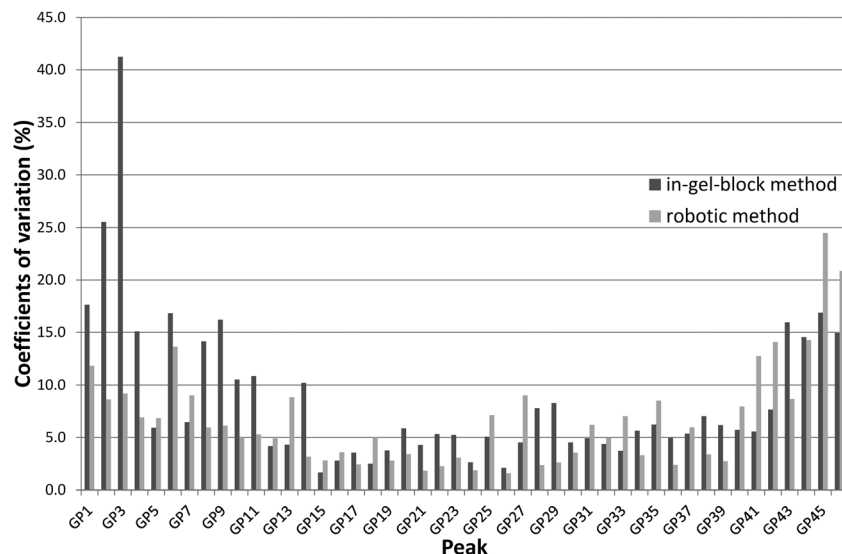
Fig. 6 Comparison of the glycan area's coefficients of variation for eight samples between the automated assay and the in-gel-block (IGB) method. Samples were prepared automatically or manually with the IGB method and analyzed by UPLC. GP: glycan peak number.

Conclusion

Most glycan sample preparation workflows developed to date are impacted considerably by low sample throughput, lack of automation and extremely high consumables costs, significantly hampering the urgently needed progress in glycomics research. We addressed these key issues and developed a concise, automated workflow for whole serum N-glycan separation and quantification. To the best of our knowledge, this is the first low-cost, automated high-throughput assay for UPLC-based whole serum glycomics and should enable efficient profiling of glycans for genome-wide association studies and clinical disease research, all of which are active research areas in our laboratory.

Acknowledgements

References

1 J. D. Marth and P. K. Grewal, *Nat. Rev. Immunol.*, 2008, **8**, 874–887.

2 K. Ohtsubo and J. D. Marth, *Cell*, 2006, **126**, 855–867.

3 Y. Y. Zhao, M. Takahashi, J. G. Gu, E. Miyoshi, A. Matsumoto, S. Kitazume and N. Taniguchi, *Cancer Sci.*, 2008, **99**, 1304–1310.

4 J. E. Huffman, A. Knezevic, V. Vitart, J. Kattla, B. Adamczyk, M. Novokmet, W. Igl, M. Pucic, L. Zgaga, A. Johannson, I. Redzic, O. Gornik, T. Zemunik, O. Polasek, I. Kolcic, M. Pehlic, C. A. Koeleman, S. Campbell, S. H. Wild, N. D. Hastie, H. Campbell, U. Gyllensten, M. Wuhrer, J. F. Wilson, C. Hayward, I. Rudan, P. M. Rudd, A. F. Wright and G. Lauc, *Hum. Mol. Genet.*, 2011, **20**, 5000–5011.

5 G. Lauc, A. Essafi, J. E. Huffman, C. Hayward, A. Knezevic, J. J. Kattla, O. Polasek, O. Gornik, V. Vitart, J. L. Abrahams, M. Pucic, M. Novokmet, I. Redzic, S. Campbell, S. H. Wild, F. Borovecki, W. Wang, I. Kolcic, L. Zgaga, U. Gyllensten, J. F. Wilson, A. F. Wright, N. D. Hastie, H. Campbell, P. M. Rudd and I. Rudan, *PLoS Genet.*, 2010, **6**, e1001256.

6 G. Lauc, J. E. Huffman, M. Pucic, L. Zgaga, B. Adamczyk, A. Muzinic, M. Novokmet, O. Polasek, O. Gornik, J. Kristic, T. Keser, V. Vitart, B. Scheijen, H. W. Uh, M. Molokhia, A. L. Patrick, P. McKeigue, I. Kolcic, I. K. Lukic, O. Swann, F. N. van Leeuwen, L. R. Ruhaak, J. J. Houwing-Duistermaat, P. E. Slagboom, M. Beekman, A. J. de Craen, A. M. Deelder, Q. Zeng, W. Wang, N. D. Hastie, U. Gyllensten, J. F. Wilson, M. Wuhrer, A. F. Wright, P. M. Rudd, C. Hayward, Y. Aulchenko, H. Campbell and I. Rudan, *PLoS Genet.*, 2013, **9**, e1003225.

7 K. Marino, J. Bones, J. J. Kattla and P. M. Rudd, *Nat. Chem. Biol.*, 2010, **6**, 713–723.

8 B. Adamczyk, T. Tharmalingam-Jaikaran, M. Schomberg, A. Szekrenyes, R. M. Kelly, N. G. Karlsson, A. Guttman and P. M. Rudd, *Carbohydr. Res.*, 2014, **389**, 174–185.

9 K. R. Reiding, D. Blank, D. M. Kuijper, A. M. Deelder and M. Wuhrer, *Anal. Chem.*, 2014, **86**, 5784–5793.

10 G. Zauner, A. M. Deelder and M. Wuhrer, *Electrophoresis*, 2011, **32**, 3456–3466.

11 T. Tharmalingam, B. Adamczyk, M. A. Doherty, L. Royle and P. M. Rudd, *Glycoconjugate J.*, 2013, **30**, 137–146.

12 H. Stockmann, B. Adamczyk, J. Hayes and P. M. Rudd, *Anal. Chem.*, 2013, **85**, 8841–8849.

13 R. Saldova, A. Asadi Shehni, V. D. Haakensen, I. Steinfeld, M. Hilliard, I. Kifer, A. Helland, Z. Yakhini, A. L. Borresen-Dale and P. M. Rudd, *J. Proteome Res.*, 2014, **13**, 2314–2327.

14 K. P. Coss, C. P. Hawkes, B. Adamczyk, H. Stockmann, E. Crushell, R. Saldova, I. Knerr, M. E. Rubio-Gozalbo, A. A. Monavari, P. M. Rudd and E. P. Treacy, *J. Proteome Res.*, 2014, **13**, 385–394.

15 E. S. Collins, M. C. Galligan, R. Saldova, B. Adamczyk, J. L. Abrahams, M. P. Campbell, C. T. Ng, D. J. Veale, T. B. Murphy, P. M. Rudd and O. Fitzgerald, *Rheumatology*, 2013, **52**, 1572–1582.

16 L. Royle, M. P. Campbell, C. M. Radcliffe, D. M. White, D. J. Harvey, J. L. Abrahams, Y. G. Kim, G. W. Henry, N. A. Shadick, M. E. Weinblatt, D. M. Lee, P. M. Rudd and R. A. Dwek, *Anal. Biochem.*, 2008, **376**, 1–12.

17 Y. Kita, Y. Miura, J. Furukawa, M. Nakano, Y. Shinohara, M. Ohno, A. Takimoto and S. Nishimura, *Mol. Cell. Proteomics*, 2007, **6**, 1437–1445.

18 M. A. Comunale, T. S. Mattu, M. A. Lowman, A. A. Evans, W. T. London, O. J. Semmes, M. Ward, R. Drake, P. R. Romano, L. F. Steel, T. M. Block and A. Mehta, *Proteomics*, 2004, **4**, 826–838.

19 D. I. Papac, J. B. Briggs, E. T. Chin and A. J. Jones, *Glycobiology*, 1998, **8**, 445–454.

20 J. Furukawa, Y. Shinohara, H. Kuramoto, Y. Miura, H. Shimaoka, M. Kurogochi, M. Nakano and S. Nishimura, *Anal. Chem.*, 2008, **80**, 1094–1101.

21 M. Takeuchi, N. Inoue, T. W. Strickland, M. Kubota, M. Wada, R. Shimizu, S. Hoshi, H. Kozutsumi, S. Takasaki and A. Kobata, *Proc. Natl. Acad. Sci. U. S. A.*, 1989, **86**, 7819–7822.

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