Analyst

Dynamic Article Links

Cite this: *Analyst*, 2011, **136**, 4670

www.rsc.org/analyst

COMMUNICATION

High throughput plasma *N*-glycome profiling using multiplexed labelling and UPLC with fluorescence detection†

Ana Knežević,‡[a] Jonathan Bones,‡[b] Stjepan Krešimir Kračun,[c] Olga Gornik,[d] Pauline M. Rudd[b] and Gordan Lauc*[ad]

Received 1st August 2011, Accepted 14th September 2011
DOI: 10.1039/c1an15684e

A rapid glycomic profiling method is described wherein *N*-glycans from plasma samples individually labelled with aniline, 2-aminobenzamide and 2-aminoacridone are mixed, co-injected and separated in the same HILIC-fluorescence run. Transfer of the multiplexed method to UPLC-fluorescence permits an increase in sample throughput from 24 to 864 plasma samples per day.

A high-throughput hydrophilic interaction liquid chromatography with fluorescence detection (HILIC-fluorescence) based glycan analysis platform was recently described that facilitated the first large-scale profiling studies of plasma *N*-glycan variation in human populations.[1–3] A critical feature of the analysis platform is that sample preparation is performed in an automation friendly manner in 96-well plates. The subsequent HILIC-fluorescence oligosaccharide separation has become the time limiting step in the analytical process. This is a significant obstacle since modern high-throughput methods, particularly in the field of genomics, enable rapid analysis of hundreds to thousands of samples. To achieve synergy with other 'omics' the speed of glycan analysis needs to improve significantly.

Driven by the desire for increased analytical speed, we report a method that enables simultaneous HILIC-fluorescence analysis of oligosaccharides from multiple plasma samples. Glycans labelled with different fluorophores can be mixed together, co-injected and separated in a single HILIC-fluorescence run with no cross-interference from the fluorophores due to significant differences in their excitation and emission maxima. To further increase sample throughput the triple dye approach was also employed in conjunction with UPLC-fluorescence using a 1.7 μm HILIC stationary phase. The combination of the mixed fluorophore multiple sample injection accompanied by the reduction in chromatographic run time afforded by UPLC enabled up to a 36-fold increase in sample throughput. This significant increase in the ability to process a greater number of samples on a daily basis facilitates the entry of glycomics into the high throughput genomic and proteomic profiling arenas and opens up the possibilities of large scale profiling studies that were previously not possible.

For this multi-label method to be successful, the following criteria required adherence: (i) fluorophores need to have non-overlapping emission and excitation spectra; (ii) fluorophores should not compete or interact in any way with each other during liquid chromatographic separation; and (iii) the chemical properties of the fluorophores (size, structure and polarity) should be similar enough to enable parallel separation without changes in gradient conditions. 2-Aminobenzamide (2-AB), 2-aminoacridone (2-AmAc) and aniline were selected as the fluorophores of choice in the current study in accordance with the above criteria. Maximal excitation and emission wavelengths for aniline, 2-AB and 2-AmAc were experimentally determined as ex250/em340 for aniline, ex330/em440 for 2-AB and ex428/em580 for 2-AmAc using a standard solution of maltoheptaose labelled with each fluorophore *via* reductive amination with sodium cyanoborohydride in 70 : 30 DMSO : acetic acid at 65 °C for two hours, as shown in Fig. 1(A). The emission wavelength chosen was not necessarily λ_{max} but the wavelength at which there was minimal interference from the emission of the other fluorophores in the other detection channels. A standard glycan mixture was next prepared by mixing 3.47 mM maltotetraose, 1.8 mM maltopentaose, 0.95 mM maltohexsaose and 3.37 mM maltoheptaose. Equal quantities of this glycan mixture were labelled with aniline, 2-AB and 2-AmAc and subsequently separated using UPLC on the 1.7 μm HILIC stationary phase (Waters BEH Glycan, 50 × 2.1 mm i.d.) with multiwavelength fluorescence detection. The UPLC instrument used throughout the current study was a Waters Acquity UPLC consisting of a binary solvent manager, a sample manager and a FLR fluorescence detector (Waters Corporation, Milford, MA, USA). As shown in Fig. 1(B), oligosaccharides labelled with aniline, 2-AB and 2-AmAc demonstrated <1% spectral interference in the monitored fluorescence channels of the other labels using the experimentally determined emission wavelengths. Expanded chromatograms are presented in Fig. S1–S3 in the accompanying ESI†. Fig. 1(B) clearly demonstrates that all three fluorophores chosen are spectrally separated and essentially optically transparent to each other with negligible cross-detection of samples in different detection channels of the

[a]*Genos Ltd, Laboratory for Glycobiology, Planinska 1, Zagreb, Croatia. E-mail: glauc@pharma.hr*
[b]*NIBRT Dublin-Oxford Glycobiology Laboratory, National Institute for Bioprocessing Research and Training, Dublin 4, Ireland*
[c]*Copenhagen Center for Glycomics, Department of Cellular and Molecular Medicine, University of Copenhagen, DK-2200 Copenhagen, Denmark*
[d]*Faculty of Pharmacy and Biochemistry, Department of Biochemistry and Molecular Biology, University of Zagreb, Croatia*

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c1an15684e

‡ These two authors contributed equally.

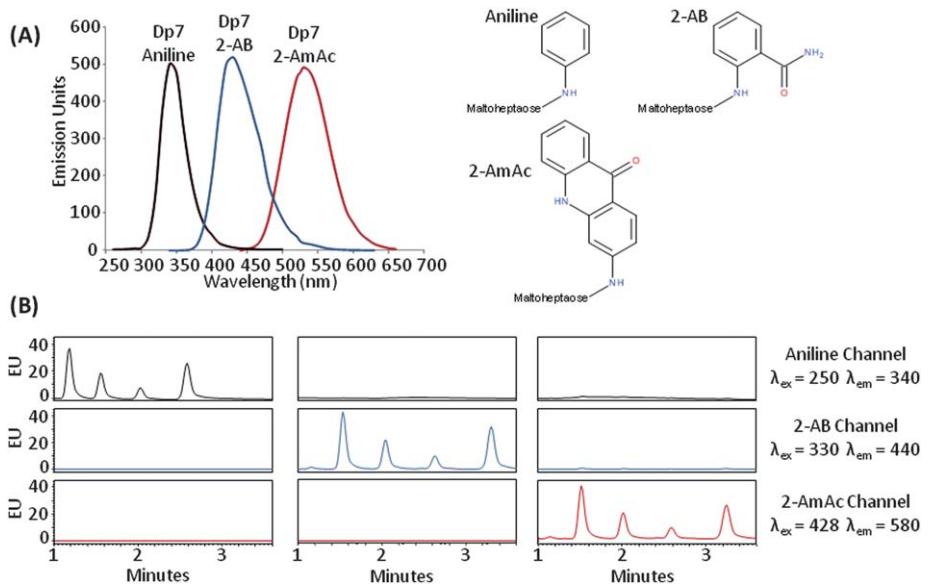

Fig. 1 (A) Experimentally determined emission spectra for the maltoheptaose conjugated fluorophores for the determination of the most appropriate detection wavelengths to minimise spectral cross-talk. Spectra were recorded using the 3D scan functionality of the FLR fluorescence detector. (B) Separation of malto-oligomers (tetraose to heptaose) with monitoring across all three detection channels in each instance for the demonstration of no unwanted detection interference. Separation was performed on a Waters Acquity UPLC instrument and a Waters BEH Glycan 50 × 2.1 mm i.d., 1.7 μm column.

multi-wavelength fluorescence detector. Limits of detection were also experimentally determined as 3.6, 2.0 and 6.1 fmol for maltotetraose labelled with aniline, 2-AB and 2-AmAc, respectively. The slightly higher limit of detection for the 2-AmAc labelled oligosaccharide is due to monitoring 2-AmAc fluorescence not at λ_{max} but under conditions of minimal spectral interference in the other detection channels.

Possible interference and unwanted mass loading effects on the chromatographic efficiency were next investigated. Retention time reproducibility was evaluated by five consecutive injections of the same mixture of the standard malto-oligosaccharides labelled with all three fluorophores. Experimentally determined variations in retention time were less than 1% RSD. Reproducibility for quantitation was also evaluated using repeat injections of the malto-oligosaccharides. Deviations of less than 1% RSD were again noted based upon relative peak area based quantitation. The high levels of reproducibility for both retention time and relative peak area observed in both cases demonstrate that glycans labelled with the three fluorophores did not interfere with each other when co-injected on column. Furthermore no loss of separation efficiency or resolution was recorded in any of the chromatograms indicating no sample mass loading effects on the chromatographic process.

The selected fluorophores differ moderately in both size and polarity. Despite these differences no alterations in selectivity for the labelled oligosaccharides were recorded in any of the resulting standard or sample chromatograms. This suggests that retention on the HILIC phase is governed predominantly *via* interaction of the glycan with the amide stationary phase with minimal contribution to retention from the attached neutral labels. Moreover, the fluorophores appear to govern the degree of retention based upon their interaction with the high solvent environment in the mobile phase. It was observed that glycans labelled with aniline were less retained than glycans labelled with 2-AmAc which were in turn less retained than glycans labelled with 2-AB, respectively. If the retention order was based solely on the polarity of the labels the expected retention order would have been that glycans labelled with 2-AmAc (log *P* 2.95) would elute before glycans labelled with aniline (log *P* 0.90) which in turn would elute before glycans labelled with 2-AB (log *P* 0.35).[4] This was not experimentally observed. Therefore, other functional groups such as the amide group in 2-AB and the morpholine ring in 2-AmAc do interact with the polar stationary phase *via* hydrogen bonding or other interactions and cause the deviation from the expected retention order.

To investigate the practicality of the multiplexed glycomic profiling method using complex samples, the three dye on-column approach was applied to the simultaneous analysis of healthy human plasma *N*-glycans using standard HILIC-fluorescence conditions as shown in Fig. 2A. The plasma *N*-glycome represents one of the most complicated and analytically challenging oligosaccharide mixtures and consists of bi-, tri- and tetra-antennary glycans with variable sialylation as well as core and outer arm fucosylation.[1,3] Run time and resolution were maintained; thus the data obtained after integration are comparable to that obtained during our previous large scale plasma *N*-glycan studies wherein a single fluorophore was used, however, now with the advantage of a three-fold increase in analytical speed.[2,3] The simultaneous use of multiple fluorescent labels is a routine in DNA analysis, but to the best of our knowledge, it has not been applied for HPLC analysis of glycans. Identical technology could be applied for capillary electrophoretic separations of glycans with LIF detection wherein using charge matched dyes with different spectral properties would facilitate application of the concept in an analogous manner as outlined for HILIC-fluorescence herein.

To further increase sample throughput the three dye on-column method for plasma *N*-glycome profiling was transferred from HPLC to UPLC using the recently introduced 1.7 μm HILIC stationary phase using conditions of maintained HPLC performance but at

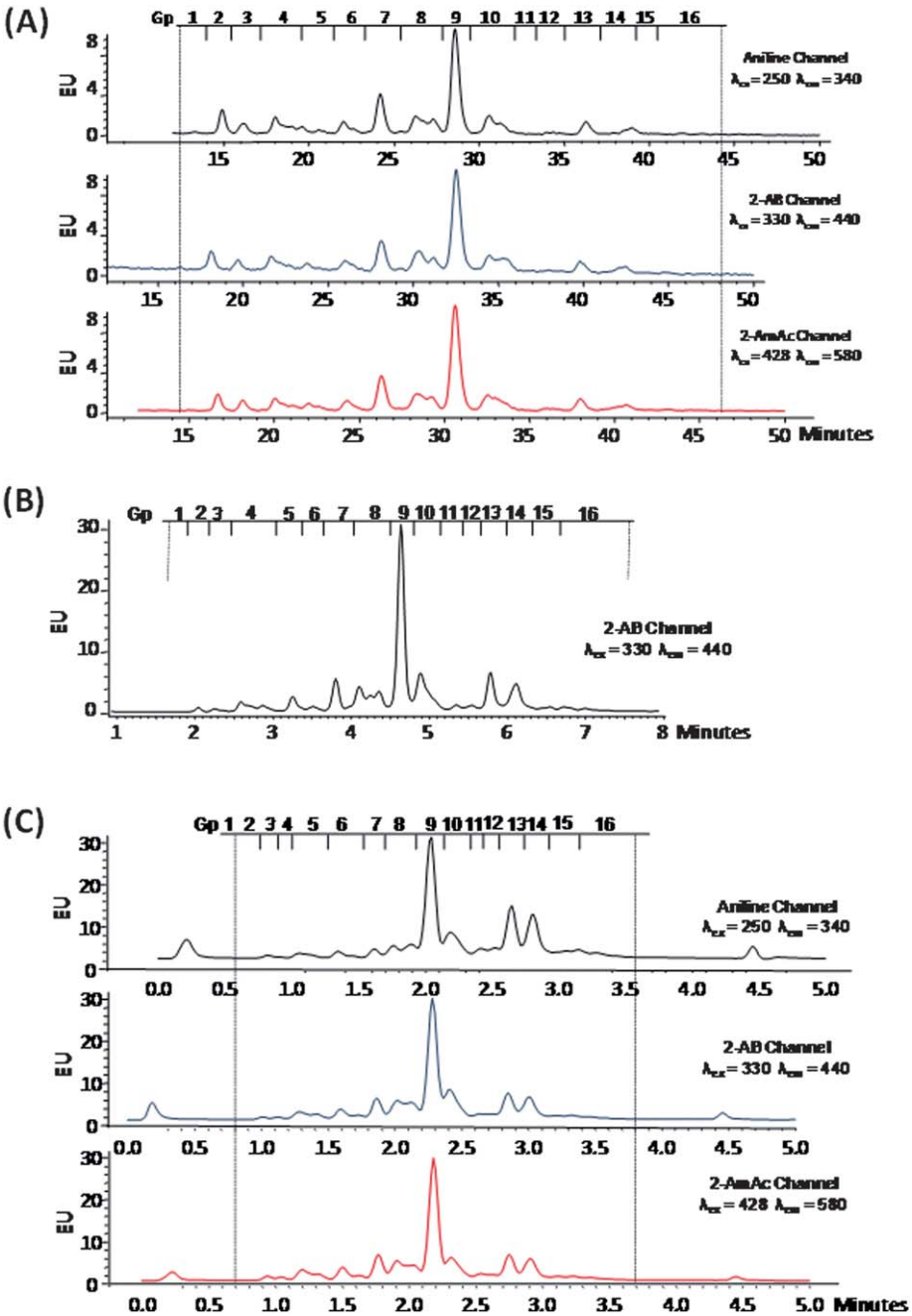

Fig. 2 (A) Co-injection and separation of the three differently labelled plasma *N*-glycan samples using our standard HPLC HILIC-fluorescence method using a Waters Alliance HPLC instrument and a TSKgel Amide 80, 150 × 4.6 mm i.d., 3.0 μm column, gradient 65–53% acetonitrile in 48 minutes, flow rate 0.48 ml min^{-1}. The GP numbering refers to the grouping of peaks as used in our previous glycome profiling studies.[2,3] (B) Transfer of the method to UPLC using a 1.7 μm HILIC stationary, shown is the resulting separation of 2-AB labelled plasma *N*-glycans, a Waters Acquity UPLC instrument and a Waters BEH Glycan 50 × 2.1 mm i.d., 1.7 μm column, gradient 70–53% acetonitrile in 7.8 minutes, flow rate 0.56 ml min^{-1}. (C) Co-injection and separation of the three differently labelled plasma *N*-glycan samples using UPLC-fluorescence in the multiwavelength mode, Waters Acquity UPLC instrument and a Waters BEH Glycan 50 × 2.1 mm i.d., 1.7 μm column, gradient 70–53% acetonitrile in 3.9 minutes, flow rate 1.12 ml min^{-1}.

maximised analytical speed.[5,6] These criteria were specifically chosen to guarantee comparability with data generated in our previous studies.[2,3] As depicted in Fig. 2B, UPLC offers a significant reduction in the chromatographic run time without any loss of selectivity or efficiency. Fig. 2C depicts the plasma *N*-glycans labelled with the three different fluorophores using a faster gradient wherein the flow rate was doubled and all gradient parameters were altered accordingly to maintain the gradient slope. As can be seen from Fig. 2C it was possible to separate a mix of the three differently labelled plasma *N*-glycome samples, which represents one of the most challenging glycan sample types, in a gradient time of 3.9 minutes whilst maintaining the desired performance for use in subsequent profiling

studies. A point of note with regard to Fig. 2C is that when using the multiple wavelength functionality of the fluorescence detector the sampling rate is automatically reduced by the chromatography control software from 20 Hz in single channel mode to 1 point per second in multiple channel mode. This decrease in the sampling frequency represents an instrumental limitation to the application of the described methodology as chromatographic features that elute faster than can be detected by the sampling rate of the detector get smoothed into a neighbouring peak. Considering that the maximum sample number per day was 24 using the traditional single dye HPLC scale HILIC-fluorescence analysis. Using the faster analytical run-times afforded by UPLC in conjunction with co-injection of the three differently labelled glycan pools, sample throughput numbers of 432 samples per day using the gradient method as depicted in Fig. 2B or 864 samples per day using the gradient method as depicted in Fig. 2C are now a reality.

Conclusions

The three dye on-column approach offers a significant increase in high throughput glycan analysis. The method described is specifically tailored for large scale population profiling studies or targeted glycomics rather than studies where discovery of altered glycosylation is the focus. This paper also calls for improvements in the scan rate and dynamic range of fluorescence detectors to match analytical performance of the instrumentation with the described multiplexed separation speed.

Acknowledgements

This work was supported by grant #309-0061194-2023 (to G.L.) from the Croatian Ministry for Science, Education and Sport and the FP7 HighGlycan grants from the EC. We thank Dr Gerrit Gerwig of the University of Groningen, Holland for the GC-MS analysis. JB and PMR acknowledge funding from the European Union FP6 GLYFDIS, FP7 GlycoBioM, FP7 GlycoHIT research programmes and Waters Corporation for the donation of the Acquity UPLC instrument.

Notes and references

1 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.

2 A. Knezevic, O. Gornik, O. Polasek, M. Pucic, I. Redzic, M. Novokmet, P. M. Rudd, A. F. Wright, H. Campbell, I. Rudan and G. Lauc, *Glycobiology*, 2010, **20**, 959–969.

3 A. Knezevic, O. Polasek, O. Gornik, I. Rudan, H. Campbell, C. Hayward, A. Wright, I. Kolcic, N. O'Donoghue, J. Bones, P. M. Rudd and G. Lauc, *J. Proteome Res.*, 2009, **8**, 694–701.

4 I. V. Tetko, J. Gasteiger, R. Todeschini, A. Mauri, D. Livingstone, P. Ertl, V. A. Palyulin, E. V. Radchenko, N. S. Zefirov, A. S. Makarenko, V. Y. Tanchuk and V. V. Prokopenko, *J. Comput.-Aided Mol. Des.*, 2005, **19**, 453–463.

5 J. Ahn, J. Bones, Y. Q. Yu, P. M. Rudd and M. Gilar, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2010, **878**, 403–408.

6 J. Bones, S. Mittermayr, N. O'Donoghue, A. Guttman and P. M. Rudd, *Anal. Chem.*, 2010, **82**, 10208–10215.