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Isolation and Purification of Glycans from Natural Sources for Positive Identification

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Abstract

The great structural diversity of glycans demands powerful analytical methodologies, such as different combinations of capillary separations with mass spectrometry (MS), to identify the correct structures involved in key glycan interactions of biological importance. Precise structural assignments, in turn, necessitate the availability of pure authentic glycans as absolute analytical standards. It is particularly evident with the cases of glycan isomerism, which are seemingly involved in the search for glyco-biomarkers of human diseases. While novel synthetic approaches are being developed toward the acquisition of new glycan standards, it is still prudent, feasible, and profitable to consider the isolation of pure glycans from some hitherto unexplored natural sources. It is demonstrated here that recycling high-performance liquid chromatography (R-HPLC) can accomplish isolation of isomeric glycans to be used as analytical standards or valuable reagents in the fabrication of glycan arrays for biomarker discovery.

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Introduction

Among the challenges of modern glycoscience is still a limited understanding of the structure–function relationship of different glycoconjugate molecules. While glycans are highly ubiquitous components of the biomolecules facilitating molecular recognition on cellular surfaces and in different levels of the immune system's function, the structural intricacies of these biologically important processes remain somewhat unclear, if not overwhelming. Glycosylation is a very complex process, often resulting in the molecules or arrays of molecules with very intricate structural forms and a very high information content [1 – 3]. Yet, we currently lack the adequate procedures to: (a) chemically resolve the structural complexity of individual glycans or measure their quantitative proportions; and (b) decipher the real biological meaning of glycosylation patterns, as based on the instrumentally measured and interpreted data. While the means to measure very small amounts of glycans and glycopeptides in biological materials have advanced remarkably during the last several years [for recent reviews, see references 4 – 6], structural elucidation and authentication of glycoconjugates have been hampered by the limited availability of glycans and glycopeptides as standard compounds that the scientific community could use in research investigations. Recently, this has been discussed in the U.S. National Research Council report [7], which calls for a concerted effort by synthetic carbohydrate chemists and analytical scientists, among others, to provide and authenticate important glycoconjugate molecules. The availability of these compounds in the future is likely to impact clinical diagnostic procedures and to improve our understanding of carbohydrate–protein and carbohydrate–carbohydrate interactions, in general.

Among the most promising approaches to probe the glycoconjugate interactions with other biologically relevant molecules have been glycan arrays [8 – 11], in which various glycan molecules are chemically immobilised to appropriate surfaces as "microdots" on a chip, thus representing diverse structures and their amounts. The glycan arrays have played a major role in the discoveries of antiglycan antibodies, galectins with different glycan binding specificities, and siglecs. Although up to several hundreds of synthetic glycans have now been available to construct glycan arrays in different laboratories [12], many more will need to become available to fill the "gaps" representing the total glycomes of different species, which are conservatively estimated to exist in up to at least several thousand structures in mammals [3]. Recent advances in the chemical and enzymatic synthesis [13 – 15] will be undoubtedly crucial in this regards, while additional efforts, as demonstrated below, could involve a yet different route to pure glycan availability, specifically, to their isolation from readily available biological materials. Appropriate bulk fractionation schemes and recovery of properly tagged glycans at high purity in the microgram-to-milligram scale need to be developed for this task.

Toward Disease Biomarker Discoveries: Recent Methodological Developments

Unusual types of protein glycosylation connected with human diseases have been the subject of investigations for several decades. However, only in recent times has it been possible to link aberrant glycan structures to pathological conditions such as congenital disorders, cardiovascular diseases, and malignancy [16, 17]. Methodological advances in measuring different oligosaccharides and their analytical profiles (quantitative glycan profiling at high sensitivity) have been of substantial value in the recognition of definitive glycan structures, with mass spectrometry (MS) and its various tandem techniques being recognised as the leading technology [for recent reviews, see references 4 – 6] in the biomarker discovery field. For the last several years, our principal motivation to develop sensitive bioanalytical instrumentation and glycomic profiling methodologies has been to explore the differences between glycosylation in healthy and in cancer conditions. While distinguishing other inflammatory diseases and cancer is currently somewhat problematic [18], glycomic profiling shows a distinct promise for the future of diagnostic and prognostic measurements [17, 19].

In cancer-related investigations through glycomics and bioinformatics, common physiological fluids (blood serum and plasma, cysts, ascitic fluids, saliva, cerebrospinal fluid, etc.) can nominally be analysed, but additionally, tumour tissues and cell lines may also provide convenient samples. While MS is uniquely sensitive to detect different glycans in these biological materials, this powerful method alone cannot determine unequivocally structural features of certain carbohydrates such as the abundance of structural isomers (glycans with the same molecular mass, but different positions or linkages of a substituted monosaccharide). Nonetheless, glycomic MS profiles can be highly informative in terms of different glycosylation patterns recorded for various cancer cohort groups [20 – 22], where the profiles obtained at high sensitivity (Figure 1) can be recorded and statistically evaluated for the patient cohorts numbering typically between 20 to 50 individuals. Various detected and quantitatively measured glycans can further be statistically evaluated for different cohort groups and judged by the usual clinical criteria (ANOVA, ROC values, etc.) whether or not they are worthy further investigation as biomarker candidates.

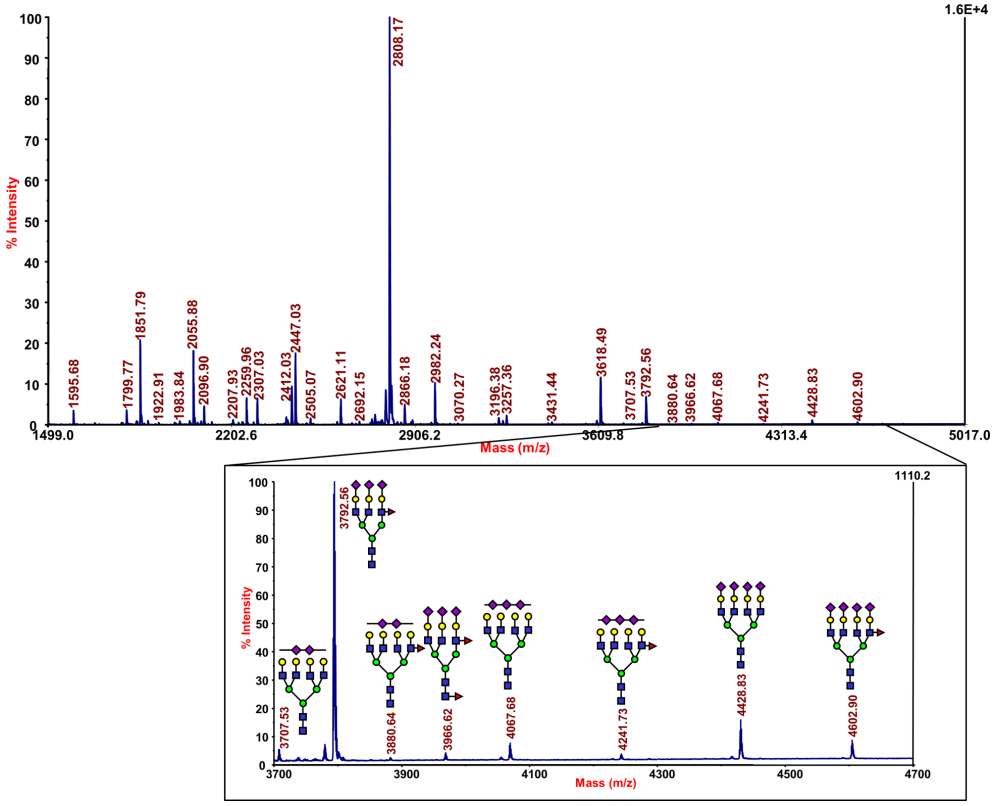


Figure 1. Example of an MS oligosaccharide profile derived from a blood serum sample measured for an ovarian cancer patient. The series of high-mass glycans (insert) are diagnostically important. Reprinted with permission from reference [21]. Copyright 2012 American Chemical Society.

An example of such a plot is shown in Figure 2 with a quantitative comparison between the levels of a fucosylated triantennary *N*-glycan in 20 ovarian cancer patients (elevated) and age-matched control samples [21]. Statistical comparisons of different glycan profiles suggest that a range of tri- and tetra-antennary glycans, with varying degrees of sialylation and fucosylation, deserve further attention for their distinctly elevated levels in ovarian cancer patients [21]. While this particular comparison appears clinically significant due to its high ROC value, the MS measurements alone do not indicate which of the two possible isomers, if either, is more associated with cancer. However, sample treatment with an appropriate exoglycosidase mixture, followed by MS, can identify a specific isomer [21, 22]. In most other comparisons [21 – 23], the outer-arm fucosylation isomer appears significant.

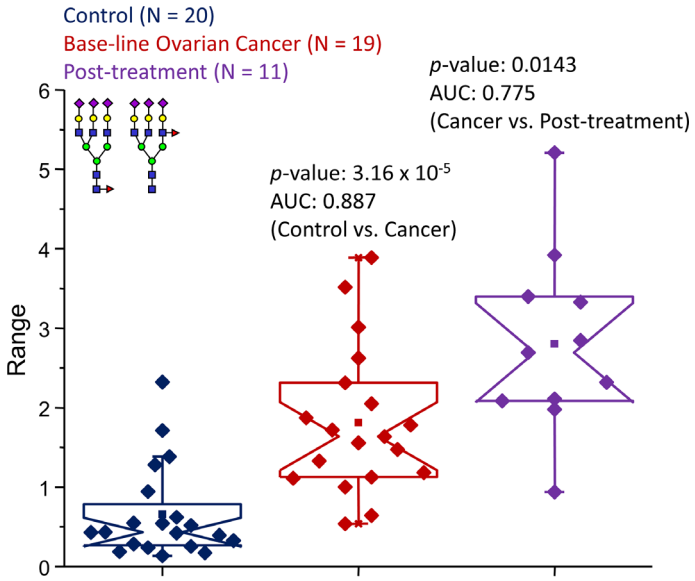


Figure 2 Notched-box plot for a fucosylated triantennary/trisialylated glycan measured through an MS-based profiling; it is not clear from MS, which isomer is involved. After the sample treatment with appropriate exoglycosidase mixture (followed by MS, both isomers were found, but only the glycan with the antennael fucosylation appeared to correlate with the cancer condition). Reprinted with permission from reference [21]. Copyright 2012 American Chemical Society.

However, many of the key *N*-glycans that distinguish healthy from disease samples are mostly evident as trace components whose authenticity must still be confirmed and their exact structures have to be determined. Unfortunately, as yet, these compounds are not synthetically available. Similar glycan types have been observed in the investigations of lung cancer [22] and colorectal cancer [23]. These represent just a few cases of a pressing need for pure glycans to be used as analytical standards.

Analytical Problems of Glycan Isomerism

Whereas a different binding of glycan isomers to a protein is easily envisaged due to different interactions of more or less sterically accessible polar groups, there are limitations to assess sugar isomerism even through techniques as powerful as mass spectrometry. Complementary technologies, such as NMR spectrometry could be additionally informative, but the tiny quantities of sugars extracted from the biological materials of interest limited a reliable structural work. The above-mentioned example of the determination of a fucosyl substitution (Figure 2), whether a core- or branch-substituted, represents only a small sampling of the structural problems to be encountered in the present-day glycobiology.

Yet another biomarker-related glycan isomerism case concerns different linkages of a sialic acid attachment in both simple and multi-antennary glycans. It has been suggested [24] that a conversion between α2,3- and α2,6-linkage could be indicative of certain cancer conditions. The recently developed derivatisation procedure [25] converts α2,3-linked sialic acid residues to lactone structures and α2,6-linked moieties to amides, so that appreciably different molecular masses can be measured through MS. These trends could be seen for a limited number of measurements in breast cancer sera [26], but a bit more complicated scenario is suggested through a comparison of the sets of lung cancer sera and those of control patients with different smoking histories (Figure 3) [22]. While these trends are suggestive of structural complexities concerning these differently sialylated and fucosylated glycans, there is currently no direct way to verify these preliminary findings and assign the position of sialic acids and their substitutions on different antennae for such triantennary glycans. Short of the availability of the authentic synthetic glycans, isolating these compounds from unique biological materials appears desirable for additional structural investigations.

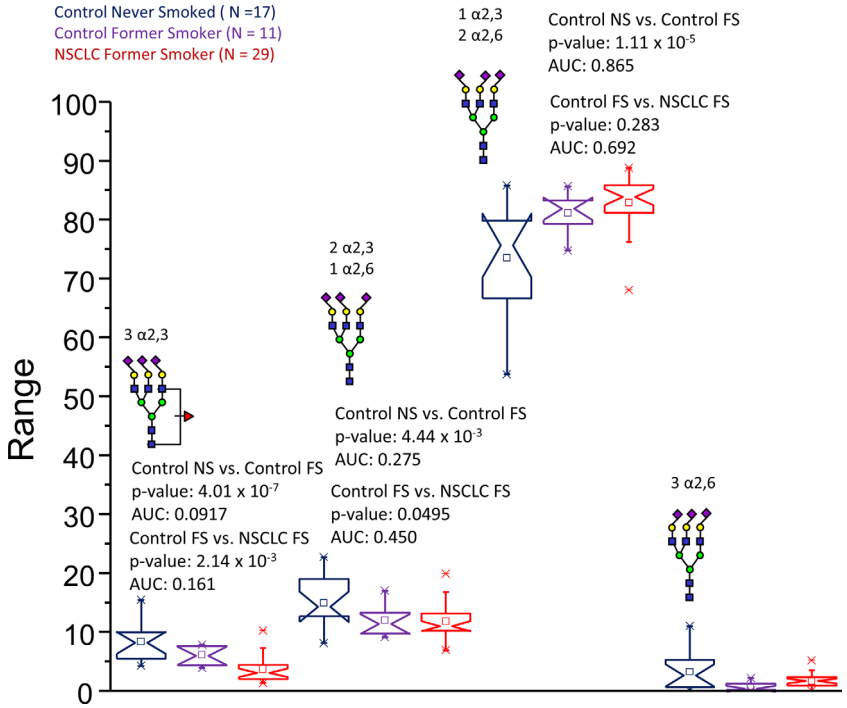


Figure 3. MS measurements of a series of triantennary fucosylated glycans using a derivatisation procedure [25] distinguishing differently sialylated glycans in different patient cohorts. Data adapted from reference [22].
NSCLC = non-small cell lung carcinoma.

Analytical separation science can also make a significant contribution to solve at least some glycan isomerism problems: Some isomers appear separable by special chromatographic columns (through HILIC and graphitised carbon packings) prior to their recording by MS and tandem MS [for a review, see reference 5]. Additionally, capillary electrophoresis (CE) with its exquisite resolving power appears uniquely capable to separate isomeric glycans due to their differences in hydrodynamic radii [27, 28]. To become detectable in CE, carbohydrates must be tagged with a fluorophore prior to their recording by laser-induced fluorescence. Unfortunately, the CE-based separation techniques are not easily combined with MS for a positive identification of glycans at this time. Once again, the availability of authentic electromigration standards could yield substantial progress in structural verification of glycan isomers.

Recycling HPLC

In 1998, Lan and Jorgenson [29] demonstrated the power of recycling HPLC through fully resolving phenylalanine from its deuterated analogue in a 90-min run. Until very recently [30], this methodology has not been applied to the isolation/purification of compounds, including sugars originating from biological sources. To utilise this preparative methodology in glycoscience applications, we decided to investigate several types of UV-absorbing tags as chromophores for detection and performed recycling chromatography through the use of twin columns located on opposite sides of a high-pressure switching valve (Figure 4). This procedure allows the control of optimised ‘‘effective column’’ lengths, so that the analytes experience repeated redirecting of the effluent of one column to the inlet of the other. The overall resolution by the HPLC system is thus controlled in a highly reproducible manner, causing the glycans of closely related structures to become separated from each other and collected at appropriate times. The chromatographic aspects of this procedure are described in a recent publication [30].

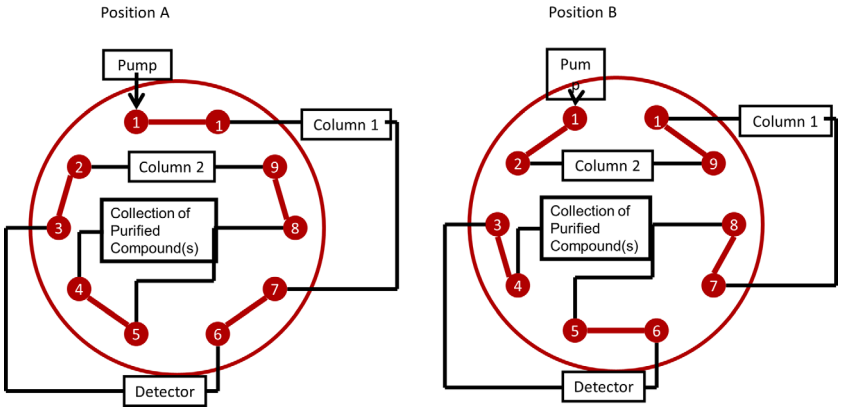


Figure 4. Valve schematics and column configuration used for recycling HPLC employed in glycan purification. Reprinted with permission from reference [30]. Copyright 2013 American Chemical Society.

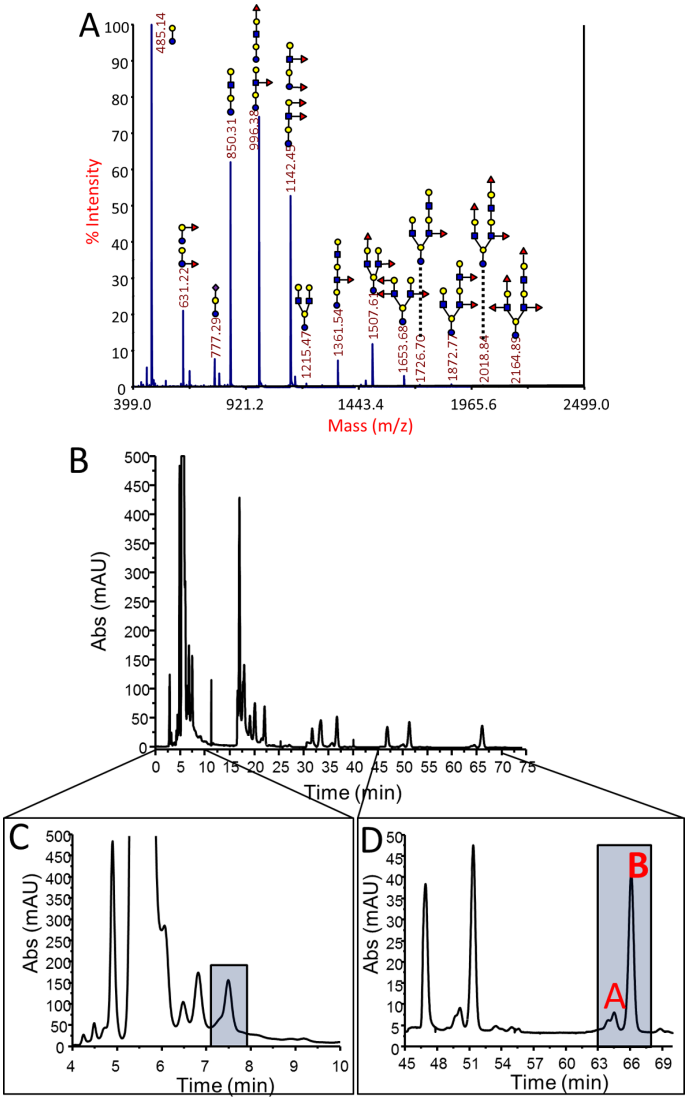
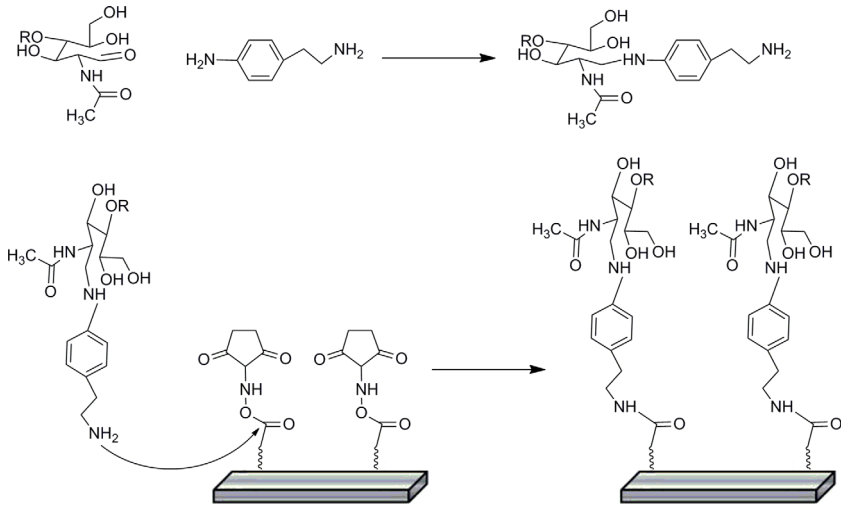


Figure 5. (A) MALDI MS profile of free oligosaccharides present in human breast milk. **(B)** R-HPLC chromatogram for the isolation and purification of human milk oligosaccharides. **(C)** a single chromatographic peak (highlighted) that **(D)** split into two peaks (A and B) due to recycling. Modified and reprinted with permission from reference [30]. Copyright 2013 American Chemical Society.

We have demonstrated the merits of recycling HPLC with the resolution of branching isomers, linear isomers differing in their linkages, and other forms of isomerism [30]. To provide unusual glycan structures to the glycobiology community as analytical standards in microgram-to-milligram amounts, it will be necessary to identify the biological sources with a sufficient abundance of the respective glycoproteins and develop effective bulk isolation

strategies and relatively inexpensive glycan cleavage methods prior to the use of the finer separations by HPLC. Among the readily available sources of free oligosaccharides is human breast milk (total carbohydrate content estimated around 5 – 15 mg/mL). Besides its major carbohydrate, lactose, up to 200 different structures with different monosaccharide sequences, linkages, and substitutions have been discovered in this biological material [31]. We have shown [30] how recycling chromatography could be applied to this biological material toward isolation of sugars with closely related structures. Figure 5A shows a brief characterisation of carbohydrates from a pooled breast milk sample (a MALDI-MS profile), while Figure 5B demonstrates a selected cut of the recycling purification in which a further selected fraction (Figure 5C) was further isolated, and subsequently resolved into fractions A and B (Figure 5D). Both peaks, while subsequently characterised by MALDI-MS and MS/MS, indicated the presence of differently fucosylated isomers [30], which could be individually recovered preparatively, and if needed, utilised as pure analytical standards.

An important future use of pure glycans is their attachment to the surfaces of the glycan array assemblies. Although, traditionally, synthetic glycans have been utilised in this capacity, glycans isolated from natural sources can be applied as well. This effort is meant to complement the current activities by others who synthesise various oligosaccharides for the same purpose. Toward the goals of our investigations, we have chosen 4-(2-aminoethyl)-aniline as a molecule with both aromatic and aliphatic amines, with two significantly different pKa values. This allows us to attach the reducing carbohydrate through the reductive amination to the aromatic amine of our chromophore, while the aliphatic amine facilitates attachment to the appropriately derivatised array surface, as seen in Scheme 1.



Scheme 1. Tagging procedure to a) detect glycans during R-HPLC and b) attach purified analytes on activated glass surfaces.

A verification of the feasibility of Scheme 1 is demonstrated in Figure 6, where HPLC of tagged glycans derived from human α_1-acid glycoprotein first separates a triantennary/trisialylated glycan and its fucosylated version (Figure 6a), as verified by their mass spectra (Figure 6b and c) and their detection, after immobilisation on an *N*-hydroxysuccinimide-fuctionalised glass slide, by appropriate fluorescently labelled lectins (Figure 6 d and e) [30]. Similarly, we were able to attach high-mannose glycans isolated from ribonuclease B (and resolved through recycling HPLC) in a glycan array-like microdot deposition procedure (Alley, W.R. Jr., Huflejt, M.E., Novotny, M.V., unpublished experiments).

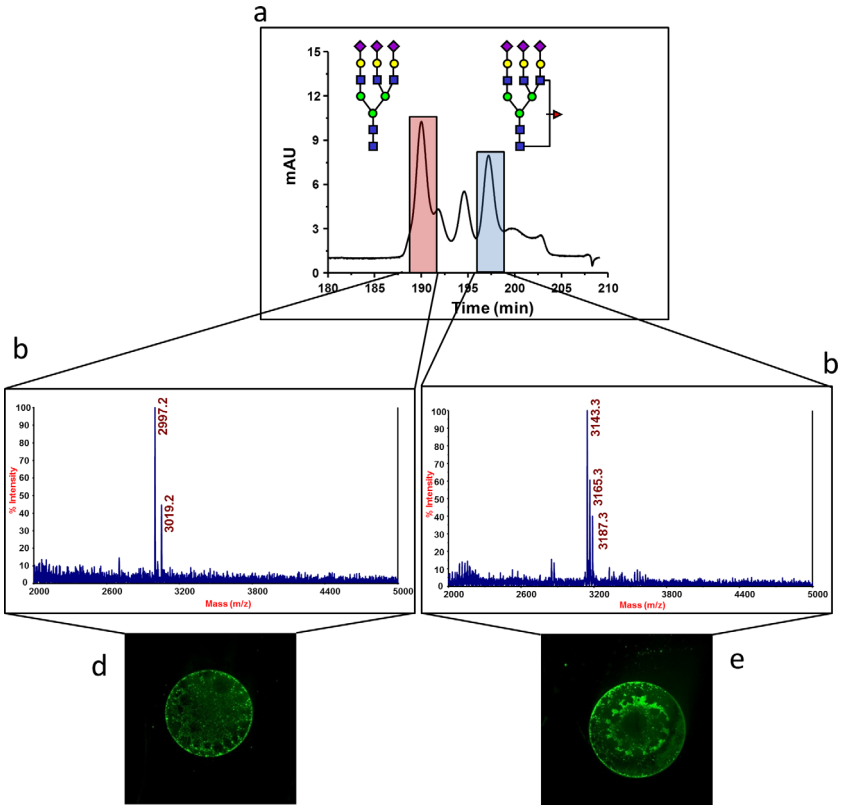


Figure 6. (a) R-HPLC chromatogram of a triantennary-trisialylated glycan and its fucosylated analogue derived from human α1-acid glycoprotein; **(b)** and **(c)** negative-mode MALDI mass spectra for the triantennary-trisialylated glycan and its fucosylated version, respectively; **(d)** and **(e)** triantennary-trisialylated glycan and its fucosylated analogue, respectively, immobilised on a glass surface and stained with an appropriate fluorescently-labeled SNA or AAL lectins, respectively. Reprinted with permission from reference [30]. Copyright 2013 American Chemical Society.

Recycling HPLC may also be an attractive purification alternative for synthetically or biosynthetically-derived glycans. While it has been recently demonstrated [15] that very complex structures can be reliably assembled through a sophisticated synthesis, the

compounds' purity assessment will likely necessitate a combination of orthogonal analytical techniques. As shown in Figure 7 with a synthesis product, a triantennary glycan with a "mixed population" sialylation (one $\alpha 2-6$ and two $\alpha 2-3$ sialyl substitutions) after 9 column passes, the collected cut is nearly pure, and a single symmetrical peak is seen in the 18th column cut. However, capillary electrophoresis (CE), a technique capable of isomer separations, can still reveal a peak-splitting phenomenon (Mitra, I., Jacobson, S.C., Alley, W.R., Jr., Novotny, M.V., unpublished experiments).

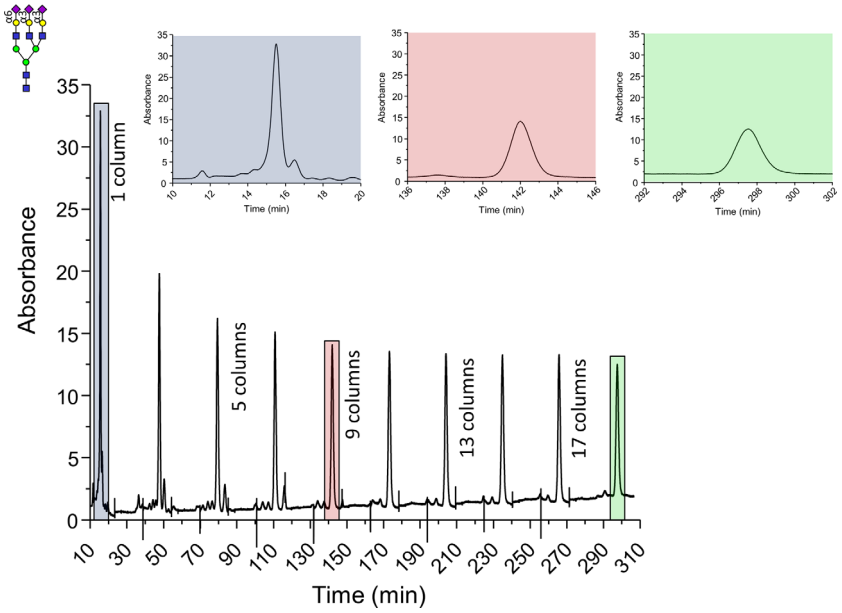


Figure 7. Purification of a synthetic glycan through HILIC recycling HPLC. The sample was obtained through courtesy of Dr. Geert-Jan Boons.

Glycan Isolation from Unusual Materials

Historically, extracting relatively large quantities of glycans from complex biological materials for the sake of structural elucidation was necessary in much of the pioneering studies in glycobiology. Conversely, the last two decades, following the advances in biomolecular MS combined with the continuous column miniaturisation efforts, have led to high-sensitivity glycan profiling capabilities [4], which necessitate only small volumes of physiological fluids. While interesting glycan structures are often suggested by their mass spectra, there is a need for their authentication concerning their isomerism. Once again, isolation from much larger volumes of biological media (containing, unfortunately, numerous "ballast proteins") may become necessary, needing, in turn, larger-scale chromatographic fractionations.

Among the suitable sources of unusual glycan structures, biological exudates (actively secreted fluids of cancer patients) may be strongly considered because (a) they are particularly rich in protein content (> 30 mg/ml concentrations), likely containing the glycoproteins with aberrant glycan structures due to malignancy and inflammation; and (b) large volumes of the fluids are typically treated as waste products in a clinical environment. In one example of a glycan profile (Figure 8) of an ascitic fluid from an ovarian cancer patient, we can observe through MS multiply-fucosylated and sialylated structures. However, these are only tentatively assigned structures without a positive identification of a number of possible isomers. As yet, authentic glycans of this type cannot be produced synthetically, but their isolation and testing for biological activity through a glycan array arrangement becomes a distinct possibility.

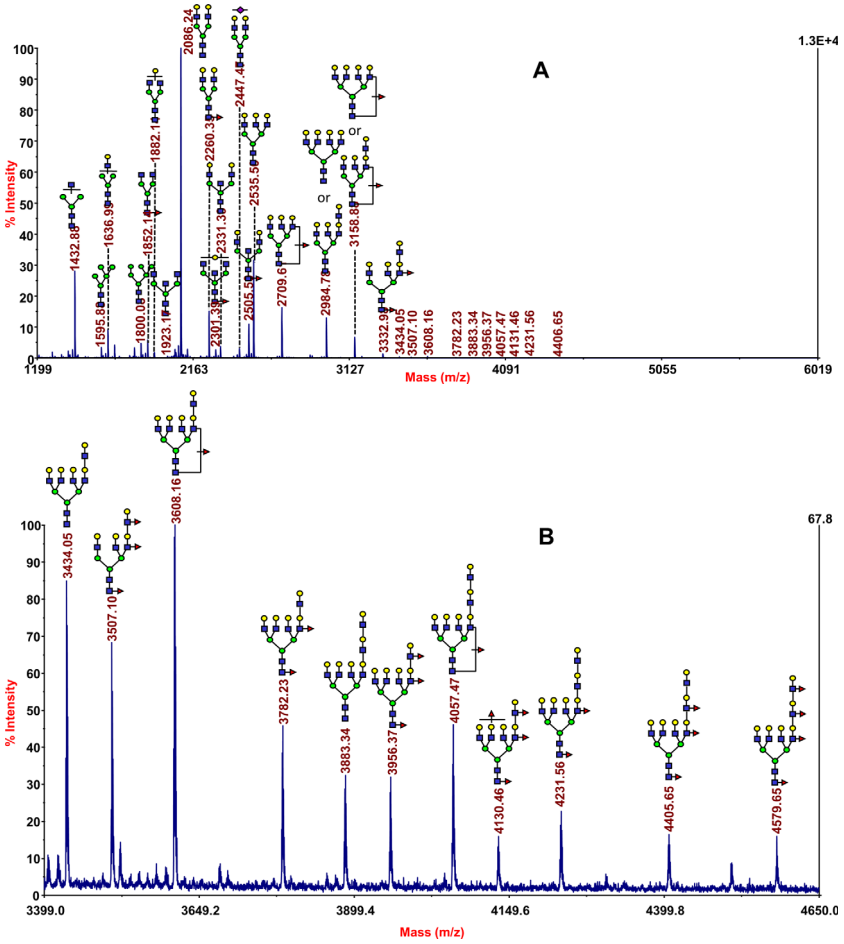


Figure 8. MALDI-MS profile of permethylated N-glycans extracted from glycoproteins of an ascitic fluid sample of an ovarian cancer patient **(A)**; **(B)** represents a high-mass end of the profile. Sample obtained through courtesy of Dr. Daniela Matei, Indiana University, School of Medicine, Indianapolis, IN.

Conclusion

Modern techniques of analytical glycoscience, through its inclusive fields of glycomics, glycoproteomics and glycobioinformatics, have significantly enriched our perception of the ubiquity of glycoconjugate structures in nature and the apparent complexity of their interactions with other biomolecules. While MS has now become a key technology to measure even trace quantities of the easily identifiable glycans, it cannot address directly the isomerism questions of, for example, fucosyl and sialyl substitutions in putative cancer biomarker molecules. A wider availability of authentic glycans for the benefits of glycoscience community must be addressed by efforts in glycan synthesis as well as the isolation and purification of glycans derived from natural sources. The previously under-utilised technique of recycling HPLC has now been demonstrated as a beneficial route to isolate unusual glycans, including isomeric molecules, for the sake of standard availability and future use in glycan array technologies.

Acknowledgements

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