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One Filter, One Sample, and the *N*- and O-Glyco(proteo)me: Toward a System to Study Disorders of Protein Glycosylation

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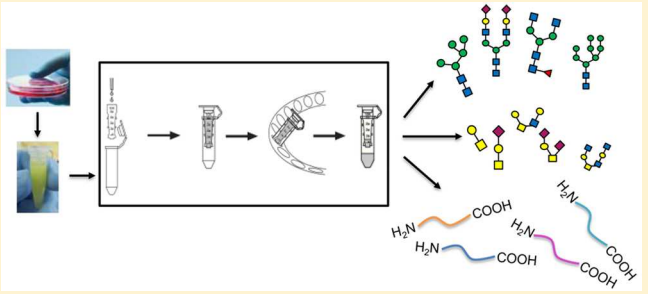
Supporting Information

ABSTRACT: A method has been developed for release/isolation of O-glycans from glycoproteins in whole cell lysates for mass spectrometric analysis. Cells are lysed in SDS, which is then exchanged for urea and ammonium bicarbonate in a centrifugal filter, before treating with NH_4OH to release O-glycans. Following centrifugation, O-glycans are recovered in the filtrate. Sonication achieves O-glycan release in 1 h. Combining the established protocol for filter-aided *N*-glycan separation, here optimized for enhanced PNGase F efficiency, with the developed O-glycan release method allows analysis of both *N*- and O-glycans from one sample, in the same filter unit, from 0.5 to 1 million cells. The method is compatible with subsequent analysis of the residual protein by liquid chromatography−mass spectrometry (LC−MS) after glycan release. The medium throughput approach is amenable to analysis of biological replicates, offering a simple way to assess the often subtle changes to glycan profiles accompanying differentiation and disease progression, in a statistically robust way.



Glycans, carbohydrate chains glycosidically linked to proteins and lipids, coat all cell surfaces and play a central role in cellular and physiological processes such as protein targeting and immune modulation. Glycans also play important roles in disease. Alterations in glycosylation have been observed in cancers,[1] Alzheimer's disease[2,3] inflammatory conditions[4] and in congenital disorders of glycosylation (CDGs). CDGs are a large class of rare genetic diseases in which alterations in various forms of glycosylation cause a variety of systemic pathologies.[5−8] Protein glycosylation is also a major consideration in the pharmaceutical industry, as glycoproteins are used in a multitude of therapeutic applications, including therapeutic monoclonal antibodies[9,10] and vaccines.[11−14] The exact nature of glycans present on therapeutic glycoproteins is critical, influencing efficacy, immunogenicity,[15−17] stability[18,19] and pharmacokinetics.[20,21] Glycans are important as drug targets.[22] It is therefore important to understand glycan biosynthesis and to have convenient methods to assess glycan structures, in order to help further understand disease and genetic disorders linked to errors in glycosylation, to potentially help in both disease diagnostics and in development of biotherapeutics.

The biosynthetic code for glycans remains enigmatic because, unlike protein and nucleic acid biosynthesis, there is no template for glycan biosynthesis. Eukaryotic glycan biosynthesis is managed by a large number of glycosyltransferase and glycosidase enzymes (located in the endoplasmic reticulum (ER) and the Golgi apparatus) and is dependent on their expression levels, location, and activity. These can change in response to alterations in the cell's environment, differentiation or proliferation state, or as a consequence of intercellular communication. Consequently, the overall distribution of glycan structures, and hence the glycan profiles, can vary quite significantly depending on the tissue in which they are expressed as well as local conditions, making analysis of protein-linked glycan structures both challenging and important. As structures cannot be predicted from a template, they have to be determined experimentally in each distinct case. To determine which of the changes in glycan profiles are significant, and which changes are just the effects of biological variation, analysis of multiple biological samples in parallel is needed. It is not necessary to carry out ultimate deep glycan profiling to make this assessment, but rather to uncover overall shifts in relative glycan proportions. This makes it possible to avoid the use of very large numbers of cells that would be necessary for deep profiling, but that at the same time limit the feasibility of analyzing appropriate replicates.[23,24]

Enzymes and biosynthetic pathways leading to *N*- and O-protein glycosylation have been defined[25−27] in a variety of experimental systems, enabling understanding of the range of

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possible glycan structures of both glycosylation types. While we have an idea of the glycan structures expected, the consequences of the loss of even a single enzyme are not always trivial to predict. Much more difficult, if possible at all, is to predict consequences of glycan biosynthetic enzyme mislocalization within the Golgi. There have been a number of studies into how enzyme localization influences glycan processing in the Golgi. It is clear that enzymes are sorted using transport vesicles, which are targeted to their appropriate locations during the vesicle tethering process. This is mediated by the conserved oligomeric Golgi (COG) protein complex, which has been shown to play a central role in maintaining the fidelity of glycosylation in all eukaryotic cells.[28−31]

Glycomic workflows frequently use separate protocols and/or large quantities of sample/cells[32,33] to be able to look at both *N*- and O-glycans and for proteomic studies. Since some genetic diseases can occur as a result of a genetic defect that affects *N*- and O-glycosylation simultaneously, whereas some defects only affect one glycan type, analysis of both *N*- and O-glycans from the same sample in an integrated protocol is ideal. Such a one-sample-one-pot method has the advantage of reducing the amount of material needed to carry out such studies and offers the potential, after *N*- and O-glycan release, to analyze the remaining protein, enabling a complementary proteomic comparison between samples. Recently, a method named filter-aided *N*-glycan separation or FANGS, has been developed for the release and isolation, using a centrifugal filter, of *N*-glycans from glycoproteins solubilized from whole cell lysates.[34] FANGS is a simple protocol making use of filter-aided sample preparation (FASP) for SDS removal, first described by Manza et al.[35] and then by Wiśniewski et al.[36] for exchange of SDS with urea. In FANGS, following SDS removal, the glycoprotein extract is incubated with PNGase F to release the *N*-glycans. *N*-Glycans are collected after centrifugation. FANGS has been successful using only 3.5×10^5 cells, equivalent to one well of a six well culture plate and so is applicable to low cell numbers and therefore can be used to analyze multiple samples in parallel. Analogous methods are, however, still needed to release and isolate O-glycans from cultured cells for mass spectrometric analysis.

For O-glycosylation, there is no enzyme that shows a broad specificity for O-glycan release to compare with PNGase F,[37] and so chemical methods are the most appropriate for pan-specific O-glycan release. Reductive β-elimination involving treatment of the glycoprotein with NaOH and $NaBH_4$ is one of the best-established methods for releasing O-glycans from glycoproteins.[37] O-glycans can also be released by treating glycoproteins with NH_4OH which has the advantage of being volatile and so is easily removed under reduced pressure.[38]

Conditions for releasing glycans from glycoproteins/peptides generally involve overnight or longer incubation. There are reports of using microwave assistance to speed up incubation times in a range of chemical and enzymatic reactions. Both domestic microwaves and microwave reactors have been used to assist tryptic digestion of proteins as well as to release *N*-glycans and O-glycans from purified glycoproteins such as bovine fetuin and mucins in 20−120 min.[39−42] Sonication, used widely in industry and research, is also used to accelerate reactions in organic synthesis, stimulate enzymes in enzymatic synthesis, and is also used to disrupt cell membranes without damaging the cell contents.[43] However, there appears to be nothing in the literature to suggest sonication has been assessed for use in the release of glycoprotein glycans. Both microwave irradiation and sonication have thus been investigated in this study for their potential to speed up O-glycan release and reduce sample preparation times.

Here we present a filter-based approach, making use of a centrifugal filter to carry out O-glycan release using NH_4OH as well as exploiting advantages of an optimized FASP[35,36] approach, eFASP,[44] which uses alternative reagents to FASP. We show how O-glycan release can be achieved in 1 h with the aid of sonication, without loss of labile sialic acids, and giving results comparable with those from the well-accepted overnight incubation methods. A streamlined protocol is presented for a one-sample-one-pot approach, to release both *N*- and O-glycans (performed as *N*- then O-glycan release or vice versa) from the same sample, in the same sample pot, by combining an optimized FANGS protocol with the O-glycan release method, also offering the potential to subsequently analyze the protein remaining in the filter. The method developed has been demonstrated using the standard glycoprotein porcine stomach mucin and has then been applied to the study of mesenchymal stromal cells (MSCs), using only 0.5−1 million cells.

EXPERIMENTAL PROCEDURES

Fully detailed experimental procedures are described in the Supporting Information.

Extraction of Glycoproteins from Porcine Bladder Urothelial Tissue. Bladders were obtained from an abattoir (A Traves & Son Ltd.) and processed the morning they were collected. Urothelium was removed from lumen of dissected bladders by scraping with a scalpel.

hTERT Mesenchymal Stromal Cell (MSC) Culture. hTERT MSCs are immortalized cell lines generated from primary human mesenchymal stromal cells by lentiviral transduction with a human telomerase reverse transcriptase gene and subsequent single cell cloning.[45] Cells were cultured in Dulbecco's Modified Eagle Medium (high glucose, pyruvate, no glutamine) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% Gluta-Max-I. Cultures were grown in a humidified incubator at 37 °C in 5% CO_2 in air in 10 cm Petri dishes and harvested at around 80% confluency.

Optimized Method for Preparation of Glycoprotein Standards, Urothelial Cell Samples, and MSCs for Glycan Release. Porcine stomach mucin (Sigma-Aldrich, U.K.) was solubilized in eFASP lysis buffer.[44] For urothelial and MSC samples, eFASP lysis buffer was added at 10× the cell pellet volume. Samples were lysed, reduced, and alkylated as described.[44] Glycoprotein samples were then diluted with exchange buffer (8 M urea, 100 mM ammonium bicarbonate) in a 40:1 (v:v) ratio of exchange buffer to sample solution. The sample solution was transferred to a filter unit (Amicon Ultra-0.5 centrifugal filter unit, nominal mass cut off 30 kDa) and centrifuged for 5 min, and the filtrate discarded. The sample retained above the filter membrane was rinsed three times with 300 μL of exchange buffer and 50 mM ammonium bicarbonate (centrifuging after each rinse for 5 min), discarding the filtrate. The filter unit was transferred to a clean collection tube ready for *N*- or O-glycan release.

Glycan Release. To release O-glycans, glycoproteins were treated with 300 μL of NH_4OH at 45 °C for 16 h, as described,[46,38] directly in the filter unit. Alternatively, on treating with NH_4OH, samples were sonicated, in the filter unit, at 45 °C for 5 min then left in the water bath for 10 min to avoid overheating, followed by another 5 min of sonication. The sonication/rest cycle was repeated giving a total sonication

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time of 20 min and total intervening rest time of 40 min. Following incubation, the filter device was centrifuged to collect the O-glycans in the filtrate. The O-glycan filtrate was dried using a vacuum centrifuge. *N*-Glycans were released as described using FANGS.[34] Glycans were permethylated prior to mass spectrometric (MS) analysis and relative quantification.[47]

Tryptic Digestion. The protein remaining in the filter unit following *N*- and O-glycan release was treated with trypsin at 37 °C for 16 h. Following incubation the filter was centrifuged to collect the tryptic peptides. Samples were acidified with 0.1% TFA before LC−MS/MS analysis.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). RNase B samples were denatured by heating at 95 °C for 5 min in the presence of 2% SDS. SDS-PAGE was performed using discontinuous polyacrylamide gels with a 12% separating gel layer in a standard Tris-glycine buffer. Gels were electrophoresed at 180 V and stained with Coomassie brilliant blue.

Glycan Analysis. Dried permethylated glycans were dissolved in 10 μL of acetonitrile. (For relative quantitation of glycans, samples were spiked with an internal standard, deuteropermethylated maltotetraose.) Sample spots were prepared by mixing matrix solution (20 mg/mL 2,5-dihydrobenzoic acid in 50% aqueous acetonitrile) with sample solution in a 2:1 (v:v) ratio. A volume of 2 μL of the mixture was transferred to a MALDI target plate and dried under vacuum. Mass spectra were acquired in positive ion mode on a 9.4 T solariX Fourier tranform ion cyclotron resonance mass spectrometer (Bruker Daltonics), with a smartbeam-II laser. Spectra were acquired using ftmscontrol 2.0 and processed with DataAnalysis 4.0. Product ion spectra were acquired using a Nanospray Flex ion source on an Orbitrap Fusion hybrid mass spectrometer (Thermo Scientific). Positive ESI-MS and MS^2 product ion spectra were acquired in the Orbitrap using Xcalibur 4.0 software (Thermo Scientific). Fragmentation was induced using either collision induced dissociation or higher energy collision dissociation.

Peptide Analysis. Samples were loaded onto an UltiMate 3000 RSLCnano HPLC system (Thermo Scientific) equipped with a PepMap 100 Å C_{18}, 5 μm trap column (300 μm × 5 mm Thermo Scientific) and an Acclaim PepMap RSLC, 2 μm, 100 Å, C_{18} RSLC nanocapillary column (75 μm × 150 mm, Thermo Scientific). The nanoLC system was interfaced with an Orbitrap Fusion hybrid mass spectrometer with a Nanospray Flex ionization source. Positive ESI-MS and MS^2 product ion spectra were acquired. Peak lists were generated using Mascot Distiller (version 5, Matrix Science). MGF files were searched against the porcine subset of the UniProt database (34 316 sequences; 14 316 901 residues), with a decoy database, using a locally running copy of the Mascot program (Matrix Science Ltd., version 2.5.1).

RESULTS AND DISCUSSION

A method has been developed, based on FANGS, to release and isolate O-glycans from glycoproteins for MS analysis. The method makes use of a centrifugal filter as a simple reaction vessel in which glycoproteins can be treated with NH_4OH solution to release O-glycans by β-elimination. Following centrifugation, glycans are retrieved in the filtrate, as they pass through the filter membrane while proteins are large enough to be retained above the membrane. Isolated O-glycans are permethylated for MS analysis. The method can be conveniently combined with the established FANGS protocol, to release, isolate, and analyze both *N*- and O-glycans using one sample, in the same filter unit, enabling analysis of the whole glycome.

Method Development/Optimization. O-glycans were released from 20 pmol of porcine stomach mucin (a well characterized, commercially available glycoprotein), by dissolving in SDS and then exchanging SDS for urea in a centrifugal filter unit (as for FASP[36] and FANGS[34]). O-Glycans were released, in the filter, by treatment with NH_4OH. After collecting and permethylating the glycans, a matrix-assisted laser desorption/ionization-Fourier transform ion cyclotron resonance (MALDI-FTICR) mass spectrum was obtained, revealing expected glycan signals[48−50] between m/z 500 and 2214 (Figure S-1), confidently assigned, as mass accuracies for the majority were below 1 ppm (Table S-1).

A recent publication describes enhanced filter-aided sample preparation[44] (eFASP), using alternative reagents to those first described by Manza et al.[35] and later in FASP by Wisniewski et al.,[36] reporting increased sensitivity and sample recovery for proteomics. To see whether eFASP sample preparation could also yield improved results for our glycomics applications, samples of mucin were prepared for O-glycan release, using eFASP for SDS solubilization and removal/exchange, to compare with FASP. After O-glycan release and permethylation, a MALDI-FTICR mass spectrum was obtained, revealing expected O-glycans for mucin[48] between m/z 500 and 2214. Data from the two sample preparation methods show the same O-glycans were released, but better signal-to-noise was observed for the sample prepared using eFASP reagents, Figure S-1.

Alkylation in FASP/FANGS is carried out using 50 mM iodoacetamide in the filter unit, for 15 min. In comparison, in eFASP the recommended alkylation step takes 1 h at 37 °C, with 50 mM iodoacetamide (in the authors' standard protocol) or with 4-vinylpyridine (4-VP) (in their express protocol, where the alkylating reagent is added before the sample is transferred to the filter, eliminating a washing step). We investigated the alkylation step to determine whether it is possible to keep the reaction time at 15 min, as in FASP/FANGS. Mucin samples were prepared for O-glycan release using eFASP reagents. Samples were alkylated for 15 min or 1 h, using iodoacetamide or 4-VP, and O-glycans were released and permethylated. Overall, 4-VP treatment gave spectra with better signal-to-noise than those from samples prepared using iodoacetamide, and there appeared to be no discernible difference in the results following alkylation for 15 min or 1 h (Figure S-2), suggesting 15 min with 4-VP is suitable for our application.

Reducing Incubation Time for O-Glycan Release. Classic methods for releasing O-glycans generally use incubation for 16−18 h. In order to reduce the time for glycan release, sonication in a sonic bath and microwave irradiation using a laboratory microwave reactor were investigated.

Sonication times of 5, 10, 15, and 20 min were tested to aid O-glycan release. Glycan signals could be observed after only 5 min of sonication (data not shown), but it was difficult to avoid the sonic bath temperature rising above 45 °C on sonication for longer periods. 45 °C was chosen for direct comparison with overnight incubation and with the intention of avoiding hydrolysis of labile glycans. Sonicating for 5 min, followed by holding the sample in the sonic bath for 10 min without sonicating, and then repeating rounds of sonication/rest ensured the temperature remained at 45 °C. Using a total of

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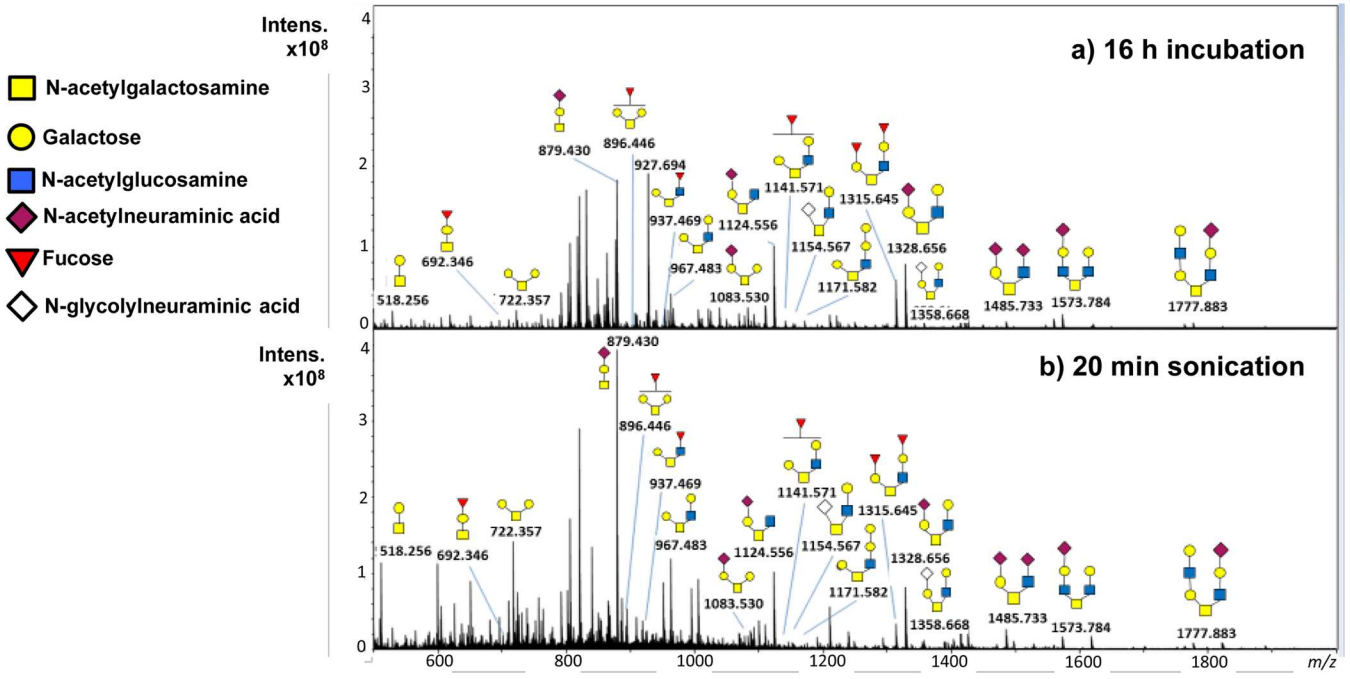

Figure 1. MALDI-FTICR mass spectrum of permethylated O-glycans (ionized as $[M + Na]^+$) released from porcine bladder urothelium using (a) 16 h incubation, (b) 20 min sonication (1 h total release time) at 45 °C. Displayed glycan structures are intended to indicate composition only.

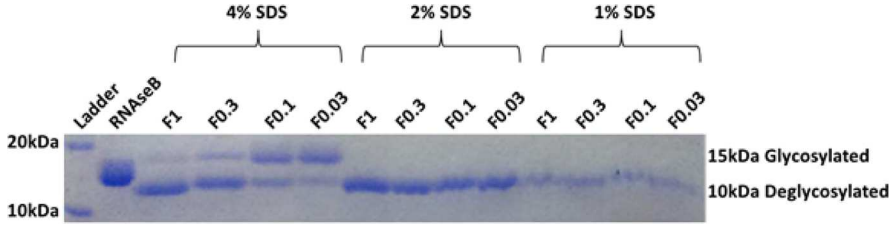

Figure 2. SDS-PAGE analysis of 4 μg of RNase B following FANGS-based sample preparation and subsequent *N*-glycan release with 37 °C overnight incubations using PNGase F amounts of 1, 0.3, 0.1, and 0.03 U in a 10 μL volume. Prior to FANGS, the lysis buffer contained varying SDS w/v concentrations of 4%, 2%, and 1% during denaturation.

20 min sonication interspersed with 40 min rest (a total O-glycan release time of 1 h) gave glycan profiles that were comparable with those obtained on overnight incubation at 45 °C, Figure S-3. Sonication was thus chosen as the method to pursue because it is compatible with glycan release in the filter unit. When using the microwave reactor, samples had to be transferred from the filter unit to a microwave tube and back, which would incur inevitable sample loss. In addition, because the sonication was carried out in a sonic bath, multiple samples can be handled side-by-side, and so the approach is amenable to handling multiple samples in parallel, in line with the aims of the developed method.

After optimizing O-glycan release using porcine stomach mucin, the method was applied to porcine bladder urothelial cells to demonstrate its application to a cell sample. Samples of porcine bladder urothelium were prepared for O-glycan release following our optimized eFASP/FANGS protocol, and O-glycans were released by treating with NH_4OH in the filter unit using overnight incubation (16 h) or four separate 5 min sonications (interspersed with 10 min rests). Glycans were permethylated, and on obtaining a MALDI-FT-ICR mass spectrum, O-glycan signals were identified in both the overnight incubation and sonication-aided release samples, between *m*/*z* 518 and 1777, confidently assigned based on mass accuracies (Table S-1). Nonfucosylated/sialylated glycans were observed at *m*/*z* 518, 722, 967, and 1171. Fucosylated glycans were identified at *m*/*z* 692, 896, 937, 1141, 1315, and 1328 and sialylated glycans were identified at *m*/*z* 879, 1083, 1124, 1154, 1328, 1358, 1485, 1573, 1777, Figure 1. Even those glycans of low abundance could be readily identified in the sonicated sample (*m*/*z* 692, 1083, 1358, 1485, 1573, 1777). The sonication method left labile sialic acid moieties intact and showed comparable signal-to-noise for each of the sialylated glycan structures with those in samples prepared using the overnight method, Figure 1. It was concluded that the sonication-based method was applicable not only to a simple soluble glycoprotein standard but also to O-glycans released from cells.

One-Sample, One-Pot: The *N*- and O-Glycans. Both *N*- and O-glycans are important when studying disease, and so access to a protocol allowing analysis of the whole glycome from one sample handled in the same reaction pot is desirable, especially one based around the use of centrifugal filters that enable use of small cell numbers as well as low volumes of reagents. The workflows for releasing *N*-glycans and O-glycans were thus combined, to test whether *N*-glycans and O-glycans could both be released from the same sample in the same reaction vessel.

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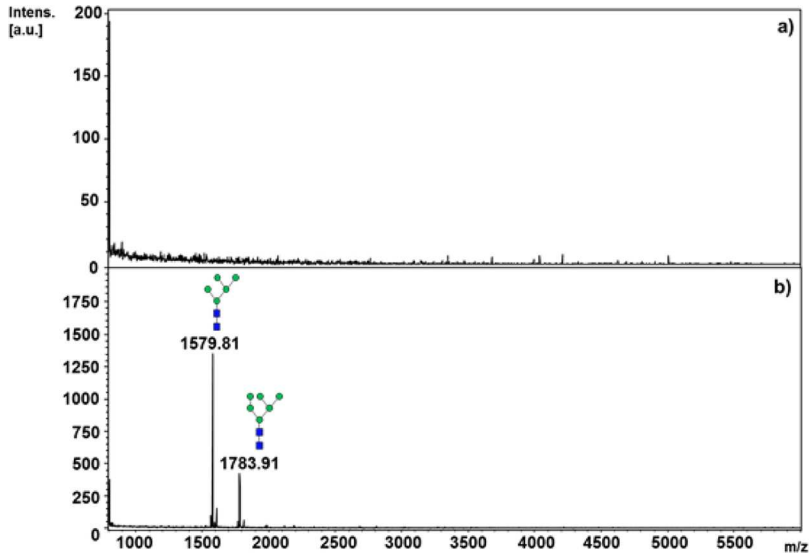

Figure 3. Positive ion mode MALDI mass spectra of permethylated FANGS-released *N*-glycans from 40 μg of RNase B using 0.003 U PNGase F and 2% SDS containing lysis buffer prior to FANGS: (a) using 10× dilution in urea-buffer following lysis and (b) using 20× dilution in urea-buffer following lysis.

Before combining the glycan release protocols, the FANGS protocol was optimized to enhance the efficiency of PNGase F digestion. Given the critical micelle concentration (CMC) of SDS is approximately 0.1%, if the SDS used for glycoprotein extraction is not sufficiently diluted with urea buffer, it would be expected to form micelles that would be retained by the filter membrane due to their size; the resulting levels of residual SDS could reduce the efficiency of PNGase F. The lysis buffer used in FANGS contains 4% SDS, and therefore the 10-fold dilution described in the FASP and FANGS protocols generates a 0.4% SDS solution, which is above the CMC. To test whether this SDS concentration does indeed have an inhibitory effect, RNase B was denatured in buffers with SDS concentrations of 1%, 2%, and 4%. Subsequently, the solution was diluted 20-fold with urea-Tris buffer and washed using a centrifugal concentrator filter as in FANGS. The resulting RNase B solution was then removed from the filter, divided into equal sized aliquots, and digested with decreasing amounts of PNGase F to assess the efficiency of cleavage. While the sample treated with 4% SDS is not fully cleaved even by 1 Unit PNGase F, reducing the SDS concentration to 2% followed by 20-fold dilution was sufficient to require as little as 0.003 Unit enzyme for full cleavage (Figure 2).

The next aim was to test the use of considerably reduced levels of PNGase F in the modified FANGS procedure to generate good quality glycan mass spectra. This is important for medium throughput applications in which the cost of PNGase F could become prohibitive. RNase B was denatured in a solution containing 2% SDS (a detergent amount sufficient when using a purified soluble protein rather than a cell suspension). When FANGS was performed following 10-fold dilution, no observable glycan peaks were obtained following digestion using 3 mUnits of enzyme. In contrast, the same amount of enzyme used with FANGS following 20-fold dilution of the SDS produced good glycan peaks (Figure 3). The spectrum shows only the most abundant *N*-glycan signals due to the more than 2000-fold lower PNGase F level than in the standard FANGS protocol.[34] The conclusion is that in the case of cell extracts, where 4% SDS is needed for full solubilization of the lipid and protein content, 40-fold dilution of the SDS solution should be used to achieve optimal PNGase F activity.

Porcine bladder urothelium was treated using the optimized sample preparation workflow based on eFASP, including the 1:40 dilution, and then treated with PNGase F for 16 h at 37 °C in the filter unit. Following incubation, the filter was centrifuged to collect the released *N*-glycans. The filter was transferred to a clean collection tube and O-glycans were subsequently released with NH_4OH treatment, aided by 20 min sonication (4 × 5 min bursts, interspersed with 4 × 10 min rests) at 45 °C. O-Glycans were retrieved on centrifugation of the filter unit. *N*- and O-Glycans were permethylated. *N*-Glycans were observed from *m*/*z* 967 to 2966, corresponding mainly to oligomannose species, with some complex type *N*-glycans, some of which were sialylated and others fucosylated. O-Glycans were observed between *m*/*z* 518 and 1777 (Figure 4), the same as for the urothelial O-glycan sample from which *N*-glycans had not previously been released. Focusing on *m*/*z* 1500−3000 in the O-glycan spectrum, it is clear that there are no signals corresponding to *N*-glycans, suggesting that all the *N*-glycans were washed out of the filter before O-glycan release. However, there are signals observed in both the *N*- and the O-glycan spectra at *m*/*z* 967, *m*/*z* 1141, and *m*/*z* 1171. Although these signals could formally be due to carry over of *N*-glycans into the O-glycan sample, these three signals are observed in a spectrum of O-glycans that had been prepared without previously releasing *N*-glycans. This, together with the fact that none of the other more abundant *N*-glycans are observed contaminating the O-glycan sample, suggests that it is unlikely that the *m*/*z* 967, 1141, and 1171 signals derive from *N*-glycan carryover, and this was demonstrated on product ion analysis of these species in the two glycan fractions, Figures S-4−S-6.

To show that the developed method can be used as a truly one pot method to study glycans and protein, after both the *N*- and O-glycans had been released and isolated, the remaining protein in the filter unit was treated with trypsin. The tryptic peptides were collected in the filtrate after centrifugation and analyzed by LC−MS/MS. Mascot searching matched 5005 peptides and identified 1301 proteins, FDR 1% (Supporting Information). These results were compared with those from a

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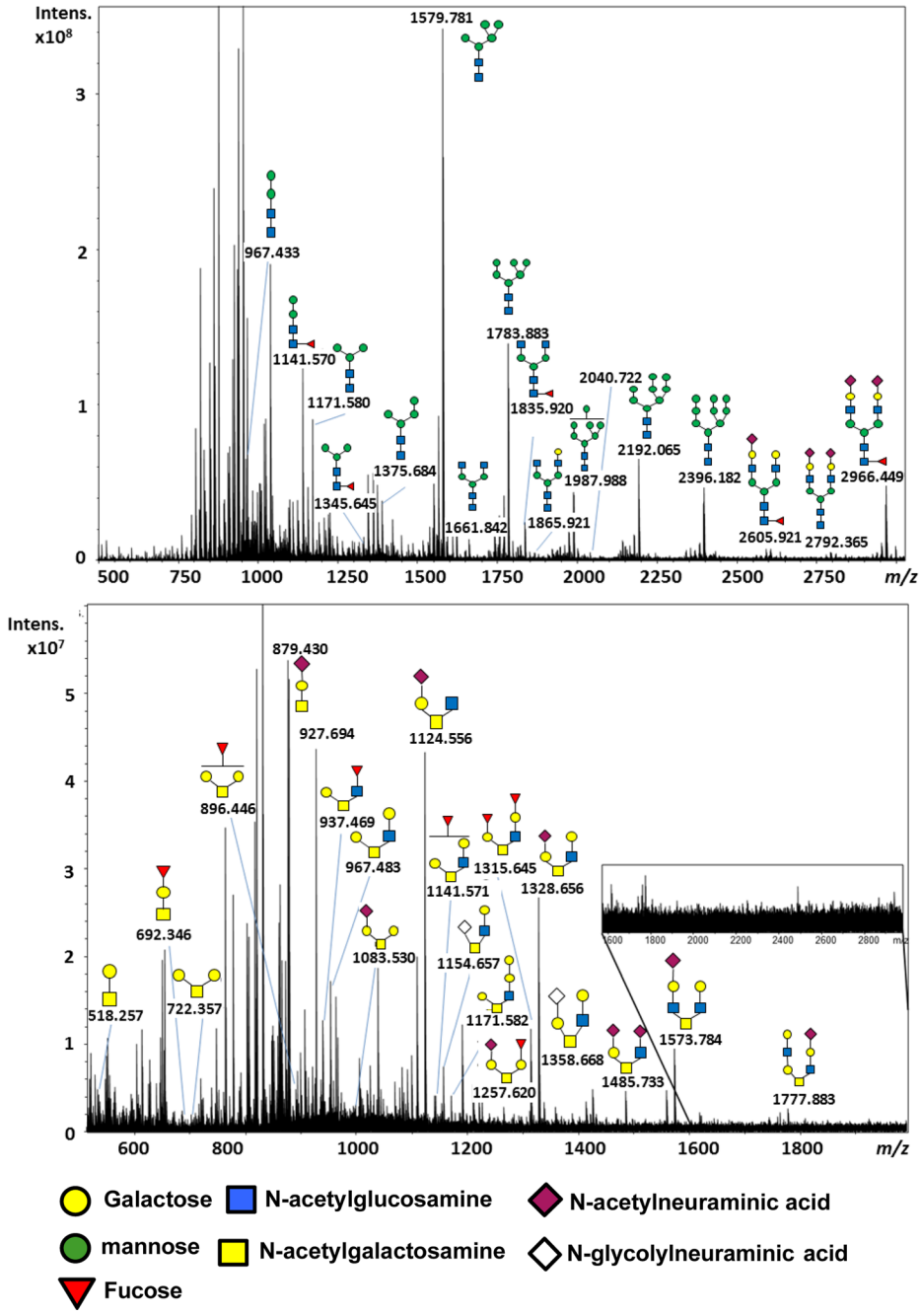

Figure 4. MALDI-FTICR mass spectrum of permethylated glycans released from porcine bladder urothelium, ionized as $[M + Na]^+$: (a) *N*-glycans released before O-glycans and (b) O-glycans released after *N*-glycans in the same filter unit from the same sample. The displayed glycans indicate composition only.

porcine urothelium sample treated with trypsin without prior removal of the glycans. Mascot searching matched 1348 proteins and 5323 peptides, FDR 1% (Supporting Information). Proteins identified were similar across the two sets of data, with 848 proteins the same in the two sets of data. Although this experiment was only carried out once, as a proof of concept, the results suggest that the proteomic analysis is not substantially compromised by prior glycan release.

Application to Study of Cultured Cells. MSCs are adult stem cells that have the ability to differentiate into adipocytes, chondrocytes, and osteoblasts to make fat, cartilage, or bone tissue.[51,52] The ability to differentiate into different cell types means there is a potential use of MSCs in regenerative medicine,[53,54] for use in cell replacement[55] and in tissue engineering.[56] Their ability to differentiate into different lineages also provides an excellent opportunity to study the connections between glycan biosynthesis and cellular differentiation. The potential for genetic manipulation within the immortalised primary lines has also the potential to generate cellular models of CDGs, for example, by introducing COG mutations to observe differences in glycan profiles.

MSC lines used in this study have been immortalized using human telomerase reverse transcriptase, designated hTERT-MSCs,[45] and undergone single cell cloning. This removes heterogeneity seen in primary cell populations for a more reliable comparison of changes in glycosylation across sample

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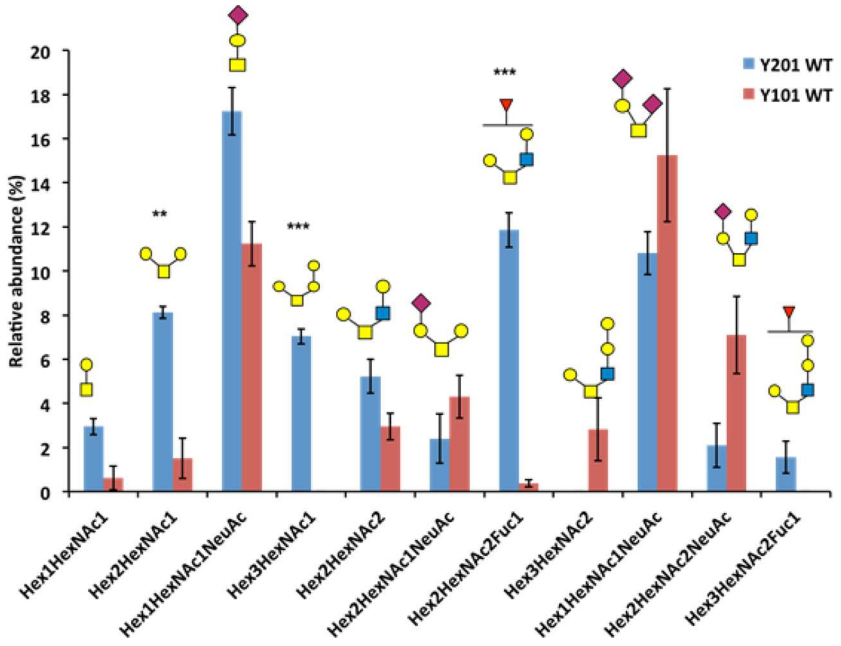

Figure 5. O-glycan profiles of Y201 and Y101 WT MSCs. Relative abundance expressed as a percentage of each glycan signal compared to a spiked internal standard signal. Statistical analysis was carried out using one-way ANOVA with Holm Sidak posthoc test (n = 5) (* for P = <0.05, ** for P = <0.01 and *** for P = <0.0001). Glycan structures intended to indicate composition only.

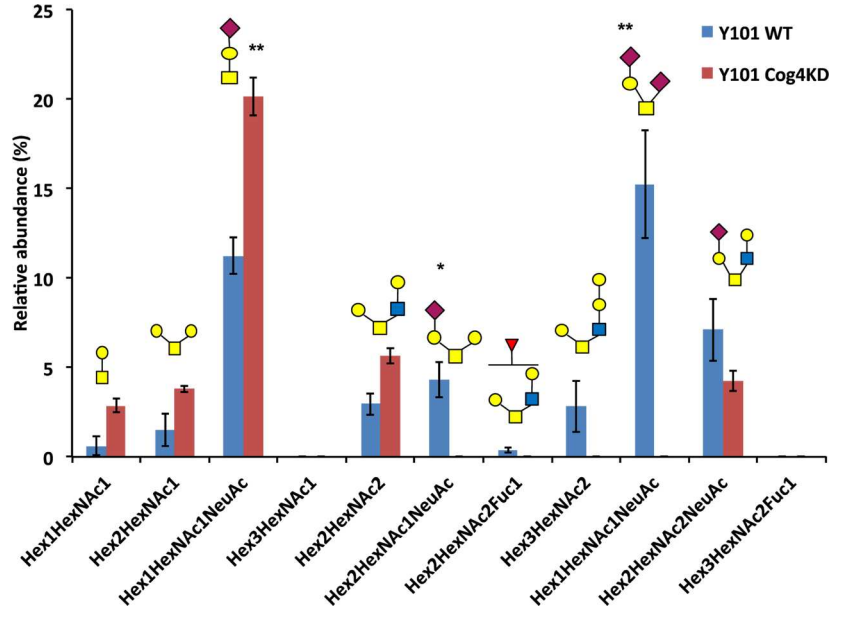

Figure 6. Comparing O-glycan profiles of Y101 WT MSCs with Cog4KD MSCs. Relative abundance calculated by expressing each glycan signal as a percentage of the spiked internal standard signal. Statistical analysis was carried out using a one-way ANOVA with Holm Sidak posthoc test (n = 5) (* for P = <0.05, ** for P = <0.01). Glycan structures intended to indicate composition only.

replicates. Two hTERT-MSC cell lines have been used, Y201 and Y101, to allow comparison of two lines with somewhat different functional properties. These cell lines have different abilities to differentiate; while Y201 cells can form all three MSC-derived lineages, Y101 cells have limited adipogenic capacity but can readily form osteoblasts.

Profiling of *N*-glycans of MSCs has been investigated and reported.[57,33] *N*-glycan profiling of the immortalized cell lines Y201 and Y101 has been reported by Wilson and colleagues.[58] What is currently lacking is analysis of the O-glycan profile of such cells to determine how the O-glycan profile changes after differentiation or genetic modification of these cell lines.

The developed O-glycan release method was applied to wild-type (WT) Y201 and Y101 MSCs to test the procedure with cultured cell samples. Cells were grown in a 10 cm culture dish, before being harvested. O-Glycans were released and permethylated. O-Glycans were identified from their characteristic signals at m/z 518, 722, 879, 967, 1083, 1141, 1171, 1240, and 1328 for the Y101 cell line and at m/z 518, 722, 879, 926, 967, 1083, 1141, 1240, 1328, and 1345, for the Y201 cell line, Figure S-7. The signals were confidently assigned based on their

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mass accuracies, which were below 1 ppm (Table S-3). Samples were spiked with an internal standard, deuteropermethylated maltotetraose, to enable relative quantification of the glycans to see if there was any difference in glycan relative abundances between the two cell lines. On comparing the resulting glycan profiles, three O-glycans were significantly higher in relative abundance in the Y201 than the Y101 cell line. These were $Hex_2HexNAc_1$ (P = 0.0028**), $Hex_3HexNAc_1$ (P = <0.0001***), and $Hex_2HexNAc_2Fuc_1$ (P = <0.0001***). Statistical analysis was carried out using a one-way ANOVA with Holm Sidak posthoc test (n = 5) (* for P = <0.05, ** for P = <0.01, and *** for P = <0.0001), Figure 5. These differences in the abundance of O-glycans could perhaps be involved in the differing potentials of the two cell lines to differentiate. The glycan identified containing three hexose residues (presumably galactose) attached to the GalNAc core is an unusual structure, previously identified[59] on calf thyroid membrane glycoprotein GP-3.

Since the developed O-glycan release method worked well on the wild-type MSC cell lines, Cog4KD MSCs were also analyzed. These cells are derived from the Y101 cell line by knocking down the Cog4 subunit of the COG complex using stable expression of a Cog4 specific shRNA (K. Wilson, P. Genever, and D. Ungar, unpublished data). O-Glycans were released from Cog4KD MSCs to see whether there were any changes to the O-glycan profile when compared with wild-type cells. O-Glycans were identified at m/z 518, 722, 879, 967, and 1328, Figure S-8, confidently assigned based on their mass accuracies, which were all below 1 ppm (Table S-4) and intensities compared again using the approach of spiking with deuteromethylated maltotetraose.

For glycan structures $Hex_2HexNAc_1$ and $Hex_2HexNAc_2$, both presumably bearing a terminal galactose, there is an increase in the relative abundance in the Cog4KD cells, compared with the wild-type cells, Figure 6. This could be attributed to loss of ability to transfer a sialic acid to galactose in $Hex_2HexNAc_2$, shown by an absence of $Hex_2HexNAc_1NeuAc_1$, a significant change when compared with the wild-type cells (P = 0.0962*). However, there is a significant increase in abundance of $Hex_1HexNAc_1NeuAc_1$, (P = 0.0069**). The large increase in abundance of $Hex_1HexNAc_1NeuAc_1$ could be due to the reduced ability to transfer a second sialic acid to the growing glycan chain, shown by the absence of $Hex_1HexNAc_1NeuAc_2$ in the Cog4KD sample, a change compared with the WT sample that is significant (P = 0.0028**). There is also a loss of the fucosylated O-glycan, $Hex_2HexNAc_2Fuc_1$, although this glycan is only relatively low abundance in the WT sample.

A study by Reynders et al. analyzed N-glycans in CDG patient cells with a mutation to Cog4, resulting in defective Cog4 protein, and found there was a decrease in sialylated N-glycans.[60] It has also been reported that in disorders affecting both N- and O-glycans, sialylation is decreased in both glycan types.[8] One study analyzed O-glycans, by MS, from serum and plasma samples of patients with mutations to Cog4 and Cog7 and found decreased sialylation in both. The first described example of an inborn error in metabolism affecting both N- and O-glycosylation reported a decrease in sialylated N- and O-glycans, where the O-glycosylation status was determined by analyzing apoplioprotein C-III (a protein which is only O-glycosylated), using isoelectric focusing.[61] Moreover, N-glycan profiling as well as PNA-lectin staining of the Cog4KD cells suggested a defect in sialylation (K. Wilson, P. Genever, and D. Ungar, unpublished data). PNA binds to terminal Gal-GalNAc, and since binding of this lectin only occurs for unsialylated structures, the increase in binding for PNA could suggest a decrease in sialylated structures. All the evidence described above adds confidence to the results from this O-glycan profiling, using only a small number (~1 million) of cultured cells.

Since the developed one-pot method was successfully used to analyze porcine bladder urothelium, the MSC wild-type and Cog4KD samples, that had previously had the O-glycans removed were subsequently treated with PNGase F to release the N-glycans. Released N-glycans were permethylated and spiked with the internal standard to carry out relative quantification of the N-glycans. N-glycans were identified between m/z 967 and m/z 3054 for the WT MSCs, showing the presence of both oligomannose and complex-type N-glycans. For Cog4KD MSCs, N-glycans were identified between m/z 967 and 2396, also showing the presence of both oligomannose and complex-type N-glycans, Figure S-9.

For both samples, oligomannose type N-glycans appear to be most abundant, but the abundance of these glycans is lower in the Cog4KD sample. There is an increase in abundance of the glycans at lower masses ($HexNAc_2Hex_2$, $HexNAc_2Hex_2Fuc_1$, and $HexNAc_2Hex_3$) for the Cog4KD. Sialylated glycans present in WT MSCs are not detected in the Cog4KD sample. There is also a decrease in abundance of complex-type glycans that contain a terminal hexose (presumably galactose). These results are in agreement with previous results generated by Wilson and colleagues, who carried out N-glycan profiling of Y101 MSCs[58] and Cog4KD MSCs (K. Wilson, P. Genever, and D. Ungar, unpublished data) as well as results described by Reynders et al.,[60] which showed a decrease in sialylated N-glycans in Cog4 CDG patients. Interestingly, the current data suggest that the Cog4KD cells contain fewer N-glycans overall than the WT.

CONCLUSIONS

A simple method has been developed for release and isolation of O-glycans from glycoproteins from whole cell lysates. The O-glycan release can be carried out in 1 h with the aid of sonication, not previously applied to the release of glycoprotein glycans; this offers a significant improvement on traditional incubation times of 16−18 h. In addition, both N- and O-glycans can be studied from one sample, in the same filter unit, by combing the established protocol for filter-aided N-glycan separation, optimized for enhanced PNGase F efficiency, with subsequent O-glycan release (or vice versa) on a realistic number of cultured cells (1×10^6). Furthermore, the method allows subsequent proteomic analysis of the protein remaining after glycan release. By virtue of its applicability to medium throughput studies, our approach offers a simple way to assess the often subtle changes to glycan profiles seen in differentiation and disease progression, in a statistically robust way using biological replicates.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b00143.

Full experimental details and additional experimental data (PDF)

Peptide analysis and MASCOT search results (XLSX)

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Notes

The authors declare no competing financial interest.
Data sets are available to download fom MassIVE (MSV000080973) and ProteomeXchange (PXD004290).

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REFERENCES

(1) Ohyama, C. *Int. J. Clin. Oncol.* **2008**, *13*, 308−313.
(2) Palmigiano, A.; Barone, R.; Sturiale, L.; Sanfilippo, C.; Bua, R. O.; Romeo, D. A.; Messina, A.; Capuana, M. L.; Maci, T.; Le Pira, F.; Zappia, M.; Garozzo, D. *J. Proteomics* **2016**, *131*, 29−37.
(3) Schedin-Weiss, S.; Winblad, B.; Tjernberg, L. O. *FEBS J.* **2014**, *281*, 46−62.
(4) Gornik, O.; Lauc, G. *Dis. Markers* **2008**, *25*, 267−278.
(5) Ferreira, V.; Briones, P.; Vilaseca, M. *Carbohydr. Chem.* **2012**, *38*, 124−155.
(6) Freeze, H. H.; Chong, J. X.; Bamshad, M. J.; Ng, B. G. *Am. J. Hum. Genet.* **2014**, *94*, 161−175.
(7) Jaeken, J. *J. Inherited Metab. Dis.* **2011**, *34*, 853−858.
(8) Leroy, J. G. *Pediatr. Res.* **2006**, *60*, 643−656.
(9) Hudak, J. E.; Bertozzi, C. R. *Chem. Biol.* **2014**, *21*, 16−37.
(10) Beck, A.; Wurch, T.; Bailly, C.; Corvaia, N. *Nat. Rev. Immunol.* **2010**, *10*, 345−352.
(11) Astronomo, R. D.; Burton, D. R. *Nat. Rev. Drug Discovery* **2010**, *9*, 308−324.
(12) Huang, Y.-L.; Hung, J.-T.; Cheung, S. K. C.; Lee, H.-Y.; Chu, K.-C.; Li, S.-T.; Lin, Y.-C.; Ren, C.-T.; Cheng, T.-J. R.; Hsu, T.-L.; Yu, A. L.; Wu, C.-Y.; Wong, C.-H. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 2517−22.
(13) Seeberger, P. H.; Werz, D. B. *Nature* **2007**, *446*, 1046−1051.
(14) Song, C.; Sun, S.; Huo, C.-X.; Li, Q.; Zheng, X.-J.; Tai, G.; Zhou, Y.; Ye, X.-S. *Bioorg. Med. Chem.* **2016**, *24*, 915−920.
(15) Raju, T. S. *Curr. Opin. Immunol.* **2008**, *20*, 471−478.
(16) Jefferis, R. *Arch. Biochem. Biophys.* **2012**, *526*, 159−166.
(17) Kanda, Y.; Yamada, T.; Mori, K.; Okazaki, A.; Inoue, M.; Kitajima-Miyama, K.; Kuni-Kamochi, R.; Nakano, R.; Yano, K.; Kakita, S.; Shitara, K.; Satoh, M. *Glycobiology* **2007**, *17*, 104−118.
(18) Fang, J.; Richardson, J.; Du, Z.; Zhang, Z. *Biochemistry* **2016**, *55*, 860−868.
(19) Mimura, Y.; Church, S.; Ghirlando, R.; Ashton, P. R.; Dong, S.; Goodall, M.; Lund, J.; Jefferis, R. *Mol. Immunol.* **2000**, *37*, 697−706.
(20) Goetze, A. M.; Liu, Y. D.; Zhang, Z.; Shah, B.; Lee, E.; Bondarenko, P. V.; Flynn, G. C. *Glycobiology* **2011**, *21*, 949−959.
(21) Alessandri, L.; Ouellette, D.; Acquah, A.; Rieser, M.; LeBlond, D.; Saltarelli, M.; Radziejewski, C.; Fujimori, T.; Correia, I. *MAbs* **2012**, *4*, 509−520.
(22) Tarp, M. A.; Clausen, H. *Biochim. Biophys. Acta, Gen. Subj.* **2008**, *1780*, 546−563.
(23) North, S. J.; Huang, H.-H.; Sundaram, S.; Jang-Lee, J.; Etienne, a T.; Trollope, A.; Chalabi, S.; Dell, A.; Stanley, P.; Haslam, S. M. *J. Biol. Chem.* **2010**, *285*, 5759−5775.
(24) Bateman, A. C.; Karamanska, R.; Busch, M. G.; Dell, A.; Olsen, C. W.; Haslam, S. M. *J. Biol. Chem.* **2010**, *285*, 34016−34026.
(25) Kornfeld, R.; Kornfeld, S. *Annu. Rev. Biochem.* **1985**, *54*, 631−664.
(26) Sørensen, T.; White, T.; Wandall, H. H.; Kristensen, A. K.; Roepstorff, P.; Clausen, H. *J. Biol. Chem.* **1995**, *270*, 24166−24173.
(27) Tran, D. T.; Ten Hagen, K. G. *J. Biol. Chem.* **2013**, *288*, 6921−6929.
(28) Kingsley, D. M.; Kozarsky, K. F.; Segal, M.; Krieger, M. *J. Cell Biol.* **1986**, *102*, 1576−1585.
(29) Miller, V. J.; Sharma, P.; Kudlyk, T. A.; Frost, L.; Rofe, A. P.; Watson, I. J.; Duden, R.; Lowe, M.; Lupashin, V. V.; Ungar, D. *J. Biol. Chem.* **2013**, *288*, 4229−4240.
(30) Miller, V. J.; Ungar, D. *Traffic* **2012**, *13*, 891−897.
(31) Fisher, P.; Ungar, D. *Front. Cell Dev. Biol.* **2016**, *4*, 15.
(32) Kudelka, M. R.; Antonopoulos, A.; Wang, Y.; Duong, D. M.; Song, X.; Seyfried, N. T.; Dell, A.; Haslam, S. M.; Cummings, R. D.; Ju, T. *Nat. Methods* **2016**, *13*, 81−86.
(33) Heiskanen, A.; Hirvonen, T.; Salo, H.; Impola, U.; Olonen, A.; Laitinen, A.; Tiitinen, S.; Natunen, S.; Aitio, O.; Miller-Podraza, H.; Wuhrer, M.; Deelder, A. M.; Natunen, J.; Laine, J.; Lehenkari, P.; Saarinen, J.; Satomaa, T.; Valmu, L. *Glycoconjugate J.* **2009**, *26*, 367−84.
(34) Rahman, S. A.; Bergstrom, E.; Watson, C. J.; Wilson, K. M.; Ashford, D.; Thomas, J. R.; Ungar, D.; Thomas-Oates, J. E. *J. Proteome Res.* **2014**, *13*, 1167−1176.
(35) Manza, L. L.; Stamer, S. L.; Ham, A.-J. L.; Codreanu, S. G.; Liebler, D. C. *Proteomics* **2005**, *5*, 1742−1745.
(36) Wiśniewski, J.; Zougman, A.; Nagaraj, N.; Mann, M. *Nat. Methods* **2009**, *6*, 359−363.
(37) Carlson, D. M. *J. Biol. Chem.* **1968**, *243*, 616−626.
(38) Rademaker, G. J.; Pergantis, S. A.; Blok-tip, L.; Langridge, J. I.; Kleen, A.; Thomas-oates, J. E. *Anal. Biochem.* **1998**, *257*, 149−160.
(39) Zhou, H.; Briscoe, A. C.; Froehlich, J. W.; Lee, R. S. *Anal. Biochem.* **2012**, *427*, 33−35.
(40) Tzeng, Y. K.; Chang, C. C.; Huang, C. N.; Wu, C. C.; Han, C. C.; Chang, H. C. *Anal. Chem.* **2008**, *80*, 6809−6814.
(41) Maniatis, S.; Zhou, H.; Reinhold, V. *Anal. Chem.* **2010**, *82*, 2421−2425.
(42) Furukawa, J.; Piao, J.; Yoshida, Y.; Okada, K.; Yokota, I.; Higashino, K.; Sakairi, N.; Shinohara, Y. *Anal. Chem.* **2015**, *87*, 7524−7528.
(43) Mason, T. *Chem. Soc. Rev.* **1997**, *26*, 443−451.
(44) Erde, J.; Loo, R.; Loo, J. *J. Proteome Res.* **2014**, *13*, 1885−1895.
(45) James, S.; Fox, J.; Afsari, F.; Lee, J.; Clough, S.; Knight, C.; Ashmore, J.; Ashton, P.; Preham, O.; Hoogduijn, M.; Ponzoni, R. D. A. R.; Hancock, Y.; Coles, M.; Genever, P. *Stem Cell Rep.* **2015**, *4*, 1004−1015.
(46) Rademaker, G. J.; Haverkamp, J.; Thomas-Oates, J. *Org. Mass Spectrom.* **1993**, *28*, 1536−1541.
(47) Wada, Y.; Azadi, P.; Costello, C. E.; Dell, A.; Dwek, R. A.; Geyer, H.; Geyer, R.; Kakehi, K.; Karlsson, N. G.; Kato, K.; Kawasaki, N.; Khoo, K.-H.; Kim, S.; Kondo, A.; Lattova, E.; Mechref, Y.; Miyoshi, E.; Nakamura, K.; Narimatsu, H.; Novotny, M. V.; Packer, N. H.; Perreault, H.; Peter-Katalinic, J.; Pohlentz, G.; Reinhold, V. N.; Rudd, P. M.; Suzuki, A.; Taniguchi, N. *Glycobiology* **2007**, *17*, 411−422.
(48) Wang, C.; Fan, W.; Zhang, P.; Wang, Z.; Huang, L. *Proteomics* **2011**, *11*, 4229−4242.

DOI: 10.1021/acs.analchem.7b00143
Anal. Chem. XXXX, XXX, XXX−XXX

(49) Karlsson, N. G.; Nordman, H.; Karlsson, H.; Carlstedt, I.; Hansson, G. C. *Biochem. J.* **1997**, *326*, 911−917.
(50) Wang, C.; Zhang, P.; Jin, W.; Li, L.; Qiang, S.; Zhang, Y.; Huang, L.; Wang, Z. *J. Proteomics* **2017**, *150*, 18−30.
(51) Prockop, D. J. *Science (Washington, DC, U. S.)* **1997**, *276*, 71−74.
(52) Pittenger, M. F.; Mackay, A. M.; Beck, S.; Jaiswal, R. K.; Douglas, R.; Mosca, J. D.; Moorman, M. a.; Simonetti, D. W.; Craig, S.; Marshak, D. *Science (Washington, DC, U. S.)* **1999**, *284*, 143−147.
(53) Uccelli, A.; Moretta, L.; Pistoia, V. *Nat. Rev. Immunol.* **2008**, *8*, 726−736.
(54) Barry, F. P.; Murphy, J. M. *Int. J. Biochem. Cell Biol.* **2004**, *36*, 568−584.
(55) Sueblinvong, V.; Suratt, B. T.; Weiss, D. J. *Clin. Chest Med.* **2007**, *28*, 361−379.
(56) Jaipaew, J.; Wangkulangkul, P.; Meesane, J.; Raungrut, P.; Puttawibul, P. *Mater. Sci. Eng., C* **2016**, *64*, 173−182.
(57) Hamouda, H.; Ullah, M.; Berger, M.; Sittinger, M.; Tauber, R.; Ringe, J.; Blanchard, V. *Stem Cells Dev.* **2013**, *22*, 3100−13.
(58) Wilson, K. M.; Thomas-Oates, J. E.; Genever, P. G.; Ungar, D. *Front. Cell Dev. Biol.* **2016**, *4*, 52.
(59) Edge, A. S. B.; Spiro, R. G. *Arch. Biochem. Biophys.* **1997**, *343*, 73−80.
(60) Reynders, E.; Foulquier, F.; Teles, E. L.; Quelhas, D.; Morelle, W.; Rabouille, C.; Annaert, W.; Matthijs, G. *Hum. Mol. Genet.* **2009**, *18*, 3244−3256.
(61) Wopereis, S.; Grunewald, S.; Morava, E.; Penzien, J. M.; Briones, P.; Garcia-Silva, M. T.; Demacker, P. N. M.; Huijben, K. M. L. C.; Wevers, R. A. *Clin. Chem.* **2003**, *49*, 1839−1845.

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