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Screening of Glycosylation Patterns in Serum Using Natural Glycoprotein Microarrays and Multi-Lectin Fluorescence Detection

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Protein glycosylation has been implicated in key biological processes including immunological recognition, cellular adhesion, protein folding, and signaling as well as disease progression. Although several methods are available to assess glycosylation of protein structures, none of them is able to screen complex biological samples at a global as well as an individual scale. A novel strategy presented here uses an all-liquid phase enrichment and prefractionation methodology coupled to glycoprotein microarray technology using a multiple lectin-based, biotin−streptavidin detection scheme. Selective detection of glycan structures was made possible by employing multiple lectins to screen glycoprotein standards as well as serum samples from normal subjects or patients with chronic pancreatitis or pancreatic cancer. Interestingly, in some instances, a greater degree of glycosylation was seen in proteins that were underexpressed based on the reversed-phase chromatogram alone. Studies with standard proteins established the limits of detection to be in the 2.5−5-fmol range. Studies on serum samples showed differences in glycosylation patterns, particularly with respect to sialylation, mannosylation, and fucosylation, in normal, pancreatitis, and cancer sera. By coupling glycoprotein enrichment and fractionation with a microarray platform, we have shown that naturally occurring glycoproteins from human serum can be screened and characterized for different glycan structures, thereby allowing one to do comparative studies that monitor individual glycosylation changes within a glycoproteome representing different biological states. This approach may be useful to identify potential biomarkers in cancer.

Glycoproteins are the most heterogeneous group of modifications known in proteins. Glycans show a high structural diversity reflecting inherent functional diversity. N- and O-oligosaccharide variants on glycoproteins (glycoforms) can lead to alterations in protein activity or function that may manifest itself as overt disease.[1,2] Many clinical biomarkers and therapeutic targets in cancer are glycoproteins,[3−5] such as CA125 in ovarian cancer, Her2/neu in breast cancer, and prostate-specific antigen (PSA) in prostate cancer. The human epidermal growth factor receptor 2 (Her2/neu) is a transmembrane glycoprotein where the presence of Her2 overexpression appears to be a key factor in malignant transformation and is predictive of a poor prognosis in breast cancer. CA125 is a mucin commonly employed as a diagnostic marker for epithelial ovarian cancer. Although CA 125 has been used as an ovarian cancer marker for a long time, many of its O- and N-glycan structures have only recently been characterized.[6] PSA is secreted primarily by prostate epithelial cells into the seminal plasma. It is one of the best characterized examples of a secreted glycoprotein used in cancer diagnostics, and its glycoforms have been described.[7]

The alteration in protein glycosylation that occurs through varying the heterogeneity of glycosylation sites or changing glycan structure of proteins on the cell surface and in body fluids has been shown to correlate with the development or progression of cancer and other disease states.[8] It has been reported that the glycosylation of PSA secreted by the tumor prostate cell line LNCaP differs significantly from that of PSA from seminal plasma (normal control). These carbohydrate differences allow a distinction to be made between PSA from normal and tumor origins and suggest a valuable biochemical tool for diagnosis.[9] Characterization of the N-glycans from human pancreatic ribonuclease (RNase 1) isolated from healthy pancreas and from pancreatic adenocar-

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cinoma tumor cells (Capan-1 and MDAPanc-3) revealed completely different glycosylation patterns.[10] These glycosylation changes in a tumor-secreted protein reflect fundamental changes in the enzymes involved in the glycosylation pathway. Therefore, a method for systematic and quantitative analysis of glycoproteins would be very useful for the detection of new potential therapeutic targets.[2]

There are ~100 human glycan-binding proteins i.e., lectins according to genomic analysis.[11] In addition, the variety of lectin protein folds suggests that there may be additional lectin groups not yet discovered.[11,12] A high-throughput technique that can assess a diverse range of glycosylation states would facilitate research in this area. Furthermore, global screening of glycoprotein profiles in varied biological states can also potentially provide valuable information regarding key pathways that make that state unique.[13,14] Protein microarrays have proven to be useful as a high-throughput screening method for whole-cell lysates, fractionated proteomes, tissues, and antigen−antibody reactions.[15−18] Protein microarrays have evolved from earlier work with membranes which showed that it is possible to detect antibodies by depositing them on membrane surfaces and subsequently detecting them by some form of incubation of the membrane with antigens. These studies also showed that protein deposition by noncontact means is more reproducible than contact printing mechanisms.[19,20]

Increased interest in glycoproteomes has also sparked related research in the microarray field. A majority of current efforts have focused on carbohydrate microarrays.[11,14,21] In this approach, various glycan-type structures are arrayed on a range of surface chemistries such as nitrocellulose, glass, and dextran-type surfaces after which they are screened in parallel for binding. Such studies are critical in assessing antibody specificity to glycans and determining currently uncharacterized glycosylation structures that elicit responses in cells.[11,14,22] However, oligosaccharides are difficult to synthesize due to varied stereochemistries, limited availability of enzymes for alternate synthesis strategies, and problems with purification when isolating naturally occurring oligosaccharides. Furthermore, the low mass and hydrophilic nature of most oligosaccharides make noncovalent immobilization difficult for some glycans.[14] This problem has been overcome by successful covalent attachment of glycans to solid surfaces using film-coated photoactivable surfaces[23] and array coupling via flexible linker molecules.[24] Although carbohydrate arrays provide valuable information about carbohydrate-interacting proteins, they do not allow us to directly study changes in glycosylation in real biological systems.

Current technologies for glycan analysis such as mass spectrometry,[25] lectin affinity chromatography,[26,27] and western blotting are time-consuming and some, such as mass spectrometry, require expertise and are technically difficult.[28] Lectin arrays can be used for rapid profiling of glycan expression patterns of various glycoproteins. Current studies using lectin arrays have focused on assessing specificity of arrayed lectins[29,30] as well as changes in lectin binding of whole-cell *Escherichia coli* lysates that have undergone a treatment with sialyllactose to see changes in bacterial adhesion to cells.[23] Data from lectin arrays can be useful in determining the most appropriate lectins for glycoprotein enrichment as well as removal of undesirable glycoproteins. However, the lectin array platform does not allow one to screen whole glycoproteomes in a way that can enable one to study both changes in overall glycoprotein patterns and changes in an individual protein's glycan expression within that glycoproteome.

A novel strategy presented here involves modifying the lectin array approach, making it more useful as a method that can study the total as well as individual glycoprotein profiles of naturally produced glycoproteins. The strategy employs a liquid fractionated protein microarray approach to screen all glycoproteins in a complex sample on a single array. Glycoproteins are first enriched on a general lectin column and then separated by reversed-phase high-performance liquid chromatography (HPLC). The separated proteins are then arrayed on nitrocellulose slides and probed with lectins with a wide range of binding specificities. The glycoprotein−lectin interaction is assessed using a biotin−streptavidin system. As an example, we demonstrate the potential utility of this approach to identify serum biomarkers in pancreatic diseases. This method provides the ability to profile the distribution of glycans in the human glycoproteome and also to study the changes in glycan expression on a global scale and on individual glycoproteins since each glycoprotein sample is a unique spot on the array.

METHOD

Standard Preparation. Fetuin from fetal calf serum, asialofetuin from fetal calf serum, porcine thyroglobulin, bovine ribonuclease B, α-acid glycoprotein, and human transferrin were purchased from Sigma. A stock solution of 20 mg/mL was made by dissolving standards in deionized water. A dilution series was made for each of the standard glycoproteins with the following

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final concentrations: 2, 1.6, 1.2, 1, 0.8, 0.6, 0.5, 0.4, 0.2, 0.1, 0.05, and 0.025 mg/mL. The dilutions were made directly into printing buffer (composition described in Glycoprotein Microarray) to avoid drying and reconstitution in order to minimize sample loss.

Serum Samples. Serum was obtained at the time of diagnosis following informed consent using IRB-approved guidelines. Human normal serum was collected at The University of Michigan under the auspices of the Early Detection Network (EDRN). Pancreatitis serum was obtained from patients with chronic pancreatitis who were seen in the Gastroenterology Clinic at The University of Michigan Medical Center. Pancreatic cancer serum was obtained from patients with a confirmed diagnosis of pancreatic adenocarcinoma who were seen in the Multidisciplinary Pancreatic Tumor Clinic at the The University of Michigan Comprehensive Cancer Center. A 40-cm^3 sample of blood was provided by each patient. The samples were permitted to sit at room temperature for a minimum of 30 min (and a maximum of 60 min) to allow the clot to form in the red top tubes and were then centrifuged at 1300*g* at 4 °C for 20 min. The serum was removed, transferred to a polypropylene capped tube, and frozen. The frozen samples were stored at −70 °C until assayed. All serum samples were labeled with a unique identifier to protect confidentiality of the patient. None of the samples were thawed more than twice before analysis. Samples were matched for age and sex to remove this variable from the analysis.

Lectin Affinity Glycoprotein Extraction. An agarose-bound lectin, wheat germ agglutinin (WGA) was purchased from Vector Laboratories (Burlingame, CA). Agarose-bound WGA was packed into a disposable screw end-cap spin column with filters at both ends. The column was first washed with 500 μL of binding buffer (20 mM Tris, 0.15 M NaCl, pH 7.4) by centrifuging the spin column at 500 rpm for 2 min. Protease inhibitor stock solution was prepared by dissolving one complete EDTA-free protease inhibitor cocktail tablet (Roche, Indianapolis, IN) in 1 mL of H_2O. The stock solution was added to binding buffer and elution buffer at a ratio of (v/v) 1:50. A 50-μL serum sample diluted with 500 μL of binding buffer was loaded onto the column and incubated for 15 min. The column was centrifuged for 2 min at 500 rpm to remove the nonbinding fraction. The column was washed with 600 μL of binding buffer twice to wash off the nonspecific binding. The captured glycoproteins were released with 150 μL of elution buffer (0.5 M *N*-acetylglucosamine in 20 mM Tris and 0.5 M NaCl, pH 7.0), and the eluted fraction was collected by centrifugation at 500 rpm for 2 min. This step was repeated twice, and the eluted fractions were pooled.

RP-HPLC Separation of Lectin-Bound Glycoproteins. The enriched glycoprotein fraction was loaded onto a nonporous silica reversed-phase (NPS-RP) HPLC column for separation. High separation efficiency was achieved by using an ODSIII-E (4.6 × 33 mm) column (Eprogen, Inc., Darien, IL) packed with 1.5-μm nonporous silica. To collect purified proteins from NPS-RP-HPLC, the reversed-phase separation was performed at 0.5 mL/min and monitored at 214 nm using a Beckman 166 model UV detector (Beckman-Coulter). Proteins eluting from the column were collected by an automated fraction collector (model SC 100; Beckman-Coulter), controlled by an in-house-designed DOS-based software program. To enhance the speed, resolution, and reproducibility of the separation, the reversed-phase column was heated to 60 °C by a column heater (Jones Chromatography, model 7971). Both mobile phases A (water) and B (ACN) contained 0.1% v/v TFA. The gradient profile used was as follows: 5−15% B in 1 min, 15−25% B in 2 min, 25−30% B in 3 min, 30−41% B in 15 min, 41−47% B in 4 min, 47−67% B in 5 min, and 67−100% B in 2 min. Deionized water was purified using a Millipore RG system (Bedford, MA).

Glycoprotein Microarray. Purified and separated glycoproteins, or glycoprotein standards (from Standard Preparation), were printed on nitrocellulose slides (Whatman Schleicher & Schuell BioScience, Keene, NH) using a noncontact printer, Nanoplotter 2.0 (GeSiM). Prior to printing, the proteins were dried down in a 96-well plate and resuspended in 15 μL of printing buffer with stirring overnight at 4 °C. The printing buffer contained 65 mM Tris-HCl, 1% SDS, 5% dithiothreitol (DTT), and 1% glycerol. Each spotting event resulted in ∼500 pL of sample being deposited by a piezoelectric mechanism. The event was programmed to occur 5 times/spot to ensure that ∼2.5 nL was being spotted per sample. Each sample was further spotted as nine replicates. The resulting spots were ∼450 μm in diameter, and the spacing between spots was maintained at 600 μm. After printing, the slides were allowed to dry for 24 h. Blocking was achieved by incubation with 1% bovine serum albumin (BSA) and 0.1% Tween-20 in 1× phosphate-buffered saline (PBS) overnight. Blocked slides were probed with biotinylated lectin in a solution of PBS-T (0.1% Tween 20 in 1× PBS). The lectins used in the study were biotinylated peanut agglutinin (PNA), *Sambucus nigra* bark lectin (SNA), *Aleuria aurentia* (AAL), concanavalin A (ConA), and *Maackia amurensis* lectin II (MAL), all purchased from Vector Laboratories (Burlingame, CA). The working concentration of all lectins used was 5 μg/mL except for SNA, which was used at 10 μg/mL as per vendor recommendation. After primary incubation, all slides were washed with PBS-T 5 times for 5 min each. Secondary incubation was achieved with a streptavidin−AlexaFluor555 conjugate (Invitrogen, Carlsbad, CA) in a working concentration of 1 μg/mL containing 0.5% BSA, 0.1% Tween-20 in 1× PBS. After secondary incubation, the slides were washed 5 times for 5 min each in PBS-T and completely dried using a high-speed centrifuge (Thermo Electron Corp., Milford, MA). The dried slides were scanned using an Axon 4000A scanner in the green channel, and GenePix Pro 6.0 software (Molecular Devices, Sunnyvale, CA) was used for data acquisition and analysis.

Protein Digestion by Trypsin. Fractions obtained from NPS-RP-HPLC were concentrated down to ∼20 μL using a SpeedVac concentrator (Thermo) operating at 45 °C. A 20-μL sample of 100 mM ammonium bicarbonate (Sigma) was then mixed with each concentrated sample to obtain a pH value of ∼7.8. A 0.5-μL sample of TPCK-modified sequencing grade porcine trypsin (Promega, Madison, WI) was added and vortexed prior to a 12−16-h incubation at 37 °C on an agitator.

Glycan Cleavage by PNGase F and Glycan Purification. For glycan cleavage and purification, glycoproteins were dried down completely and redissolved in 40 μL of 0.1% (w/v) RapiGest solution (Waters, Milford, MA) prepared in 50 mM NH_4HCO_3 buffer, pH 7.9, to denature the protein. Protein samples were reduced with 5 mM DTT for 45 min at 56 °C and alkylated with 15 mM iodoacetamide in the dark for 1 h at room temperature. A 2-μL sample of the enzyme PNGase F (QA-Bio, Palm Desert, CA)

was added to the samples, and the solutions were incubated for 14 h at 37 °C. The glycans released were purified using SPE microelution plates (Waters) packed with HILIC sorbent (5 mg). The microelution SPE device was operated using a centrifuge with a plate adaptor (Thermo). Protein and detergent were removed during this step. Glycans were further cleaned by a graphitized carbon cartridge (Alltech, DeerWeld, IL) to remove salt. A solution of 25% ACN with 0.05% TFA was used to elute the carbohydrates.

Mass Spectrometry: Protein identification by LC−MS/MS. Digested peptide mixtures from NPS-RP-HPLC were separated by a capillary RP column (C18, 0.3 × 150 mm) (Michrom Biosciences, Auburn, CA) on a Paradigm MG4 micropump (Michrom Biosciences) with a flow rate of 5 μL/min. The gradient, started at 5% ACN, was ramped to 60% ACN in 25 min and finally ramped to 90% in another 5 min. Both solvents A (water) and B (ACN) contain 0.1% formic acid. The resolved peptides were analyzed on an LTQ mass spectrometer with an ESI ion source (Thermo, San Jose, CA). The capillary temperature was set at 175 °C, spray voltage was 4.2 kV, and capillary voltage was 30 V. The normalized collision energy was set at 35% for MS/MS. MS/MS spectra were searched using the SEQUEST algorithm incorporated in Bioworks software (Thermo) against the Swiss-Prot human protein database. One miscleavage is allowed during the database search. Protein identification was considered positive for a peptide with X_{corr} of greater than or equal to 3.0 for triply, 2.5 for doubly, and 1.9 for singly charged ions.

Glycan Structure Analysis. MS and MS^2 spectra of glycan samples were acquired on a Shimadzu Axima QIT MALDI quadrupole ion trap-TOF (MALDI-QIT; Manchester, UK). Acquisition and data processing were controlled by Launch-pad software (Karatos, Manchester, UK). A pulsed N_2 laser light (337 nm) with a pulse rate of 5 Hz was used for ionization. Each profile resulted from two laser shots. Argon was used as the collision gas for CID, and helium was used for cooling the trapped ions. The TOF was externally calibrated using 500 fmol/μL bradykinin fragment 1−7 (757.40 m/z), angiotensin II (1046.54 m/z), P14R (1533.86 m/z), and ACTH (2465.20 m/z) (Sigma). 2,5-Dihydroxybenzonic acid (25 mg/mL; LaserBio Labs) was prepared in 50% ACN with 0.1% TFA. A 0.5-μL glycan sample was spotted on the stainless steel target, and 0.5 μL of matrix solution was added followed by air-drying.

SDS−PAGE. The fractions collected from RP-HPLC were further separated by SDS−PAGE according to Laemmli,[31] run in a Mini-Protean cell (Bio-Rad, Hercules, CA) at 80 V controlled by Power Pac3000 (Bio-Rad). The proteins were visualized by staining with Sypro-ruby fluorescence dye (Molecular Probes, Carlsbad, CA). The staining was performed according to the protocol suggested by the manufacturer.

RESULTS AND DISCUSSION

Glycoprotein Microarray Strategy. The methodology, presented here and illustrated in Figure 1, is a potential approach that can be used to study differences in glycans expressed on unique glycoproteins in complex biological samples. Following the strategy, serum is first purified and enriched for glycoproteins using a general lectin column. The enriched glycoproteins are further separated on a reversed-phase HPLC column. The fractionated glycoproteins are then arrayed on nitrocellulose slides as unique protein spots after which they are screened for different glycan structures using five different lectins. The lectins are conjugated to biotin. Streptavidin conjugated to a fluorophore (Alexafluor555) is used for secondary detection. The interaction between the biotin and streptavidin is therefore indirectly used to visualize the interaction between glycoprotein and lectin. This sandwich assay approach also minimizes nonspecific interactions thereby increasing signal-to-noise ratio. Differential glycosylation patterns are subsequently observed using image analysis sofware.

Standard Glycoprotein Microarrays. To determine the feasibility of using a glycoprotein microarray to study separated prepurified glycoproteins, initial studies were done using standards with known glycan structures in order to assess the specificities of the lectins used and the quality of the processed arrays, as well as to determine the range in which a linear response was observed for the concerned standard proteins.

Five standard glycoproteins were used to assess the feasibility of a glycoprotein microarray strategy. A dilution series of each glycoprotein was made using concentrations ranging from a blank with no sample to 2 mg/mL. Each dilution was printed in nine replicates to assess the variability of spots from the same sample during a print run. Each sample spot on the array was achieved by depositing five droplets of ∼500 pL each, resulting in a total volume of 2.5 nL/spot by a piezoelectric mechanism. Consequently, the standards spotted ranged from 0 to 5 ng.

Table 1 describes the binding specificities of the biotinylated lectins used for glycan detection. Five separate lectins were used for the analysis. ConA recognizes α-linked mannose including high-mannose-type and mannose core structures. Both MAL and SNA recognize sialic acid on the terminal branches. While SNA binds preferentially to sialic acid attached to terminal galactose in an (α-2,6) and, to a lesser degree, an (α-2,3) linkage,[32] MAL detects glycans containing NeuAc-Gal-GlcNac with sialic acid at the 3 position of galactose.[33] In contrast, PNA binds desialylated exposed galactosyl (β-1,3) *N*-acetylgalactosamine. In fact, sialic acid in proximity to the PNA receptor sequence will inhibit its binding. AAL recognizes fucose linked (α-1,6) to *N*-acetylglucosamine or (α-1,3) to *N*-acetyllactosamine. Use of the combination of these five lectins should be highly successful in covering a majority of N-glycan types reported and differentiating them according to their specific structures.

(1) Lectin Specificity Studies. The specificity of purchased lectins was assessed to ensure that they did not bind nonspecifically. Five standard glycoproteins were used for this study, fetuin, asialofetuin, thyroglobulin, ribonuclease B, and transferrin. The printed glycoprotein standards were incubated with biotinylated lectins for binding. The bound biotinylated lectins were subsequently detected with streptavidin conjugated to AlexaFluor555. This sandwich-type detection scheme was employed because the very specific biotin−streptavidin interaction should improve signal-to-noise ratio significantly. Figure 2 shows the images obtained when slides were probed with each of the lectins. Background fluorescence was at a minimum with the processing conditions used. Data illustrated in Figure 3a support previously reported glycan structures corresponding to the glycoproteins used in this

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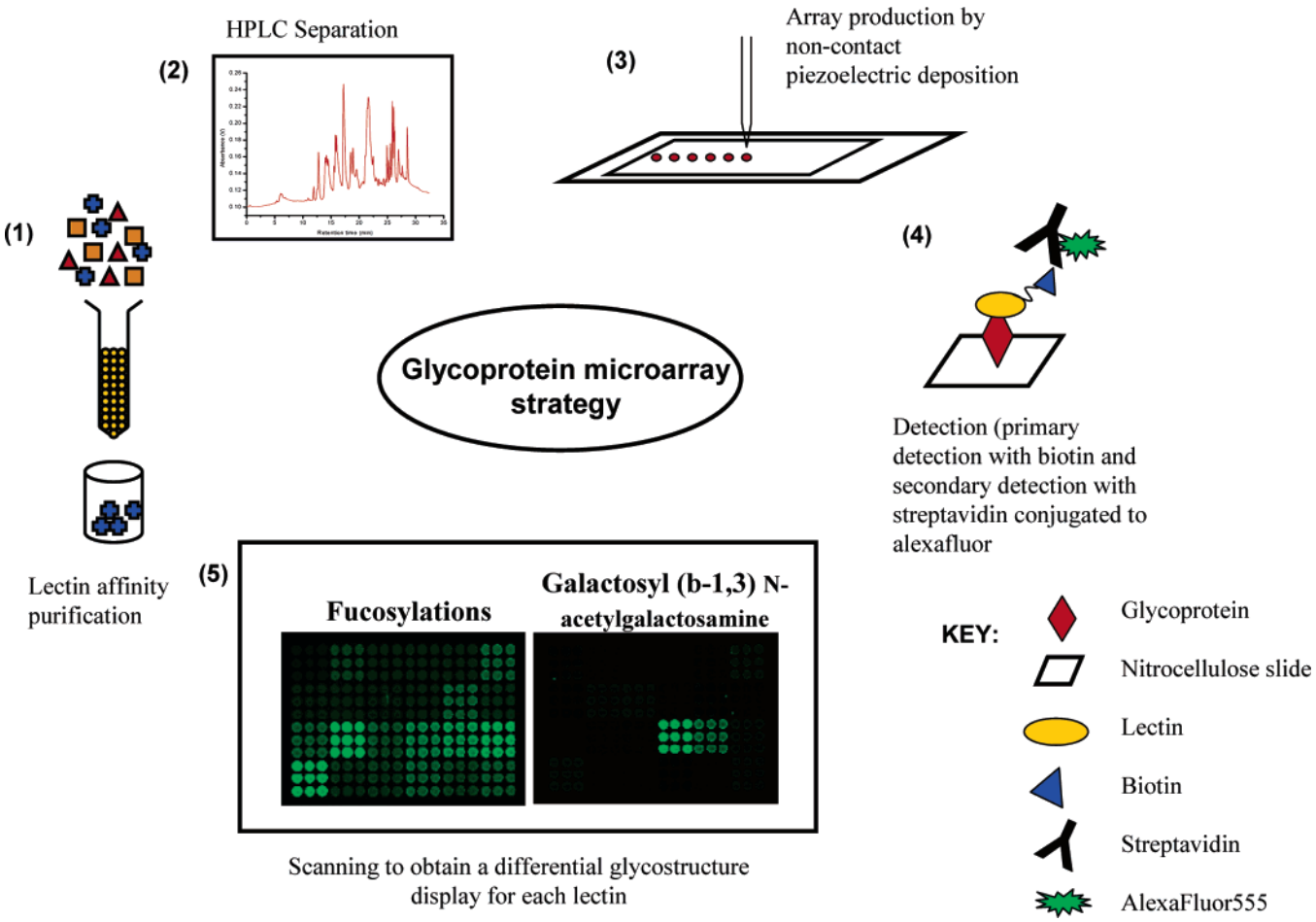

Figure 1. Proposed experimental strategy for studying serum glycoproteins. (1) Lectin purification using a general lectin column. (2) Nonporous reversed-phase HPLC separation and fraction collection. (3) Microarray production using a noncontact piezoelectric printing device. (4) Glycan detection using biotinylated lectin−streptavidin−Alexafluor555. (5) Image acquisition and spot analysis using Genepix 6.0 software.

Table 1. Biotinylated Lectins Used for Glycan Detection and Their Specificities

biotinylated lectin	glycan structure detected
concanavilin A (ConA)	α-linked mannose
Maackia amurensis II (MAL)	sialic acid in an (α-2,3) linkage
Aleuria aurantia (AAL)	fucose linked (α-1,6) to *N*-acetylglucosamine or to fucose linked (α −1,3) to *N*-acetyllactosamine
Sambucus nigra (elderberry) bark (SNA)	sialic acid attached to terminal galactose in (α−2,6), and to a lesser degree, (α-2,3), linkage
peanut agglutinin (PNA)	galactosyl (*β*-1,3) *N*-acetylgalactosamine

study. It is known that the abundant glycan structures of bovine fetuin are sialylated, bi- and triantennary complex-type N-glycans (core nonfucosylated). The sialic acid residues are found in both (α-2,3) and (α-2,6) linkages.[34] Abundant glycans in asialofetuin include asialo-bi and asialo-triantennary N-linked oligosaccharides. Dominant porcine thyroglobulin glycans include disialylated biantennary N-linked oligosaccharides with core fucose[35] and oligomannose N-linked oligosaccharide with 5−9 mannosyl residues.[36] The glycan of ribonuclease B is high mannose type, i.e., $Man_{5-9}GlcNac_2$.[37] The dominant glycan in transferrin is sialylated, biantennary complex-type N-glycan.[38]

As shown in Figure 3a, ConA binds strongly to thyroglobulin and ribonuclease B since both of their glycans contain oligomannose N-linked oligosaccharide with 5−9 mannosyl residues. Transferrin, fetuin, and asialofetuin bind weakly to ConA as mannose residues are only present in their core structure and not in the exposed branches. SNA binds fetuin, thyroglobulin, and transferrin, which have all been reported to possess sialic acid moieties on their glycans, while MAL only bound to fetuin and porcine thyroglobulin, which have sialic acid attached in an (α-2,6) position. These two lectins can therefore be used to discriminate between sialic acid residues in an (α-2,3) versus (α-2,6) linkage due to the more specific interaction of MAL. The 2−3 versus 2−6 sialylation of Lea antigens has been implicated in

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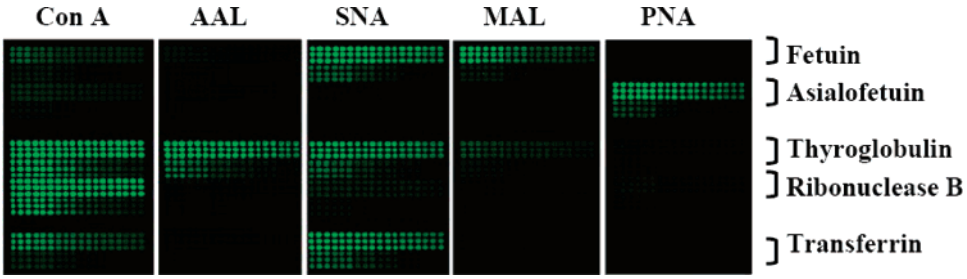

Figure 2. Scanned images of printed standard glycoproteins probed with different lectins. Each block bracketed on the right represents a dilution series of indicated standard from to 0.025 mg/mL. Each dilution has been printed as 9 replicates in a 3 × 3 block.

pancreatic cancer,[39] supporting the use of multiple lectin detection schemes in microarray formats for explicit differentiation of glycan structures. PNA bound to only asialofetuin since it is the only standard used that has desialylated, exposed galactosyl (β-1,3) *N*-acetylgalactosamine residues in its glycan structure. This lectin was also found to be the most specific lectin used. As shown in Figure 2. and Figure 3a, AAL binds strongly to porcine thyroglobulin, which is the only standard used whose main structure consists of disialylate, biantennary N-linked oligosaccharide with core fucose. There might be very low abundant fucosylated glycan attached to transferrin as reported in previous data,[38] where only 2% of tranferrin glycans are reported to be fucosylated. The abundance might be below the detection limit of this lectin since the highest concentration of transferrin spotted on the slide in this work was only 2 mg/mL, which corresponds to 0.12-fmol absolute amount of the fucosylated transferrin based on previously reported data.

(2) Lineary of Response and Detection Limits of Lectins. In all cases where standard proteins elicited response, the limit of detection was found to be between a concentration of 0.05 and 0.1 mg/mL. This corresponds to an absolute protein content of between 125 and 250 pg. On average, glycoproteins fall in the molecular mass range of ~50 kDa. Consequently, 125−250 pg translates into a 2.5−5 fmol detection limit. Mass spectrometric glycan structure determination often requires higher amounts of sample due to the need for multiple sample handling steps as well as MS^n fragmentation requirements for complete structural information. In the case of MAL where only fetuin was found to bind, the limit of detection was much higher at almost 1 mg/mL protein concentration corresponding to 2.5 ng or 50 fmol total protein content. In this study, all protein spots were measured to be ~450 μm in diameter. If the printing buffer composition is changed so that spots spread out to a lesser degree across the array surface, the density of sample per spot area could be increased, possibly resulting in lower limits of detection.

To determine the linearity of response to individual lectins for each of the standard proteins, curves were generated based on the fluorescence response of all printed spots and their replicates. In addition to the nine replicates on each slide, data points were collected from two processed slides for each lectin in order to assess the variability between slide images processed in the same manner and on the same day. It was found that all proteins showed a linear response to each of the lectins within a 0.025−1 mg/mL concentration range. However. linearity of response was optimal in a range of 0.025−0.5 mg/mL. Figure 3 shows some of the standard curves that were obtained. In the case of the response to MAL and PNA (Figure 3e and f), the curve does not appear perfectly linear. It was seen that background fluorescence was higher in these slides compared to the slides used with other lectins, possibly making the standard curve shift to lower values at lower concentration of standards.

It was also noticed that all standard curves were unique to the standard protein that was being used to generate it. This is not surprising since a lectin does not measure quantity of a protein spotted but reflects the extent to which a particular glycan structure is expressed on that protein. To illustrate this the dominant glycan structures on ribonuclease B and transferrin were determined by tandem mass spectrometry. Figure 1 in Supporting Information shows glycan structures and their corresponding mass spectra. Based on the mass spectra, it is evident that ribonuclease B has a mannose-rich glycan structure not present in transferrin. This explains the findings in Figure 3a, where even at the same concentration of standards, ribonuclease B responds to ConA to a much greater degree than transferrin. As shown in the Supporting Information, detailed information such as how the oligosaccharide residues are linked can be obtained from the fragmentation spectra of oligosaccharide. Even the isomeric structure can be differentiated in some cases. However, the whole experiment and data interpretation is complex and time-consuming. On the other hand, in a glycoprotein microarray experiment, hundreds of glycoproteins can be screened at one time, and the data are easier to analyze, although no detailed structural information can be obtained. Therefore, the microarray-based method is complementary to mass spectrometry at this point, and it can be used for high-throughput, proteomic-scale analysis. One thing worth mentioning is that although some of the standards used contained sialic acid residues on their respective glycans, MALDI-based tandem mass spectrometry was often not sufficient to determine their presence (transferrin in Figure 3 and Supporting Information Figure 1). The inability to detect sialic acid moieties on glycans due to in-source decay as ions transit from the MALDI target to the ion trap has previously been reported.[40] To stabilize the fragile sialic acid moiety, modifications such as esterification[41] and permethylation[42] need to be made on the carboxyl group; however, negative mode MALDI could allow the detection of sialylated oligosaccharides with relatively high sensitivity.

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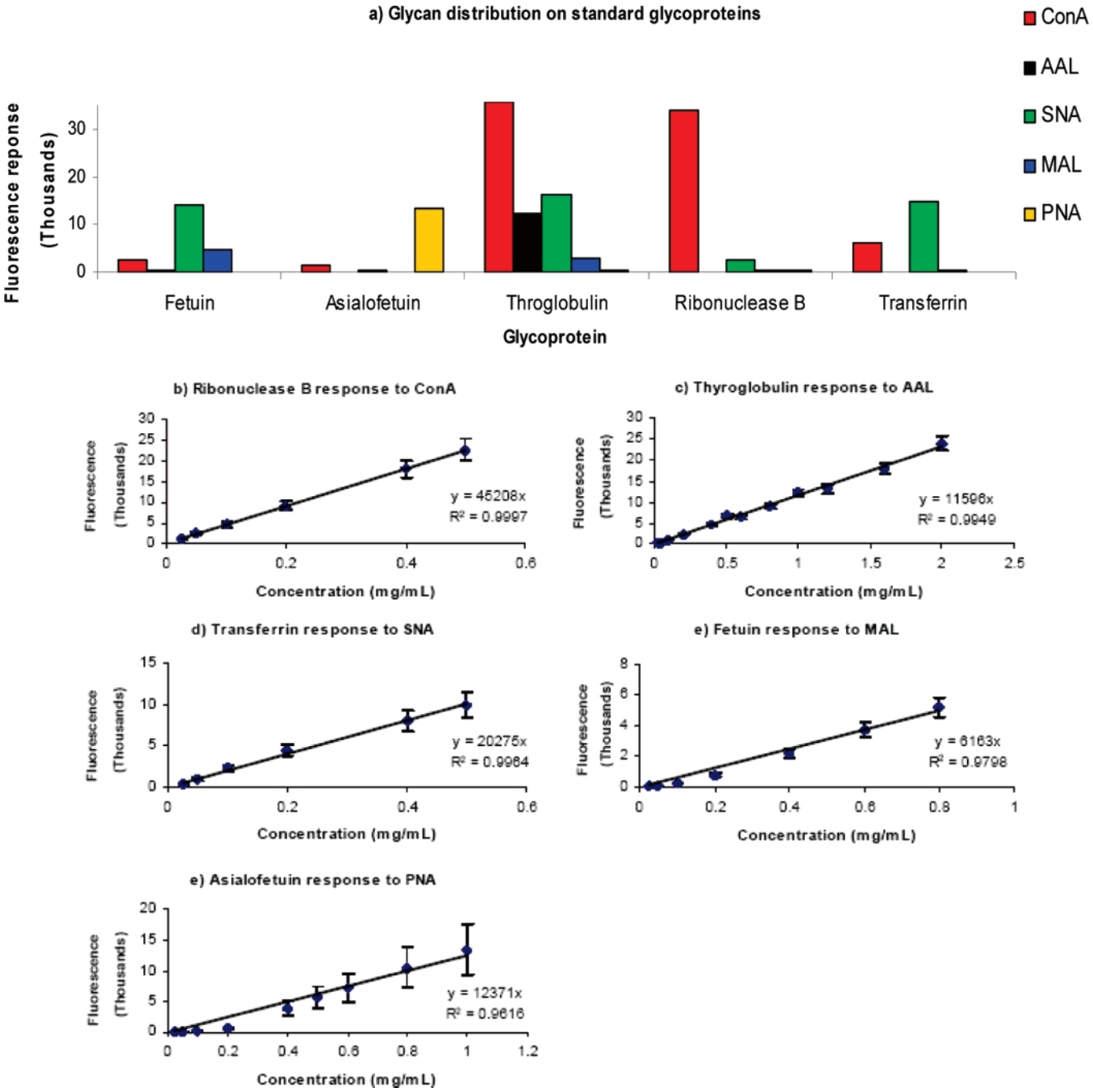

Figure 3. Linearity of response in standards. (a) Glycan distribution on standards printed at 1 mg/mL concentration. Standard curve of (b) ribonuclease B in response to ConA. (c) Thyroglobulin in response to AAL. (d) Transferrin in response to SNA. (e) Fetuin in response to MAL. (f) Asialofetuin in response to PNA using lectin concentration of 5 μg/mL.

(3) Variation between Spots and Slides. Fluorescence values for all spots were used to assess the spot variability and reproducibility. It was found that standard deviations for all proteins and their binding to lectins was within 10% of the mean value, all replicates considered. In some cases, higher standard deviations (more than 20%) were observed, e.g., in the case of response to PNA. However, upon further scrutiny it was found that these higher deviations were due to differences in slides and not due to variability in printing. This conclusion was made because when viewing replicates within a single slide, deviations were consistently within 10% of the mean (see Supporting Information Table 2). Therefore, the presented data suggest that printing occurs reproducibly and the variation between slides is most likely due to slight heterogeneity between slide surfaces and small differences in sample handling during slide processing. It would be ideal to print all replicates on the same slide to avoid differences in slide surfaces and handling.

Based on the study with standards, it can be said that glycoprotein microarrays can potentially be used to study differences in glycosylation states of individual proteins in more complex biological samples.

Studies with Serum Samples. Since studies with standards were successful in terms of reproducibility and sensitivity, an attempt was made to enrich and prefractionate glycoproteins from

Table 2. Protein IDs of Data Shown in Figures 4 and 5 As Identified by μ-LC−MS/MS with Mean Lectin Response, Standard Deviation,[a] and Change in Glycan Expression Based on Microarray Data[b]

Figure		4B	4C	5A	5B	5C	5D
protein ID		complement factor H (H factor 1)	α-1-acid glycoprotein 1 and 2	antithrombin III (ATIII)	leucine-rich α-2-glycoprotein (LRG)	α-2-macro-globulin (α-2-M).	CO3_HUMAN complement C3
% coverage		21	55, 27	31	34	41	61
MW		139034	23497, 23588	52569	38155	163175	187046
ConA	N mean	443000 (37000)	69000 (9000)	551000 (35000)	413000 (29000)	1078000 (22000)	2050000 (76000)
	C mean			660000 (31000)	992000 (45000)	29000 (2000)	340000 (9000)
	P mean	237000 (30000)	275000 (23000)				
	fold change	1.5× in N	3× in P	no change	2× in C	34× in N	5.5× in N
AAL	N mean	138000 (19000)	250000 (33000)	58000 (4000)	41000 (3000)	68000 (3000)	67000 (3000)
	C mean			65000 (4000)	79000 (5000)	5000 (300)	21000 (2000)
	P mean	69000 (5000)	61000 (8000)				
	fold change	1.6× in N	3× in N	no change	1.7× in C	13× in N	3× in N
SNA	N mean	533000 (39000)	387000 (72000)	131000 (12000)	75000 (7000)	194000 (6000)	163000 (7000)
	C mean			298000 (13000)	338000 (22000)	7000 (400)	32000 (3000)
	P mean	388000 (20000)	398000 (16000)				
	fold change	No change	No change	2× in C	4× in C	28× in N	4.5× in N
MAL	N mean	195000 (8000)	121000 (11000)	7000 (300)	7000 (300)	28000 (1000)	
	C mean			12000 (400)	13000 (400)		
	P mean	235000 (12000)	90000 (7000)				
	fold change	no change	no change	1.7× in C	1.9× in C	Only in N	no binding
PNA	N mean	32000 (5000)	78000 (8000)	5000 (100)	6000 (200)	24000 (1000)	
	C mean			5000 (100)	8000 (200)		
	P mean	39000 (9000)	55000 (9000)				
	fold change	no change	no change	no change	no change	only in N	no binding

[a] Shown in parentheses after mean lectin response. [b] All data were background subtracted and normalized based on UV peak areas. N, normal; P, pancreatitis; C, cancer.

human serum and make a glycoprotein microarray to see if differences were evident in sera from biologically distinct states. In this case, sera samples from patients who were not diagnosed with pancreatic disease or were diagnosed with chronic pancreatitis or pancreatic cancer were examined. Such a strategy could be used with a wide range of biological samples following appropriate sample preparation protocols.

As illustrated in Figure 1, serum was first purified for glycoproteins using WGA. WGA can bind oligosaccharides containing terminal *N*-acetylglucosamine or chitobiose as well as sialic acid residues, structures that are common to many serum and membrane glycoproteins. The purified and enriched glycoproteins were then separated in a second dimension by nonporous reversed-phase HPLC. This separation resolved the enriched glycoproteins into ~30 fractions. When 2.5 mg (~50 μL of raw serum) of serum proteins was enriched, ~100 μg of glycoproteins was typically recovered as determined by the Bradford protein assay. Only half of this sample was run in the second dimension. After considering recovery from the reversed-phase column and the number of fractions collected in the second dimension, it can be estimated that each fraction contained an average of 1−2 μg of protein (this amount is proportional to the height of relative peaks). An amount of 2 μg/peak could be an overestimation since peak heights varied and some peaks could contain minor components that were not resolved. All collected fractions were dried down and resuspended in 15 μL of printing buffer so that the working concentrations of the glycoproteins printed were in the range of 0.07−0.13 mg/mL (assuming a 1−2 μg content or less per peak). This range falls between the concentrations that were used for the standard glycoproteins, ensuring similarity in parameters used in both studies.

To see if there were any changes in glycosylation patterns between sera from different biological states, WGA-enriched glycoproteins from normal and pancreatitis serum were fractionated and spotted on nitrocellulose slides. The reversed-phase chromatogram of enriched glycoproteins from the two sera samples showed some differences in peak heights. In addition to confirming the concentration difference shown by the different peak heights, the glycoprotein microarray also indicated a different glycosylation pattern for the observed differences. Figure 4a shows the reversed-phase chromatogram highlighting differences between the two samples.

Based on the peak heights alone it seemed that the peak highlighted in red is 2−3 times overexpressed in normal serum compared to pancreatitis serum. However, microarray data in Figure 4b indicated that response to some of the lectins for the same peak was often more than 2−3 times in the normal serum compared to pancreatitis. To verify that this trend was due to change in glycan expression and not protein concentration, all data were normalized using integrated peak areas. After normalization, it was found that the peak concerned expressed almost twice as much mannosylation and fucosylation while all other glycan structures assessed did not change significantly. μLC−MS/MS analysis identified the peak as a complement factor H.

Additionally, the peak highlighted in orange showed another interesting trend. Although the peak height was less than 2 times higher in the pancreatitis serum compared to the normal serum, normalized response to AAL was more than 3 times higher in the normal sample as shown in Figure 4c. This suggests that the protein concerned is much less fucosylated in chronic pancreatitis. Furthermore, the protein showed a 3-fold higher expression of mannose on its glycans in normal versus pancreatitis serum as seen by the normalized fluorescence intensities with ConA. Response to SNA, MAL, and PNA was not significantly different for the same protein between the two samples. We found that normalization was necessary for a more accurate picture of

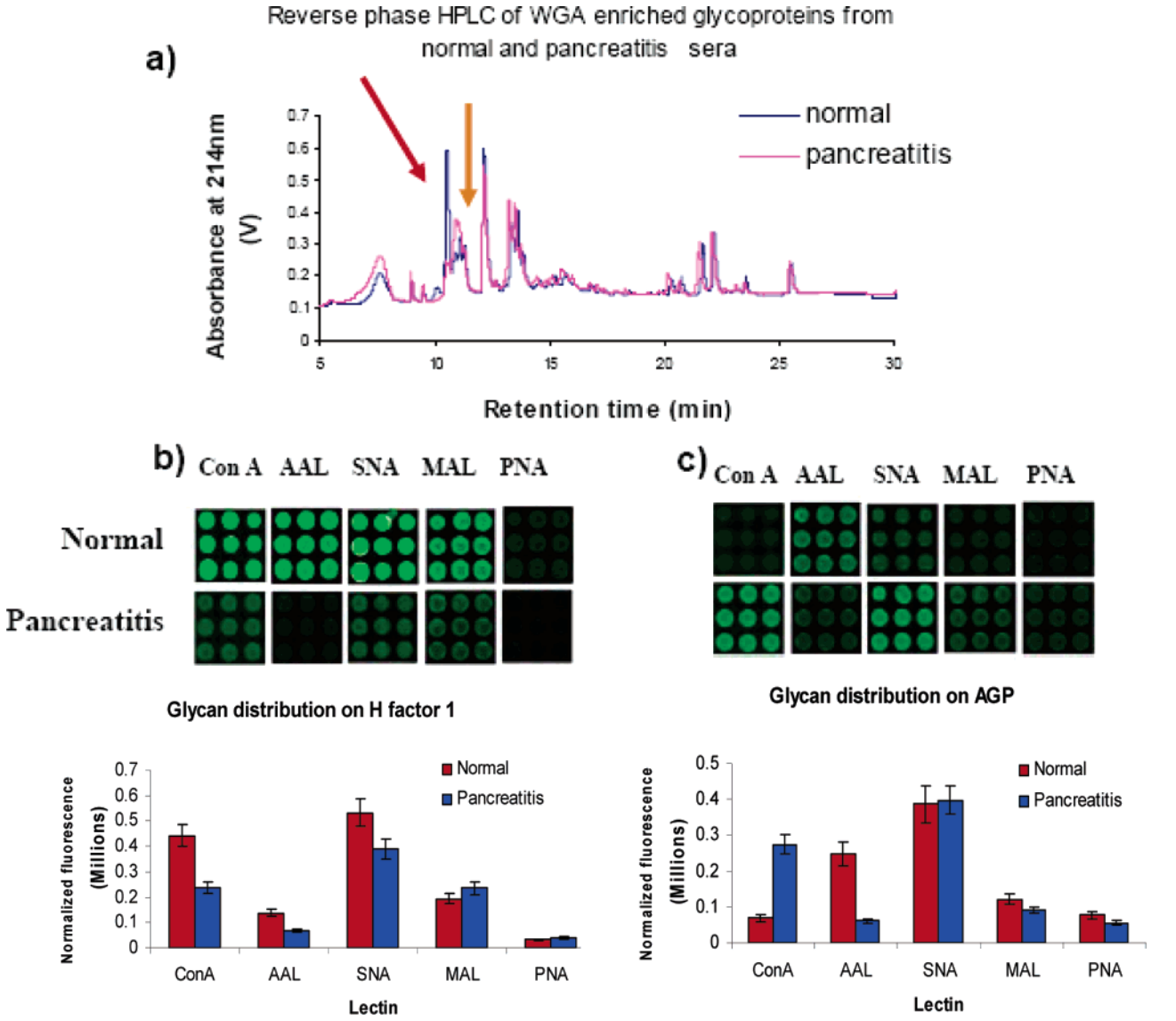

Figure 4. Identifying differences in glycosylation from sera of different biological states. (a) Reversed-phase chromatogram of enriched glycopoteins from normal and pancreatitis sera with differences highlighted. Bar graphs showing integrated fluorescence values of spots shown in array images after background subtraction and normalization based on UV peak area for peak shown with (b) red arrow and (c) orange arrow in the reversed-phase chromatograms.

differential glycan expression and in order to subtract any differences caused by overall protein abundance.

The glycoprotein shown in Figure 4c was identified by tandem mass spectrometry and found to be α_1-acid glycoproteins 1 and 2. This protein and changes in its glycosylation state have been implicated in various disease pathologies including pancreatitis, where higher levels of the protein was seen in severe pancreatitis.[43,44] Although the study, as presented in this report, cannot claim that α_1-acid glycoprotein is a significant marker of pancreatitis, it does show that this novel strategy has potential as a method that can identify such important markers.

Another separate study was done to see whether any difference was apparent in enriched glycoproteins from normal versus pancreatic cancer sera. Pancreatic cancer is currently difficult to diagnose at an early stage due to lack of early diagnostic markers and in some patients may be difficult to differentiate from chronic pancreatitis.[45,46] More differences between normal and cancer serum were found than between normal and pancreatitis. Figure 5 shows representative reversed-phase chromatograms and sections of arrays comparing normal and pancreatic cancer serum glycoproteins. In all data shown, reversed-phase chromatograms indicated similar protein amounts since peak heights and widths were comparable (In the cases where peak heights were not similar, data were normalized to account for concentration differences between samples being compared.). It can be seen from the bar graphs that sialic acid was more abundant in selected cancer serum glycoproteins compared to normal serum glycoproteins (Figure 5a and b). Specifically, antithrombin III showed

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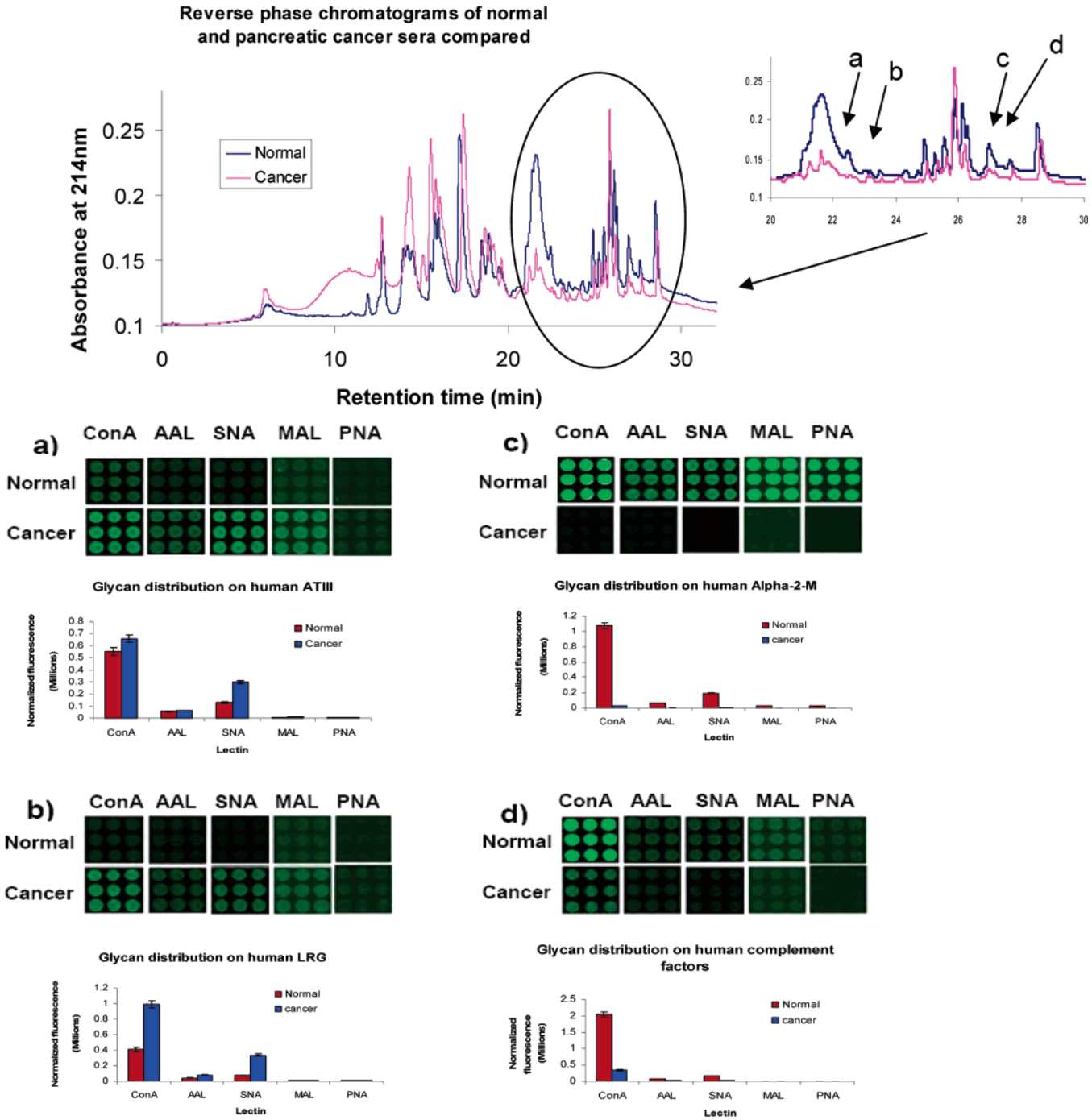

Figure 5. Reversed-phase chromatogram of enriched glycoproteins from normal and cancer sera. The chromatogram from 20 to 30 min has been enlarged for more detail on area of interest. Comparison of differential glycosylation patterns in normal vs cancer serum (a−d). All comparisons shown below had approximately the same peak area between cancer and normal sera, but glycosylation patterns were different (in cases where peak areas were different, the data were normalized by peak area to remove concentration effects). Each illustration shows sections of microarray images of a protein's binding to the lectins indicated. Bar graphs show integrated fluorescence values of spots shown in the array images after background subtraction and normalization based on UV peak area.

a 2-fold higher expression of α-2,6-linked sialic acid (as shown by SNA data in Figure 5a) while all other glycans assessed did not change between normal and cancer sera. Also, leucine-rich α-2-glycoprotein showed a 2-fold higher expression of mannose and a 2-fold higher expression of fucose in addition to a 4-fold higher expression of α-2,6-linked sialic acid (Figure 5b). Conversely, some peaks showed higher mannosylation and sialylation in normal serum compared to cancer serum (Figure 5c and d). α-2-Macroglobulin had 34-fold more mannosylation and 28-fold more sialylation (Figure 5c) while human complement factor showed 5.5-fold higher mannosylation and 4.5-fold higher sialylation in normal compared to cancer sera (Figure 5d). Table 2 summarizes lectin and mass spectrometry data for all analyzed proteins from serum samples that are discussed in Figures 4 and 5. Details about MS/MS data and peptides identified are presented in Supporting Information Table 1.

While a particular glycosylation was more abundant in cancer versus normal for some proteins, for example, (α-2,6) sialylation

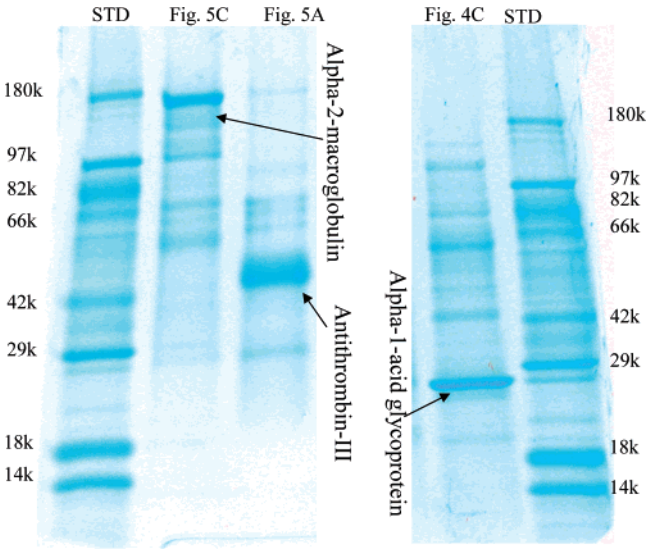

Figure 6. Gel images of a standard (STD) mixture and fractions that contained α-2-macroglobulin, antithrombin III, and α-1-acid glycoprotein to show purity of analyzed fractions. It can be seen that although these components are a major part of the fraction, there are other proteins also present in each fraction. Once an interesting protein is identified based on results with microarrays, a gel can be run for a more exhaustive analysis to confirm which protein is causing a response to lectin in such cases.

in antithrombin III, the trend was reversed for other proteins, such as (α-2,6) sialylation in complement factors. If all the proteins were studied together without prefractionation, such differences would not be highlighted because equal but opposite responses would cancel each other out. The two-step strategy involving fractionation prior to array production presented here addresses this potential problem.

Because of the complexity of serum sample, thorough separation might not have been achieved by a single reversed-phase column postenrichment. Many low-intensity peaks are suppressed by the presence of high-intensity peaks in nondepleted serum sample.[47] To test the purity of the collected UV peak in the reversed-phase separation, SDS−PAGE was performed on selected UV peaks. Figure 6 shows the SDS−PAGE gel image of UV peaks, which were identified mainly to be α-2-macroglobulin, Antithrombin III and α-1-acid glycoprotein in Table 2. As shown in Figure 6, although there are some low concentration proteins coeluting at this retention time, α-2-Macroglobulin, antithrombin III, and α-1-acid glycoprotein are the dominant components that are responsible for the peak intensity, glycan interaction with lectins, and fluorescence intensity. It also suggests that depletion of highly abundant protein or multiple dimension separation may be necessary for the study of complex proteomes such as human serum.

Glycan differences presented in this report are based on comparison of only a single sample set. Some recent differential glycoproteomics studies using a quantitative labeling technique for quantitation have shown that sialylation varies within 2-fold in serum from different individuals.[26,27] If this were true, some of the differences where fold change was less than 2 cannot be considered significant. These changes could be due to age, sex, physical activity, and diet among other factors. Future work will aim at analyzing multiple sample sets to determine differences that are significant and eliminate those that fall within individual variations.

Although currently a proof-of-concept experiment, the strategy presented in this report can be used to identify changes in glycosylation in serum proteins that represent different biological states and may serve as a novel approach to the identification of clinically useful serum biomarkers. At present, we are investigating global changes in glycosylation profiles in sera from multiple patients with various stages of pancreatic diseases to see if significant differences are evident, particularly to identify glycosylation changes unique to patients with pancreatic cancer.

CONCLUSION

A novel strategy that can be used to profile glycosylation patterns in complex biological samples is described. Unlike previous methods that can only assay known glycoproteins by using antibody microarrays or unfractionated complex mixtures that make it difficult to distinguish between the glycoproteins that may be causing a differential response, this strategy starts with an enrichment step followed by a separation, which allows one to assess glycosylation patterns of individual proteins. This gives one the capability to monitor global glycosylation pattern changes as well as identify potential new proteins whose glycosylation changes are essential in biologically important states since each glycoprotein is a unique spot on the array. The data presented here provide an example of how this novel approach can be used to identify different glycosylation patterns in sera from patients with different diseases of the pancreas. The study also showed that glycoprotein microarray data can provide information that reversed-phase UV data cannot. Particularly, it was shown that proteins with the same retention time and similar peak heights showed an altered glycan structure distribution after normalization using integrated peak areas. The strategy can be used in large scale on biological samples to determine critical differences for diagnostics as well as large-scale glycoproteome screening.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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