Published in final edited form as:

Anal Chem. 2012 April 3; 84(7): 3269–3277. doi:10.1021/ac203336u.

Examination of Glycan Profiles from IgG-Depleted Human Immunoglobulins Facilitated by Microscale Affinity Chromatography

Martin Svoboda, Benjamin F. Mann, John A. Goetz, and Milos V. Novotny*

Department of Chemistry and National Center for Glycomics and Glycoproteomics, Indiana University, Bloomington, IN 47405

Abstract

Among the most important proteins involved in the disease and healing processes are the immunoglobulins (Igs). Although many of the Igs have been studied through proteomics, aside from IgG, immunoglobulin carbohydrates have not been extensively characterized in different states of health. It seems valuable to develop techniques that permit us to understand changes in the structures and abundances of Ig glycans in the context of disease onset and progression. We have devised a strategy for characterization of the glycans for the Ig classes other than IgG (i.e. A, D, E, and M) that contain kappa light chains, while using only a few microliters of biological material. First, we designed a microcolumn containing the recombinant Protein L that was immobilized on macroporous silica particles. A similarly designed Protein G microcolumn was utilized to first perform an on-line depletion of the IgG from the sample, human blood serum, and thereby facilitate enrichment of the other Igs. While only 3 μ L of serum were used in these analyses, we were able to recover a significantly-enriched fraction of non-IgG immunoglobulins. The enrichment properties of the Protein L column were characterized using a highly sensitive label-free quantitative proteomics LC-MS/MS approach, and the glycomic profiles of enriched immunoglobulins were measured by MALDI-TOF-MS. As a proof-of-principle, a comparative study was conducted using blood serum from a small group of lung cancer patients and a group of age-matched cancer-free individuals to demonstrate that the method is suitable for investigation of glycosylation changes in disease. The results were in agreement with a glycomic investigation of whole blood serum from a much larger lung cancer cohort.

INTRODUCTION

Analytical glycobiology assumes an important role in the currently popular search for disease biomarkers, as most human diseases appear associated with some alterations in protein glycosylation.^{1,2} Quantitative glycomic profiling of physiological fluids (e.g., blood serum or plasma) of cancer patients, for example, can potentially be utilized for diagnostically or prognostically important evaluations.^{3,4} At present, the most commonly used glycomic methodologies involve liquid chromatography (LC) with detection of fluorescently labeled sugar derivatives,^{5,6} or different types of mass spectrometry (MS).^{7–9} Generally, the analytical procedures targeting glycans in physiological fluids involve deglycosylation of complex protein/peptide mixtures, followed by the measurements of individual oligosaccharides and differential evaluation of their profiles under different conditions of sickness or health.

*Corresponding Author: Milos V. Novotny, 800 E Kirkwood, Chemistry Department, Indiana University, Bloomington, IN 47405, novotny@indiana.edu, phone: (812)-855-4532, fax: (812)-855-8300.

Asparagine-linked (N-linked) glycans originating from the total serum glycoproteome have been initially chosen in our laboratory as the glycomic profiling targets to compare blood sera of healthy individuals and cancer patients.^{3, 10–12} Significant changes in the patterns of glycosylation could be observed as a function of the disease states, from as little as a few microliters of serum, due to the high sensitivity of MS measurements on permethylated glycans.^{9, 13} While profiling comprehensive glycomes will continue to have their biomedical value, a more detailed understanding of the biological relevance of these glycosylation changes now necessitates that the glycans of interest be traced back to the glycoproteins on which they reside. We thus need reproducible, microscale strategies to target key groups of glycoproteins for their subsequent glycomic profiling.

Due to their multilateral functions in inflammation, cancer and the healing process,^{14,15} the immunoglobulins represent an important group of glycoproteins. Certain changes in glycosylation of IgG have been recognized in a number of diseases, including rheumatoid arthritis,^{16–18} hepatitis C-associated cirrhosis,¹⁹ ovarian cancer,^{20–21} and some others.²² Recently, a high-throughput analysis of IgG glycosylation was conducted on a group of 2298 individuals from three isolated populations, and, while there was a wide variability of the glycomic profiles of individuals, clear trends were observed with respect to age.²³ Furthermore, the development of recombinant IgG-based therapeutics by the pharmaceutical industry has required a thorough characterization of the glycan heterogeneity on those molecules.²⁴ In contrast, very few reports (excepting a few studies of inflammation^{25,26}) have characterized the glycosylation of the other classes of Igs, A, D, E, and M.

The strategies for extracting immunoglobulins, chromatographically, from blood serum at the microscale can involve the use of selective proteins, which are immobilized on suitable solid supports such as agarose beads, synthetic polymers in the form of monolithic columns, or silica materials. The choice of solid support can be critical to the success of microscale separations, which demand surface inertness together with a minimum non-specific adsorption of proteins. A common strategy for isolation of immunoglobulins is to use certain proteins extracted from the cell walls of microorganisms, which have the ability to specifically bind immunoglobulins. One of the most common of such proteins, Protein G, binds immunoglobulins only from class G, representing the most abundant class of serum immunoglobulins,²⁷ by interacting mainly with the Fc domain,²⁸ although there have been some reports that it also interacts in the Fab regions on the heavy chains.²⁹ Staphylococcal Protein A is another bacterial protein that binds strongly to IgG, though unlike Protein G, it also binds weakly to IgA and IgM.³⁰ Yet another bacterial protein, Protein L, binds all classes of immunoglobulins (i.e. classes A, D, E, G, and M), but unlike Protein G or Protein A, it interacts with immunoglobulins in the Fab regions ($K_d \sim 10^{-9}$ M), specifically with the kappa light chains, subtypes I, III, and IV.^{31–32} The ratio of light chains in serum immunoglobulins is two kappa chains for every lambda,²⁷ with kappa chains present in all Ig classes, though they are uncommon in serum IgD, 90% of which contain lambda chains.³³

Here, we describe an effective microfractionation strategy by utilizing two separate silica microcolumns, containing Protein G and Protein L, as a prelude for glycomic characterization of a series of immunoglobulins in very small (microliter) volumes of human serum. These silica-based microcolumns are used in a serial fashion, as described below. As a support material, silica particles provide several advantages such as rigidity (pressure resistance) that is compatible with HPLC-like and automated analytical schemes, a large surface-to-volume ratio and, perhaps most importantly, chemical inertness toward the samples of interest that limits the non-specific adsorption of proteins such as serum albumin. Following some initial experiments using the agarose-linked Protein L to evaluate the enrichment capability of the molecule, we have proceeded with silica particles for most of

the described study, since we find them more suitable for fabrication of microcolumns and future work necessitating high-pressure capabilities.

As the average concentration of IgG in human blood serum is 11 mg/mL (ranging from 6–18 mg/mL in different individuals),²⁷ which is approximately four times more than the combined amount of immunoglobulins from the other classes,²⁷ the Protein L preconcentration step was preceded by IgG capture on a similarly prepared Protein G medium. As demonstrated, it is feasible to connect both types of microcolumns in a series, so that IgG can be depleted from the serum, while the other Igs can be subsequently captured on the Protein L column. A quantitative elution of captured Igs can then be performed separately for the two microcolumns, followed by the enzymatic release of glycans to yield two complementary Ig fractions.

The serial microcolumn enrichment strategy described herein has been applied to 3- μ L aliquots of healthy (control) sera to characterize the analytical system. The specificity and repeatability of the enrichment process has been evaluated with the aid of label-free quantitative proteomic measurements on the enriched Igs, as well as by glycomic profiling of enzymatically cleaved glycans by MS. To validate this approach for profiling Ig N-glycans in a preclinical disease investigation, a small disease cohort (lung cancer) was further analyzed.

EXPERIMENTAL

Materials and Chemicals

Nucleosil 4000-7 (7 μ m, 4000 Å) silica particles functionalized with surface diols were obtained from Macherey-Nagel GmbH & Co (Düren, Germany). Protein L and BCA protein assay kit were received from Pierce Thermo Scientific (Rockford, IL). Recombinant protein G, dimethylformamide (DMF), chloroform, acetic acid and HPLC-grade water were from EMD Chemicals, Inc. (Darmstadt, Germany). Peptide-N4-(N-acetyl-beta-glucosaminyl)asparagine amidase (PNGase F) was from Northstar Bioproducts (East Falmouth, MA). Guanidine hydrochloride, methyl iodide, sodium hydroxide beads, borane-ammonia complex, acetonitrile and trypsin were acquired from Sigma (St. Louis, MO). A human serum sample pooled from healthy female donors was purchased from Innovative Research (Novi, MI) and was stored at -70°C until the time of analyses. Dithiothreitol (DTT) and iodoacetamide (IAA) were from Bio-Rad Laboratories (Hercules, CA). The MALDI matrix, 2,5-dihydroxybenzoic acid (2,5-DHB), was purchased from Alfa Aesar (Ward Hill, MA). Cellulose acetate filters were from Corning Incorporated (Corning, NY). Empty, C18 and graphitized-carbon spin columns were obtained from Harvard Apparatus (Hillston, MA). PEEK tubing, unions and stainless steel frits were purchased from Upchurch (Oak Harbor, WA).

Serum Samples from the Lung Cancer Cohort

Blood samples were collected by clinically trained personnel and processed within a few hours according to a standardized protocol. Briefly, approximately 10 mL of blood were collected into the glass red-top tubes. Samples were allowed to coagulate for 30 min and were then centrifuged at 3500 rpm for 30 min. Without delay, the sera were divided into three cryovials and frozen immediately at -70°C until the time of analysis. This study was approved by the Indiana University Institutional Review Board, while a full documentation on the patients was provided by Hoosier Oncology Group, Inc (Indianapolis, IN). Patient information and demographics are available in Tab. ST-1.

Microcolumn Fabrication

Proteins G and L were immobilized on the diol silica surface, as described previously,³⁴ with minor modifications. Briefly, 125 mg of diol silica were suspended in 5 mL of acetic acid/water (9/1, v/v), 125 mg of sodium periodate were added to oxidize the diols to aldehydes, and the mixture was stirred for 3 h at room temperature. After this time, the silica was washed excessively with water, ethanol and ether and dried under a vacuum. Aldehyde-modified silica was suspended in 5 mL of 0.1 M phosphate buffer (pH 7.5) containing 0.25 M sodium chloride. 0.5 mg of Protein G or L in 100 μ L of HPLC water was added and mixed. After addition of 0.35 mg of sodium cyanoborohydride, the mixture was stirred at 4 °C for 72 h. Next, 2.5 mg of sodium borohydride were added stepwise, over 30 min, to reduce non-reacted aldehyde groups to diols. Following addition of sodium borohydride, the suspension was subsequently stirred for 1 h in the refrigerator. Silica with the attached protein was washed 4 times with 0.1 M phosphate buffered saline (PBS), pH 7.4, containing 0.15 M sodium chloride and 0.02% sodium azide. A BCA protein assay was used to monitor coupling as it proceeded. Results indicated that protein coupling was completed at 48 h.

For packing of the silica-protein particles into the microcolumn, we used a fast protein liquid chromatograph (FPLC) system ÄKTApurifier 10 from Amersham Biosciences (Piscataway, NJ). The slurry of silica-protein was placed into a 1-mL packing reservoir; the column was attached downstream of the packing reservoir and packed in PBS at a flow rate of 100 μ L/min for 30 min. Parameters of the PEEK column were 5-cm length and 0.5 mm i.d. The column was end-capped with two 0.5- μ m mesh stainless steel frits.

Microcolumn Affinity Chromatography

Affinity chromatography experiments were performed with the ÄKTApurifier 10 equipped with a UV detector set at 280 nm. For each serum sample, 3 μ L were diluted in 50 μ L of binding buffer and filtered through a 0.45 μ m cellulose acetate filter prior to injection. For both Protein G and Protein L microcolumns, the binding buffer was 0.1 M phosphate (pH 7.4) containing 0.15 M sodium chloride and 0.02% sodium azide, and the elution buffer was 0.1 M acetic acid (pH 2.5). Following the binding step, an intermediate washing step was performed in a 55/45 mixture (binding buffer/elution buffer) to remove nonspecifically-bound sample components. The flow rate was 20 μ L/min for the binding step and 40 μ L/min for the washing and elution steps. The Unicorn v4.00 software, which was used to operate the ÄKTApurifier, was configured to identify the elution fraction with a peak-finding algorithm. The affinity-enriched fraction was collected with a P900 fraction collector. Collected fractions were immediately dried.

Agarose-based Affinity Chromatography

The adsorption and release of immunoglobulins from Protein L agarose was examined in a batch mode, in which 7.5 μ L of the serum were diluted to 30 μ L in PBS, pH 7.2. 20 μ L of agarose beads were added to each sample to preclear the components in the serum that had the strongest nonspecific interactions with the support. Following preclearing, the samples were incubated with 20 μ L of Protein L-agarose for 4 h in a 1.5-mL microtube with end-over-end rotation at 4 °C. After incubation, the tube was centrifuged and the supernatant was removed. The agarose bed was washed three times with 200 μ L of PBS. After the final wash, the supernatant was removed, and the elution buffer, a 100- μ L aliquot of 0.1 M glycine, pH 2.5, was applied. Elution was allowed to proceed for 1 h at room temperature. The slurry was centrifuged, and the supernatant was dried under a vacuum in preparation for digestion with PNGase F as described below.

Enzymatic Release of N-glycans, Trypsin Digestion of Proteins, and Spin-column Purification of Glycans and Peptides

See Supporting Information pages S3–S4, as also described in previous publications.^{9, 13}

Reduction of Glycans

Dried glycans were suspended in 20 μ L of an aqueous solution of borane-ammonia (B-A) complex (10 mg/mL). Each sample was incubated for 70 min at 60 °C and then allowed to cool to room temperature. A 1- μ L aliquot of acetic acid was added, and the mixture was incubated for 30 min at room temperature. This addition was repeated twice until no more bubbles were formed and all B-A complex was quantitatively eliminated. Subsequently, the sample was dried. Borane salts were removed in the form of their methyl esters by adding a 100- μ L aliquot of methanol three times. After each addition, the sample was dried.

Solid-phase Permethylation

Reduced N-glycans were permethylated using the static solid-phase procedure published previously.³⁵ Briefly, the sodium hydroxide beads, dispersed in acetonitrile, were loaded into empty microspin columns. Acetonitrile was displaced through washing the column twice with 200 μ L of DMF. The sample was dissolved in 100 μ L of a 60%/35%/5% DMF/methyl iodide/water solution, loaded into the column with sodium hydroxide beads and allowed to react for 15 min. In the next step, the sample was centrifuged (600 RPM, 1 min), 25 μ L of methyl iodide were added, and the sample was reapplied onto the column for an additional 15 min. After centrifugation (1500 RPM, 1 min), 300 μ L of chloroform was added for recovery of permethylated glycans. The organic solution was repeatedly washed with 800 μ L of 0.5 M NaCl and HPLC-grade water. Permethylated glycans were dried with a vacuum centrifuge.

MALDI-TOF MS

Enzymatically released and chemically derivatized N-glycans were measured using a 4800 MALDI TOF/TOF instrument from Applied Biosystems (Foster City, CA). Glycans were suspended in 5 μ L of an 80%/20% water/methanol solution. 2,5-dihydroxybenzoic acid was used as a matrix dissolved in 75%/25% water/methanol solution at a concentration of 10 mg/mL. 1 mM sodium acetate was added to ensure that glycans were ionized as the sodiated adducts. 0.5 μ L of the sample was spotted on the MALDI plate and immediately dried under the vacuum. Deposited glycans were coated with 0.5 μ L of the matrix solution and again immediately dried under the vacuum to create a homogenized thin layer. The spectra were measured in the positive-ion mode, while the m/z range was set from 1500 to 5000. A total of 2000 laser shots were applied to each spot. Details of MS data processing can be found on page S3 of the Supporting Information.

LC-MS/MS

Tryptic peptides were subjected to LC-MS analysis with an Agilent 1100 nanoflow HPLC instrument coupled to a high-resolution LTQ-FT Ultra mass spectrometer from Thermo Scientific (Waltham, MA). For separation of peptides, a capillary column packed in-house with C18 silica particles (Magic C18AQ, 200 Å, 3 μ m, Michrom Bioresources) was used. A linear gradient was applied from 3% to 80% mobile phase B over 50 min, at a flow rate of 250 nL/min. Mobile phase A consisted of 97%/3%/0.1% water/acetonitrile/formic acid. Mobile phase B was composed of 99.9%/0.1% acetonitrile/formic acid. The column eluent was nanosprayed at 1.8 kV into the mass spectrometer inlet. Ions were collected in the m/z range from 300 to 2000 Da. Data-dependent collision-induced dissociation experiments (CID) were performed on the 5 most intense ions in a precursor scan and subsequent MS/MS spectra were collected for Uniprot database searching with MASCOT. Data acquisition

was performed using the Xcalibur software. Details of mass spectral data processing can be found on pages S4–S5 of the Supporting Information. For a full explanation of label-free quantification of LC-MS data, see the reference by Mann, et al.³⁶

RESULTS AND DISCUSSION

Immunoglobulins are biologically important mammalian proteins whose functions and stability are dependent on their N-glycosylation.³⁷ Many other components of the immune system are also reliant on their pattern of glycosylation,^{38,39} and are involved in the organism's capacity to ward off a wide range of pathological conditions including infectious diseases and cancer. Inflammation, cancer, and immunity are interlinked in a set of complex molecular and cellular interactions,^{14,40} but these processes are now becoming increasingly accessible to study through the new highly sensitive tools of glycomics and proteomics. While assessment of the glycosylation levels of IgG and other commercially produced monoclonal therapeutic antibodies is routine and utilizes various technologies,^{41–42} microscale profiling of IgG glycans in blood serum has rarely been achieved. A notable exception is a very recent report,²³ where the IgG glycomes of 1609 individuals from three isolated human populations have been compared with regard to heritability and other variations through the use of LC-fluorescence detection. To even a lesser degree, glycan constituents of other Igs have been investigated.

We initially targeted a range of immunoglobulins through their interaction with a previously described bacterial protein, Protein L, known to exhibit its affinity through kappa light chains.³¹ In evaluating different sorptive media for their capacity to reproducibly attach, and later quantitatively desorb, immunoglobulins contained in $\leq 10\text{-}\mu\text{l}$ volumes of blood sera, we have used a quantitative, label-free proteomic method,³⁶ which offers the needed sensitivity for identification of retained vs. non-retained proteins. The extent of non-specific sorption and protein recovery from the microscale columns were the criteria for choosing the silica-protein L over the commercially available agarose-Protein L sorption material (see below). Since the IgG variants had the overwhelming effect on the sorption capacity of the Protein L miniature columns, our following strategy has been a two-microcolumn approach, in which the biological sample is first subjected to the silica-Protein G sorption for IgG depletion, while the remaining immunoglobulins are allowed to undergo sorption on the silica-Protein L medium. Subsequently, the retained immunoglobulins are independently desorbed from their respective beds, independently subjected to N-glycanase digestion, and our standard glycomic profiling procedure. As shown below, these in-depth glycomic profile measurements yield complementary types of information.

Agarose-Protein L Enrichment of Igs

Since Protein L-agarose was commercially available, we first tested its utility for extraction of immunoglobulins in small volumes of blood serum prior to investing efforts in the silica microcolumn fabrication process. The efficiency of Protein L in specific sorption/release was monitored by a high-resolution mass spectrometer (LTQ-FT instrument), where the extent of enrichment of the Ig fraction was monitored with the ProteinQuant software. While the details of the software are fully described in reference 36, in general terms, it utilizes the LC-MS data files to reconstruct the extracted ion chromatograms for each peptide that was assigned to a protein during MASCOT searching, and it then integrates the peak area for the peptide elution peaks. A protein area is computed by summing the peptide areas for each protein.

A comparison of the unbound and bound fractions was used to calculate an enrichment ratio (bound/unbound) for each of the identified immunoglobulin chains (Table 1). The relative signal for 8 of the 12 Ig chains quantified increased more than 4 fold, while the relative

signal from the kappa chains, which includes the kappa subtypes I, III, and IV that were specifically targeted for enrichment, increased by more than an order of magnitude. The only identified Ig chain that did not show a relative increase in signal intensity was the lambda light chain, which was likely present in the enriched fractions as a result of interactions between different Ig molecules.⁴³ Additionally, normalized serum albumin signal in the bound fraction was slightly more than half that in the unbound. These preliminary experiments indicated that Protein L was suitable for immunoglobulin enrichment through interactions with the light chains. However, to target the less abundant classes of immunoglobulins, the depletion of IgG was first necessary. As such, the silica microcolumns containing Protein G and Protein L were fabricated to allow for serial affinity enrichments, in which the IgG molecules were first captured upstream of the remaining Igs, as described below. While agarose was suitable for demonstrating the efficacy of Protein L enrichment, the silica materials were better suited for the fabrication of very small columns (~10 μ L) that would be compatible with on-line multidimensional strategies that could utilize the HPLC-like modes of separation.

Binding Capacity of Silica-based Protein G and Protein L Microcolumns

The reductive-amination coupling method is an efficient and high-yield pathway to attach proteins to the aldehyde-silica surface. For a quick analysis of the progress of the coupling reaction, we used a BCA protein assay kit for measuring free protein in the reaction environment, and we did not observe any residual Protein G or Protein L in solution after 72 hrs of coupling. For testing of our microcolumns, we used commercial human serum IgG (Sigma). Based on these experiments, the dynamic capacity of the Protein G column was determined to be 24 μ g of IgG * min^{-1} . At this rate, a flow-through peak with a normalized peak area of 2% was observed (Fig. S-1). When more than 120 μ g of IgG (in 100 μ L) was injected, the flow-through peak continued to grow, whereas the elution peak remained unchanged. The average amount of IgG in blood serum is known to be 6–18 mg/mL,²⁷ so the microcolumn should be able to deplete the IgG from at least 6 μ L of serum.

Characterization of the dynamic binding capacity of the prepared Protein L column was also accomplished using purified human serum IgG. The amount injected was 500 μ g per trial (3 times), and the elution fractions were collected into prepared microvials (Fig. 1). The amount of captured IgG was then measured by the BCA method. The Protein L column was able to bind an average of 8.02 ± 1.08 μ g of IgG* min^{-1} . Since the mean concentration of non-IgG immunoglobulins in the blood serum is 3.2 mg/mL²⁷ and 50% of immunoglobulins contain the appropriate kappa light chains, the Protein L column could be used to isolate immunoglobulins from approximately 13 μ L of the blood serum.

Microcolumn Enrichment of Igs from Blood Serum

Pooled human blood serum from females was chosen as a standard to test the repeatability of the microextraction process for isolation of immunoglobulins from low- μ L volumes of biological material for glycomic profiling by MALDI-MS. For these experiments, the silica-based columns were connected together in a series, first the Protein G column, followed by the Protein L column. 3- μ L aliquots of serum were injected into the system. After the immunoglobulins were captured, but prior to application of the elution buffer, the microcolumns were disconnected and extensively washed at pH 5.3 as a 55/45% mixture of binding and elution buffers. During the optimization studies, this ratio of the two buffers was determined to be ideal to elute nonspecifically-bound proteins without disrupting the interaction between the immunoglobulins and Protein L (or Protein G). We repeated the affinity separation of serum six times. The chromatographic curves from those six trials for both columns are displayed in Figures S-2A and S-2B. Following the affinity experiments, the collected immunoglobulin fractions were subjected to a label-free quantitative bottom-up

proteomic analysis³⁶ and to the previously described glycomic profiling,¹³ and a statistical evaluation of the data was performed as described below.

Bottom-up Proteomic Profiling of Immunoglobulin Fractions

In the Protein G fractions, protein chains belonging to the immunoglobulin family accounted for 95% of the area under the total ion chromatogram (TIC) (Tab. ST-2). Not only were all four subclasses of IgG identified, but the heavy chains of IgA and IgM were also present, though in negligible quantities relative to IgG. Because Protein G is not expected to capture these Ig classes, they could appear as a result of their interactions with IgG.⁴³

Approximately 3 % of the TIC peak area was attributable to other glycoproteins, where vitronectin, with three reported N-glycans, was the major contaminant. Notably, serum albumin, which is the most abundant protein in the blood and which is frequently observed as the most abundant, nonspecifically-bound protein in affinity experiments with serum samples, was almost entirely eliminated from the Protein G-bound samples.

In the Protein L fractions, Ig chains accounted for 73% of the TIC area, and the major structures observed were the heavy chains from IgA (Table 2). This Ig is the second most-abundant class in human blood. The third most-abundant is IgM, which was appropriately represented in our samples as well. We also detected IgD, but only in trace amounts. This was not unexpected because over 90% of serum IgD is associated with the lambda light chains,³³ and the concentration in serum is 30 ng/mL.⁴⁴ Lambda light chains were also detected in the bound fractions, albeit in low quantities. Together with the IgG1 chain, which was also observed, the presence of these two unexpected Ig chains was probably the result of interactions between different classes of Ig molecules.⁴³ The non-Ig proteins that were identified could be divided into two groups: the non-N-glycoproteins, which represented 17% of the TIC and the N-glycoproteins, which represented 10%. Considering only the identified N-linked glycoproteins, 87% of the peak area for this group was attributable to the immunoglobulins, suggesting that the glycomic profiles (discussed below) were predominantly derived from non-IgG immunoglobulins.

The most abundant glycoprotein was apolipoprotein A-I, which does not influence our N-glycan determination, since it may be glycosylated (i.e. chemically modified by a single glucose), not enzymatically glycosylated.⁴⁵ Some contributions to the glycomic profiles were attributable to the complement factors C4-A and C3, which were detected in the Protein L-enriched fractions and accounted for 3.4 and 2.5% of the TIC area, respectively. The complement factors are capable of binding the Fc domain of antigen-associated IgM.⁴⁶ Because Protein L associates with the light chains in the Fab regions, similar to antigen binding, it could facilitate complement factor association with the Fc domain. Complement factor C3 has 3 possible glycosylation sites (2 of which are occupied), and they present only high-mannose structures (Man₅₋₉GlcNAc₂). High-mannose structures are expressed on C4 as well, though the dominant glycans are complex biantennary structures with either one or two sialic acids, where the monosialylated form can be additionally fucosylated.⁴⁷

In spite of some non-Ig proteins identified, it is evident that we obtained highly enriched Ig fractions through the described procedure. In summary, non-Ig glycoproteins that could interfere in glycomic profiling accounted for 2.5% and 10% of the area under the TIC for the Protein G and L columns, respectively.

Glycomic Profiling of Eluted Fractions

The main focus of this work was to obtain the profiles of N-glycan structures associated with non-IgG immunoglobulins. This examination was performed using the procedure developed in our laboratory that comprises the use of PNGase F and solid-phase

permethylation. The benefits of this approach are the possibility of a simultaneous analysis of neutral and acidic glycans, enhanced sensitivity of glycomic measurements, and easier MS/MS interpretation.

In the Protein G fractions, 19 glycan structures (S-3), which were previously attributed to IgG,²⁵ were easily quantified. The glycomic profile was dominated by the agalactosylated, biantennary core-fucosylated structure, and its monogalactosylated and digalactosylated forms. Additionally, two of the structures were high-mannose glycans, and six were bisecting structures. These observations were in agreement with the IgG glycan profiles reported previously.²³

High-mannose structures were the most abundant glycans in the Protein L-fraction profiles (Fig. 2). These structures were previously reported for IgM and IgD,^{44, 48} both of which were captured on our column, though IgD in only a low amount. In total, sixteen N-glycans with different m/z values were identified in the Protein L-enriched fractions, fourteen of which were quantifiable. The most abundant complex glycan was the biantennary, digalactosylated structure with sialic acid, which was previously reported as the dominant glycan structure of IgA.^{26,49} This agrees with the quantitative proteomic data, which showed that the largest contribution to the TIC area for the proteins captured with the Protein L microcolumn was from IgA. The most abundant complex glycan structure on IgM was reportedly the monosialylated, core-fucosylated bisecting structure, which was the second-most abundant glycan among the complex glycan structures in our profiles.

Application to Serum Fractionation and Glycomic Profiling: Lung Cancer

As a proof-of-principle, we used the microcolumn enrichment for a preliminary investigation of a lung cancer sample set. Additionally, the cancer cohort provided a set of biological replicates, not simply technical replicates, as was the case with the commercial pooled serum that was used to initially evaluate the method. The ability to gain insightful glycomic information from sera of different individuals can be more challenging due to the biological variability of different samples.

Blood sera from six control persons and from six lung cancer patients were analyzed. The samples selected for this pilot study were all males between the ages of 57–66 and all had previously smoked. Again, 3 μ l of the sera were used, while the samples were treated in the same way as described previously. Bottom-up proteomic examinations were performed for all samples. Proteomic profiles were similar to those in standard serum samples, for both Protein G and Protein L fractions. In Protein G fractions, about 95% of the area measured under the TIC belonged to the protein chains from the immunoglobulin family, with all four subtypes of gamma heavy chains identified. In the Protein L fractions, again approximately 87% of the LC-MS signal attributable to N-glycoproteins was from non-IgG immunoglobulins. No significant differences were observed when average relative intensities of proteins in the control group were compared to intensities of the cancer group (data not shown).

In the Protein G fractions (Fig. S-4), for the cancer patients group, an elevated amount of a biantennary, agalactosylated, core-fucosylated glycan was observed, whereas a decrease was observed for the monogalactosylated and digalactosylated forms of the same core-fucosylated structure. Approximately, this increase/decrease was roughly observed in other cancer studies using different methodologies,^{20, 50} although the selective enrichment procedures developed here are likely to enhance the number of monitored glycans in future studies. Examination of glycans from the immunoglobulins captured on the Protein L column (Fig. 3) showed some differences in high-mannose structures, where their average abundance was reduced in the cancer group. These are glycans known to be strongly

expressed on IgM. The decreasing trend is worth noting, because our large clinical study on total glycomic profiles has indicated the same trend.⁵²

Among the remaining glycans quantified after Protein-L affinity were eight structures exhibiting a bisecting N-acetylglucosamine on the β -linked core mannose. Strikingly, all eight bisecting structures were higher in relative abundance in the cancer group than in the controls. A similar increasing trend in bisecting structures was described for IgA in a disease study of liver cirrhosis.⁵⁴ Since immunoglobulins are synthesized (and thus glycosylated) in B cells, changes in the nature of bisecting structures on the immunoglobulins follow a biologically distinct pathway from that utilized by the liver to promote addition of the same structures to the acute-phase proteins.⁵⁵ Because the major immunoglobulin class in the Protein L fraction was A, this difference could likely be assigned to this Ig. Moreover, IgA has been previously reported to have bisecting-type glycans on it.⁴⁹ This observation, in particular, accentuates the value of glycomic analysis of targeted fractions of glycoproteins for gaining knowledge of their specific glycosylation patterns. This proof-of-principle study will need to be followed in the future by a larger cohort of patients.

CONCLUSIONS

We demonstrated here the availability and performance of a novel microcolumn approach, through which it is feasible to obtain a significantly enriched IgG-depleted immunoglobulin fraction from the blood serum. Using the Protein G column allowed us first to capture the most abundant G immunoglobulins, while the IgG-depleted serum flowed downstream to the Protein L column to capture the less abundant Igs. The protein profiles of the Protein L-enriched fractions were evaluated by a quantitative label-free bottom-up proteomic analysis, which indicated that 87% of the LC-MS signal from N-linked glycoproteins could be attributed to the immunoglobulins. IgA and IgM accounted for the majority of the immunoglobulins enriched following IgG depletion, though IgD was also observed at trace amounts. Finally, this two-column system was successfully used for extraction of immunoglobulins from 3 μ l of blood serum from 6 cancer patients and 6 healthy persons. A comparison of the glycomic profiles of the immunoglobulins was performed, and the relative intensities of identified structures were compared between the two groups. While this study was performed on a small set of samples, it illustrates that this serial affinity chromatography approach may be used for specific glycomic profiling of serum immunoglobulins using only a few microliters of starting material.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by Grant Number U01CA128535 (awarded to MVN, with subcontract to the Hoosier Oncology Group, Inc.) from the National Cancer Institute, and Grant Number GM24349 from the National Institute of General Medical Science, U.S. Department of Health and Human Services.

References

1. Klein A. *Adv Clin Chem.* 2008; 46:51–85. [PubMed: 19004187]
2. Peracaula R, Barrabes S, Sarrats A, Rudd PM, de Llorens R. *Dis Markers.* 2008; 25(4–5):207–18. [PubMed: 19126965]
3. Kyselova Z, Mechref Y, Kang P, Goetz JA, Dobrolecki LE, Sledge GW, Schnaper L, Hickey RJ, Malkas LH, Novotny MV. *Clin Chem.* 2008; 54(7):1166–75. [PubMed: 18487288]

4. Goldman R, Resson HW, Varghese RS, Goldman L, Bascug G, Loffredo CA, Abdel-Hamid M, Gouda I, Ezzat S, Kyselova Z, Mechref Y, Novotny MV. *Clin Cancer Res*. 2009; 15(5):1808–13. [PubMed: 19223512]
5. Anumula KR. *Anal Biochem*. 2006; 350(1):1–23. [PubMed: 16271261]
6. Bones J, Mittermayr S, O'Donoghue N, Guttman A, Rudd PM. *Anal Chem*. 2010; 82(24):10208–15. [PubMed: 21073175]
7. Weiskopf AS, Vouros P, Harvey DJ. *Anal Chem*. 1998; 70(20):4441–7. [PubMed: 9796427]
8. Dallas DC, Martin WF, Strum JS, Zivkovic AM, Smilowitz JT, Underwood MA, Affolter M, Lebrilla CB, German JB. *J Agric Food Chem*. 2011; 59(8):4255–63. [PubMed: 21384928]
9. Alley WR Jr, Madera M, Mechref Y, Novotny MV. *Anal Chem*. 2010; 82(12):5095–106. [PubMed: 20491449]
10. Mechref Y, Hussein A, Bekesova S, Pungpapong V, Zhang M, Dobrolecki LE, Hickey RJ, Hammoud ZT, Novotny MV. *J Proteome Res*. 2009; 8(6):2656–66. [PubMed: 19441788]
11. Alley WR Jr, Novotny MV. *J Proteome Res*. 2010; 9(6):3062–72. [PubMed: 20345175]
12. Kyselova Z, Mechref Y, Al Bataineh MM, Dobrolecki LE, Hickey RJ, Vinson J, Sweeney CJ, Novotny MV. *J Proteome Res*. 2007; 6(5):1822–32. [PubMed: 17432893]
13. Kang P, Mechref Y, Klouckova I, Novotny MV. *Rapid Commun Mass Spectrom*. 2005; 19(23):3421–8. [PubMed: 16252310]
14. Janeway, C. *Immunobiology: the immune system in health and disease*. 6. Garland Science; New York: 2005.
15. Grivennikov SI, Karin M. *Curr Opin Genet Dev*. 2010; 20(1):65–71. [PubMed: 20036794]
16. Parekh RB, Dwek RA, Sutton BJ, Fernandes DL, Leung A, Stanworth D, Rademacher TW, Mizuochi T, Taniguchi T, Matsuta K, et al. *Nature*. 1985; 316(6027):452–7. [PubMed: 3927174]
17. van Zeben D, Rook GA, Hazes JM, Zwinderman AH, Zhang Y, Ghelani S, Rademacher TW, Breedveld FC. *Br J Rheumatol*. 1994; 33(1):36–43. [PubMed: 8162455]
18. Bond A, Alavi A, Axford JS, Bourke BE, Bruckner FE, Kerr MA, Maxwell JD, Tweed KJ, Weldon MJ, Youinou P, Hay FC. *J Autoimmun*. 1997; 10(1):77–85. [PubMed: 9080302]
19. Mehta AS, Long RE, Comunale MA, Wang M, Rodemich L, Krakover J, Philip R, Marrero JA, Dwek RA, Block TM. *J Virol*. 2008; 82(3):1259–70. [PubMed: 18045939]
20. Saldova R, Royle L, Radcliffe CM, Abd Hamid UM, Evans R, Arnold JN, Banks RE, Hutson R, Harvey DJ, Antrobus R, Petrescu SM, Dwek RA, Rudd PM. *Glycobiology*. 2007; 17(12):1344–56. [PubMed: 17884841]
21. Alley WR Jr, Vasseur JA, Goetz JA, Svoboda M, Mann BF, Matei DE, Menning N, Hussein A, Mechref Y, Novotny MV. *J Proteome Res*. 2012 accepted.
22. Huhn C, Selman MH, Ruhaak LR, Deelder AM, Wührer M. *Proteomics*. 2009; 9(4):882–913. [PubMed: 19212958]
23. Pucic M, Knezevic A, Vidic J, Adamczyk B, Novokmet M, Polasek O, Gornik O, Supraha-Goreta S, Wormald MR, Redzic I, Campbell H, Wright A, Hastie ND, Wilson JF, Rudan I, Wührer M, Rudd PM, Josic D, Lauc G. *Mol Cell Proteomics*. 2011; 10(10):M111 010090. [PubMed: 21653738]
24. Jefferis R. *Nat Rev Drug Discov*. 2009; 8(3):226–34. [PubMed: 19247305]
25. Butler M, Quelhas D, Critchley AJ, Carchon H, Hebestreit HF, Hibbert RG, Vilarinho L, Teles E, Matthijs G, Schollen E, Argibay P, Harvey DJ, Dwek RA, Jaeken J, Rudd PM. *Glycobiology*. 2003; 13(9):601–22. [PubMed: 12773475]
26. Field MC, Amatayakul-Chantler S, Rademacher TW, Rudd PM, Dwek RA. *Biochem J*. 1994; 299(Pt 1):261–75. [PubMed: 8166649]
27. O'Gorman, MRG.; Donnenberg, AD. *Handbook of human immunology*. 2. CRC Press; Boca Raton: 2008.
28. Sauer-Eriksson AE, Kleywegt GJ, Uhlen M, Jones TA. *Structure*. 1995; 3(3):265–78. [PubMed: 7788293]
29. Derrick JP, Wigley DB. *J Mol Biol*. 1994; 243(5):906–18. [PubMed: 7966308]
30. Hober S, Nord K, Linhult M. *J Chromatogr B*. 2007; 848(1):40–7.

31. Akerstrom B, Bjorck L. *J Biol Chem*. 1989; 264(33):19740–6. [PubMed: 2479638]
32. Nilson BH, Solomon A, Bjorck L, Akerstrom B. *J Biol Chem*. 1992; 267(4):2234–9. [PubMed: 1733930]
33. Spiegelberg HL. *Methods Enzymol*. 1985; 116:95–101. [PubMed: 3937029]
34. Madera M, Mechref Y, Novotny MV. *Anal Chem*. 2005; 77(13):4081–90. [PubMed: 15987113]
35. Kang P, Mechref Y, Novotny MV. *Rapid Commun Mass Spectrom*. 2008; 22(5):721–34. [PubMed: 18265433]
36. Mann B, Madera M, Sheng Q, Tang H, Mechref Y, Novotny MV. *Rapid Commun Mass Spectrom*. 2008; 22(23):3823–34. [PubMed: 18985620]
37. Kobata A. *Biochim Biophys Acta*. 2008; 1780(3):472–8. [PubMed: 17659840]
38. Crocker PR, Redelinghuys P. *Biochem Soc Trans*. 2008; 36(Pt 6):1467–71. [PubMed: 19021577]
39. Marth JD, Grewal PK. *Nat Rev Immunol*. 2008; 8(11):874–87. [PubMed: 18846099]
40. Grivennikov SI, Greten FR, Karin M. *Cell*. 2010; 140(6):883–99. [PubMed: 20303878]
41. Dang F, Kakehi K, Nakajima K, Shinohara Y, Ishikawa M, Kaji N, Tokeshi M, Baba Y. *J Chromatogr A*. 2006; 1109(2):138–43. [PubMed: 16376899]
42. Bynum MA, Yin H, Felts K, Lee YM, Monell CR, Killeen K. *Anal Chem*. 2009; 81(21):8818–25. [PubMed: 19807107]
43. Hennig C, Rink L, Kirchner H. *Lancet*. 2000; 355(9215):1617–8. [PubMed: 10821371]
44. Arnold JN, Radcliffe CM, Wormald MR, Royle L, Harvey DJ, Crispin M, Dwek RA, Sim RB, Rudd PM. *J Immunol*. 2004; 173(11):6831–40. [PubMed: 15557177]
45. Calvo C, Ulloa N, Campos M, Verdugo C, Ayrault-Jarrier M. *Clin Chim Acta*. 1993; 217(2):193–8. [PubMed: 8261628]
46. Abbas, AK.; Lichtman, AH. *Cellular and Molecular Immunology*. 5. 2003.
47. Crispin MD, Ritchie GE, Crichtley AJ, Morgan BP, Wilson IA, Dwek RA, Sim RB, Rudd PM. *FEBS Lett*. 2004; 566(1–3):270–4. [PubMed: 15147907]
48. Arnold JN, Wormald MR, Suter DM, Radcliffe CM, Harvey DJ, Dwek RA, Rudd PM, Sim RB. *J Biol Chem*. 2005; 280(32):29080–7. [PubMed: 15955802]
49. Mattu TS, Pleass RJ, Willis AC, Kilian M, Wormald MR, Lellouch AC, Rudd PM, Woof JM, Dwek RA. *J Biol Chem*. 1998; 273(4):2260–72. [PubMed: 9442070]
50. Kanoh Y, Mashiko T, Danbara M, Takayama Y, Ohtani S, Egawa S, Baba S, Akahoshi T. *Anticancer Res*. 2004; 24(5B):3135–9. [PubMed: 15510601]
51. Saldova R, Fan Y, Fitzpatrick JM, Watson RW, Rudd PM. *Glycobiology*. 2011; 21(2):195–205. [PubMed: 20861084]
52. Vasseur JA, Goetz JA, Alley WR Jr, Novotny MV. *Glycobiology*. 2012 Submitted.
53. de Leoz ML, Young LJ, An HJ, Kronewitter SR, Kim J, Miyamoto S, Borowsky AD, Chew HK, Lebrilla CB. *Mol Cell Proteomics*. 2011; 10(1):M110 002717. [PubMed: 21097542]
54. Klein A, Michalski JC, Morelle W. *Proteomics Clin Appl*. 2010; 4(4):372–8. [PubMed: 21137057]
55. Klein A, Carre Y, Louvet A, Michalski JC, Morelle W. *Proteomics Clin Appl*. 2010; 4(4):379–93. [PubMed: 21137058]

Figure 1.

Chromatograms for (triplicate) enrichment of 500 μg of IgG on a Protein L-silica microcolumn (50×0.5 mm). The sample was loaded in PBS and unbound protein was washed from the column at a flow rate of 20 $\mu\text{L}/\text{min}$. After 35 min, 100 mM acetic acid was applied at a flow rate of 40 $\mu\text{L}/\text{min}$ to elute bound IgG.

Figure 2.

Relative abundance of N-glycan structures identified in the Protein L-fractions of immunoglobulins, which were separated using an initial volume of 3 μ l of pooled female serum for each analysis. Abundance was evaluated using the in-house developed program PeakCalc. Six technical replicates were performed for the affinity chromatography experiments, while each technical replicate was spotted six times on the MALDI target. The mean relative intensity from the separate enrichment experiments was used to determine the average value and standard deviation for each glycan structure. Monosaccharides are depicted as follows: blue square represents N-acetylglucosamine, green circle is mannose, yellow circle is galactose, red triangle represents fucose, and pink diamond is sialic acid.

Figure 3.

A comparison of the relative intensities of the glycan structures from the IgG-depleted immunoglobulins. Six samples from cancer patients were compared to six samples from control, cancer-free individuals. Abundance was evaluated using the in-house developed program PeakCalc. Six technical replicates were performed for the affinity chromatography experiments, while each technical replicate was spotted six times on the MALDI target. The mean relative intensity from the separate enrichment experiments was used to determine the average value and standard deviation for each glycan structure. Monosaccharides depicted are the same as in Fig. 2.

Table 1

Label-free Comparative Proteomics for Protein L-Agarose Enrichment of Human Blood Serum.

UniProt Accession	Proteins	Mean Protein Area (%)		
		Unbound (N=3)	Bound (N=6)	B/U
P01857	Ig gamma-1 chain C region	1.96	9.27	4.7
P01859	Ig gamma-2 chain C region	1.28	6.40	5.0
P01860	Ig gamma-3 chain C region	0.03	0.44	16.0
P01861	Ig gamma-4 chain C region	0.04	0.74	18.9
P01876	Ig alpha-1 chain C region	1.14	5.15	4.5
P01877	Ig alpha-2 chain C region	0.02	0.25	10.3
P01871	Ig mu chain C region	1.01	1.55	1.5
P01591	Immunoglobulin J chain	0.02	0.04	1.8
P01834	Ig kappa chain C region	1.54	16.53	10.7
P0CG04	Ig lambda chain C region	1.13	0.76	0.7
N/A	Ig light chains V region	1.14	6.52	5.7
N/A	Ig heavy chains V region	1.03	1.04	1.0
	Total Ig	10.35	48.68	4.7
P02768	Serum albumin	41.29	21.30	0.5

Table 2

Proteomic profiles of bound fractions from Protein L enrichment of pooled blood sera.

UniProt Accession	Proteins	Protein Area (%)	SD (N=6)	CV (%)
<u>Immunoglobulins</u>				
P01876	Ig alpha-1 chain C region	31.13	2.28	7.3
P01834	Ig kappa chain C region	18.66	2.37	12.7
P01871	Ig mu chain C region	10.47	1.92	18.3
P01591	Immunoglobulin J chain	1.95	0.65	33.1
P0CG04	Ig lambda chain C region	1.88	0.18	9.4
P01857	Ig gamma-1 chain C region	1.55	0.50	32.5
P01877	Ig alpha-2 chain C region	0.96	0.11	11.9
P01880	Ig delta chain C region	0.06	0.01	18.0
	Ig kappa chains V region	4.98		
	Ig heavy chains V region	1.15		
	Total	72.78		
<u>Non-glycoproteins</u>				
P02647	Apolipoprotein A-I	7.56	0.95	12.5
P02768	Serum albumin	2.60	0.39	14.9
P02652	Apolipoprotein A-II	2.43	0.27	11.2
O43866	CD5 antigen-like protein	2.25	0.42	18.7
	Other proteins	1.79		
	Total	16.62		
<u>N-linked Glycoproteins</u>				
P0C0L4	Complement C4-A	3.40	0.72	21.3
P01024	Complement C3	2.53	0.11	4.4
P04114	Apolipoprotein B-100	1.59	0.51	31.8
	Other proteins	3.07		
	Total	10.60		