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Particle-based N-linked glycan analysis of selected proteins from biological samples using nonglycosylated binders

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ABSTRACT

Glycosylation is one of the most common and important post-translational modifications, influencing both the chemical and the biological properties of proteins. Studying the glycosylation of the entire protein population of a sample can be challenging because variations in the concentrations of certain proteins can enhance or obscure changes in glycosylation. Furthermore, alterations in the glycosylation pattern of individual proteins, exhibiting larger variability in disease states, have been suggested as biomarkers for different types of cancer, as well as inflammatory and neurodegenerative diseases. In this paper, we present a rapid and efficient method for glycosylation analysis of individual proteins focusing on changes in the degree of fucosylation or other alterations to the core structure of the glycans, such as the presence of bisecting *N*-acetylglucosamines and a modified degree of branching. Streptavidin-coated magnetic beads are used in combination with genetically engineered immunoaffinity binders, called VHH antibody fragments. A major advantage of the VHHs is that they are nonglycosylated; thus, enzymatic release of glycans from the targeted protein can be performed directly on the beads. After deglycosylation, the glycans are analyzed by MALDI-TOF-MS. The developed method was evaluated concerning its specificity, and thereafter implemented for studying the glycosylation pattern of two different proteins, alpha-1-antitrypsin and transferrin, in human serum and cerebrospinal fluid. To our knowledge, this is the first example of a protein array-type experiment that employs bead-based immunoaffinity purification in combination with mass spectrometry analysis for fast and efficient glycan analysis of individual proteins in biological fluid.

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1. Introduction

N-linked glycosylation is one of the most common post-translational modifications of proteins. The processes that glycosylate proteins are enzyme driven and can be affected by several factors, such as pregnancy, diet, alcohol abuse, or different diseases. A specific protein will in many cases retain its glycosylation after leaving the cell where it was produced. This implies that information concerning the state of the cell where the protein was produced could be gathered by sampling proteins far from the cell of origin [1]. Our knowledge of the changes to protein glycosylation as a consequence of disease has increased substantially over the last decade [1–5]. Many of the serum protein biomarkers that are used to detect or monitor cancer are glycoproteins and several changes to protein glycosylation have also been presented [1,6]. For example, the presence of bisecting *N*-acetylglucosamine (GlcNAc) and increased fucosylation of the antennae of glycans have been shown to correlate with different cancer types. Changes to the glycosylation of immunoglobulin-γ [7], alpha-1-acid glycoprotein [8] and alpha-1-antitrypsin (AAT) [5], among others, have been observed for inflammatory diseases. Change in glycosylation has also been observed in several neurodegenerative diseases, but in these conditions, cerebrospinal fluid (CSF), rather than serum, is the biological sample of choice [3,9–12]. The rationale for this is that so-called *brain-type* glycosylation occurs in proteins native to the central nervous system [13–15]. Such *brain-type* glycosylation is characterized by high levels of bisecting GlcNAc and proximal fucosylation.

Bead-based approaches for the selective extraction of a single protein from a complex biological sample have been used extensively for studies concerning protein complexes or

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interactions between proteins [16,17]. However, bead-based approaches have found limited use in the study of protein glycosylation. Most studies concerning the glycosylation of specific proteins have been performed using a combination of one- or two-dimensional gel-electrophoretic separation of proteins followed by enzymatic release of the glycans from excised spots [12,18,19]. Bead-based protocols can more easily be multiplexed and run in parallel than two-dimensional gel electrophoresis and have a higher degree of selectivity than one-dimensional gel electrophoresis. Currently, the most efficient methods for glycan pattern analysis of specific proteins use bead-based affinity extraction in combination with fluorescent labeling of enzymatically released glycans and subsequent separation and detection using multichannel capillary electrophoresis with laser-induced fluorescence detection [20]. This combination has been used to evaluate immunoglobulin A and AAT as possible biomarkers using 2415 different serum samples. Although fluorescence detection is a very good quantitative technique, it does not have the possibility of providing any additional data concerning the chemical structure of the glycan other than the elution or migration time in the chromatographic or electrophoretic separation system.

We have developed a method for glycan analysis of selected serum and CSF proteins using affinity binders immobilized on particles. Streptavidin-coupled magnetic microparticles were used for the immobilization of genetically engineered immunoaffinity binders. The captured proteins were then deglycosylated using peptide *N*-glycosidase F (PNGase-F) and desialylated using neuraminidase directly on the beads. Removing the sialic acids reduces the heterogeneity of the glycans that may arise from different degrees of sialylation, highlighting the differences corresponding to alterations to the glycan core structures. A simple clean-up and desalting step using spin-tips containing a carbon particulate phase were employed for sample preparation of the released glycans prior to MALDI-MS analysis.

2. Experimental

2.1. Materials and reagents

Pierce™ streptavidin magnetic beads, Hypersep™ porous graphitized carbon (hypercarb) spin tips 10–200 μL, CaptureSelect™ Biotin anti-AAT conjugate, and CaptureSelect™ Biotin anti-transferrin conjugate were obtained from Thermo Scientific (Waltham, MA, USA). Standard human AAT was obtained from Acris Antibodies Inc. (San Diego, CA, USA). Amicon Ultra 0.5-mL centrifugal filters, 2,5-dihydroxybenzoic acid (DHB), dimethylaniline (DMA), standard human transferrin, and the commercially available N-linked glycan standards H5N2 [$(Gal\text{-}GlcNac)_2Man_3(GlcNac)_2$] and H5N4 [$2Man_3(GlcNac)_2$] were purchased from Sigma-Aldrich (St. Louis, MO, USA). PNGase F kit for enzymatic release of N-linked glycans and α-2-3,6,8 neuraminidase kit for the release of sialic acids were obtained from New England Biolabs UK Ltd. (Hitchin, UK). Acetonitrile (analytical grade) was purchased from Rathburn Chemicals Ltd. (Walkerburn, UK). Water was purified using a Millipore water purification system to resistance >18 MΩ/cm.

2.2. Biological samples

2.2.1. Human serum sample

Reference human blood samples were acquired from healthy donors through Karolinska Institutet, Sweden. Once received, the samples were centrifuged at 1100 × *g* for 15 min and the clear serum aliquoted into microtubes and stored at −80° C until further use.

2.2.2. Human CSF sample

Neurologically healthy control CSF samples were obtained from the study conducted by Kamali-Moghaddam et al. [21]. That study, which was conducted in accordance with the Declaration of Helsinki, was approved by the Regional Ethical Review Boards of Uppsala, Sweden and Cluj, Romania, respectively, and the written consent of the individual donors were obtained.

2.3. General procedure for immunoprecipitation using magnetic beads and VHH antibody fragments

Aliquots of streptavidin-coated magnetic bead suspensions (50 μL, 10 mg/mL) were transferred to 0.5-mL tubes. By using a magnet to sediment the particles, the supernatant (storage liquid) was discharged and the beads were washed with phosphate-buffered saline (PBS) (3 × 300 μL). After washing, a solution of AAT/transferrin CaptureSelect™ camelid single domain antibody fragments (VHHs) in PBS (150 μL, 0.0167 mg/mL) was added to the magnetic beads, followed by incubation for 1 h at room temperature while mixing, to facilitate biotin–streptavidin binding. Thereafter, the magnetic beads were washed with PBS (3 × 300 μL) and incubated with sample solution (150 μL) for 1 h at room temperature while mixing, to allow the protein to be captured by the VHH antibody fragment. Optimization of the protein-binding time was performed by following the reaction with capillary electrophoresis (CE) for 2 h. During this time, small aliquots (7 μL) of the supernatant were withdrawn after 0, 5, and 30 min, and 1, 1.5, and 2.0 h.

After protein binding, the supernatant was discarded and the magnetic beads were washed with PBS (2 × 300 μL). Samples from the washing steps were saved for CE analysis. To avoid a contribution by nonspecifically bound proteins adsorbed to the plastic, a third aliquot of PBS (150 μL) was used to suspend the beads and transfer them to clean 0.5-mL tubes, followed by a fourth wash with PBS (150 μL). Enzymatic cleavage of the glycans from the bead-immobilized proteins was conducted using a mixture of PNGase F and neuraminidase (5:1 U) in H_2O (total volume 150 μL). Release of glycans was achieved after 2 h of incubation while mixing at 37 °C. For comparison, deglycosylation was also performed for 4 and 18 h.

The supernatant containing the glycans was subsequently collected and purified using porous graphitized carbon solid-phase extraction spin tips, in accordance with the manufacturer's instructions. Activation of the columns was performed by adding 0.05% TFA in ACN/H_2O (6:4) (3 × 50 μL) followed by 0.05% TFA in H_2O (3 × 50 μL). The glycan-containing supernatant was then loaded on the carbon and the column was washed with 0.05% TFA in H_2O (3 × 50 μL). Finally, the glycans were eluted with 0.05% TFA in ACN/H_2O (6:4) (4 × 50 μL). Before mass spectrometry analysis, the sample was concentrated to approximately 5 μL by solvent evaporation at room temperature using a stream of N_2. After concentration, the sample was mixed 1:1 v/v with the matrix solution (DHB/DMA; 21.52 mg/ml DHB and 4.64 mg/ml DMA in 50% ACN) and spotted (1 μL) on a MALDI plate.

2.3.1. Standard AAT protein

Standard AAT protein (0.051 mg/mL) in PBS was used as a positive control for AAT CaptureSelect™ VHH in the development of the optimized general procedure for immunoprecipitation using magnetic beads and VHH antibody fragments.

2.3.2. Standard transferrin protein

Standard transferrin protein (0.13 mg/mL) in PBS was used as a positive control for transferrin CaptureSelect™ VHH in the development of the optimized general procedure for immunoprecipitation using magnetic beads and VHH antibody fragments.

2.3.3. Protein mix A

Protein mix A was prepared by mixing AAT (0.040 mg/mL), human serum albumin (0.70 mg/mL), transferrin (0.098 mg/mL), and herceptin (0.31 mg/mL) in PBS. This mix was used as a positive control for both AAT and transferrin CaptureSelect™ VHHs in the development of the optimized general procedure for immunoprecipitation using magnetic beads and VHH antibody fragments.

2.3.4. Protein mix B

Protein mix B was prepared by mixing ceruloplasmin (0.037 mg/mL), human serum albumin (0.70 mg/mL), transferrin (0.098 mg/mL), and herceptin (0.31 mg/mL) in PBS. This mix was used as a negative control for the AAT CaptureSelect™ VHH in the development of the optimized general procedure for immunoprecipitation using magnetic beads and VHH antibody fragments.

2.3.5. Protein mix C

Protein mix C was prepared by mixing AAT (0.040 mg/mL), ceruloplasmin (0.027 mg/mL), human serum albumin (0.70 mg/mL), and herceptin (0.31 mg/mL) in PBS. This mix was used as a negative control for the transferrin CaptureSelect™ VHH in the development of the optimized general procedure for immunoprecipitation using magnetic beads and VHH antibody fragments.

2.3.6. Human serum

Human serum was diluted approximately 20 times with PBS before addition to the magnetic beads coated with either AAT or transferrin CaptureSelect™ VHH antibody fragments.

2.3.7. Human CSF

To establish the same binding conditions as for serum, the human CSF was preconcentrated approximately 20 times using Amicon centrifugal filters followed by 20-fold dilution with PBS before addition to the magnetic beads coated with either AAT or transferrin CaptureSelect™ VHH antibody fragments. This step also functions as a clean-up removing salts as well as peptides and small proteins.

2.4. MALDI-TOF-MS analysis

All of the mass spectra were acquired using a Voyager-DE™ STR mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA), equipped with a nitrogen laser (337 nm), in reflector mode and with positive ionization. Before each analysis, the instrument was calibrated using a mixture of the Man5 and NA2 glycan standards. The acquisition was performed with an accelerating potential of 20 kV, grid voltage set at 60%, and a delay time of 100 ns. The laser intensity was set at the lowest value that could produce strong glycan peaks while keeping the noise level low. Spectra were acquired in the mass range from m/z 800 to m/z 3000, with the Low Mass Gate set at m/z 700. All spectra, resolutions, and signal-to-noise ratios were processed and calculated using the Data Explorer V4 software (Applied Biosystems Inc., Foster City, CA, USA).

2.5. Capillary electrophoresis analysis

To determine the yield of the immunoaffinity extraction of the target proteins, a previously published CE method [16] was adapted and modified on a Hewlett Packard 3D CE instrument equipped with a UV/Vis diode array detector. The separation was carried out with a 0.1 M borate buffer (pH 10) as a background electrolyte in a fused silica capillary (effective length 80 cm, i.d. 50 μm), using a positive voltage of 30 kV at room temperature. This resulted in a separation current of 75 μA. The samples were injected hydrokinetically by applying pressure of 30 mbar for 30 s. The detection of the target proteins was performed at 200 nm, with a 20-nm bandwidth. To each sample 3 μL of a solution containing 295 mM sodium hydrogen carbonate and 1.3 mM sodium benzoate (volumetric internal standard) was added. When transferrin was analyzed this solution also contained 4.0 mM iron(III)chloride to induce all transferrin molecules to adopt the same charge state. Samples were further diluted to a final volume of 10 μL by the addition of deionized water. This gave final concentrations in the sample solution of 88, 1.2, and 0.4 mM respectively for sodium hydrogen carbonate, iron(III)chloride, and sodium benzoate respectively. The total length of the run under these conditions was 35 min, including the conditioning of the capillary.

3. Results and discussion

For developing the present method, streptavidin-coated magnetic microbeads were used. At an early stage, these particles were compared with N-hydroxysuccinimide-activated agarose beads for use as the affinity capture media, but nonspecific interaction of proteins with the agarose beads was more prevalent. The magnetic beads also have the advantage of being easy to handle using magnets. Commercially available VHH antibody fragments were used for both AAT and transferrin. The most common affinity reagent, immunoglobulin-γ (IgG), typically consists of two identical heavy (H) peptide chains and two identical light (L) peptide chains consisting of four and two domains respectively [22]. VHH antibody fragments are the single variable domains derived from homodimeric heavy-chain antibodies (HCAabs), originally found in Camelidae [23]. As these genetically engineered constructs are equivalent to one Fab domain in a conventional antibody, they are antigen-binding fragments. In addition to their small size (approximately 15 kDa), other properties such as a long shelf-life [24] and structural stability under harsh chemical and thermal conditions [25] make them a powerful tool for various diagnostic and therapeutic applications. Nonglycosylated binders have the advantage of facilitating the direct on-bead deglycosylation of captured proteins, thus simplifying the workflow and shortening the overall time of the procedure.

The initial tests of the affinity capture and enzymatic release steps were performed with volumes and reagents as suggested by the supplier. Miniaturization of the reactions was then performed to reduce the consumption of reagents and affinity binders. This also shortens the overall reaction time by eliminating or reducing evaporation steps prior to the MALDI sample preparation. The total time required for the full assay, including the MALDI-MS experiment, was 6 h. A schematic overview of the full procedure can be seen in Fig. 1.

3.1. Selection of target proteins

The rationale for choosing AAT and transferrin as target proteins in the development and assessment of this method is that both of these proteins have the potential to be used as glycan biomarkers. AAT is a protease inhibitor of approximately 52 kDa that can be glycosylated at three different sites and its concentration in plasma can fluctuate from 0.88 to 1.68 mg/mL, increasing three to four times during inflammatory and malignant conditions. Additionally, changes in the glycosylation pattern of this protein have been associated with cardiovascular and metabolic diseases, as well as liver malfunction [20]. Transferrin is a protein of approximately 80 kDa with a plasma concentration of 2–3 mg/mL. It is composed of 679 amino acids arranged into two domains, each of which contains an iron-binding site. The main function of transferrin is binding Fe^{3+} and transporting it through the body as a way of regulating the iron concentration in biological fluids. Glycosylation can occur at two different sites of the protein and the glycosylation state of trans-

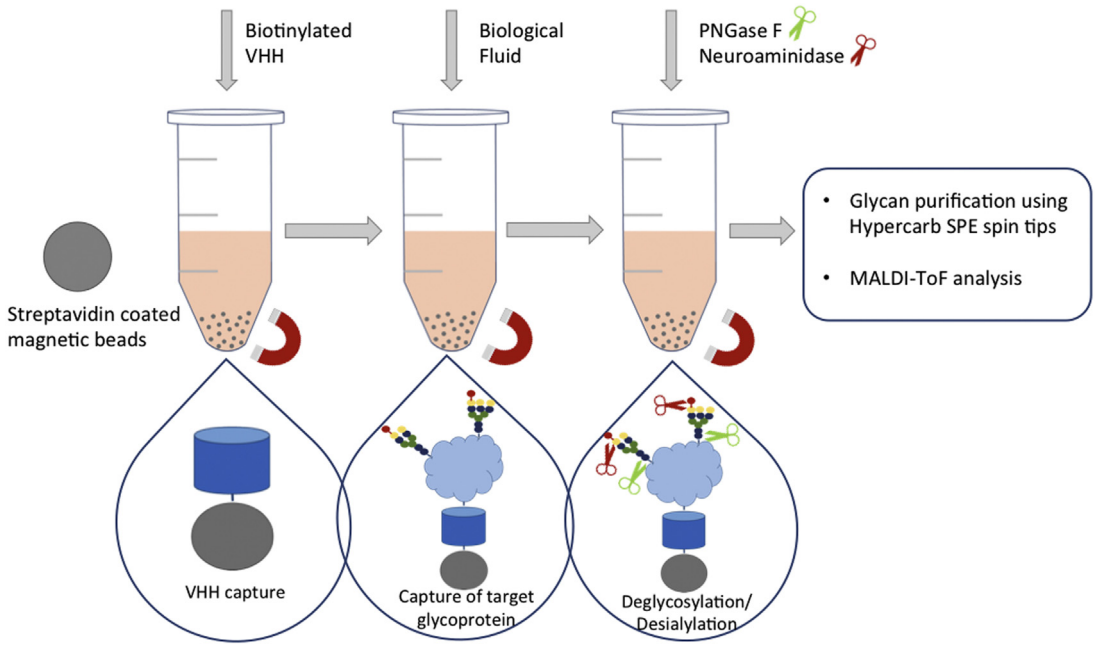


Fig. 1. A schematic overview of the bead-based method developed for glycosylation analysis of individual proteins in biological fluids.

ferrin has been used to diagnose chronic alcoholism and congenital disorders of glycosylation, among others [26,27].

3.2. Assessment of binding efficiency using CE

The time required for performing an assay depends on the protein and reagent concentrations and the strength of the interaction between the binder used and the target protein. A higher equilibrium binding constant facilitates shorter reaction times. The capture efficiency of the protein by the immunoaffinity reagent was studied using CE. CE was chosen because of the small injection volumes necessary for this technique, whereby the supernatant and wash solution were sampled on several occasions in the workflow without significant losses. Quantification of AAT and transferrin was performed using a solvent-matched calibration curve ranging from 0.08 to 0.28 mg/mL. The amounts of protein used correspond to the amounts present in diluted serum and are at approximately two to three times in excess compared with the amount of VHH used. Triplicate analysis of the supernatant of the binding step, withdrawn at specific times as mentioned above, showed that the binding of both AAT and transferrin was completed in the first 10 min of the incubation. After this time point, the concentration of both proteins in the supernatants remained stable (Supporting information, Fig. S1), indicating saturation of the VHHs by the target protein. Moreover, the analyses of the supernatant in the subsequent washing step showed that no detectable amounts of protein were released, proving the effectiveness of the binding (results not shown).

3.3. Assessment of deglycosylation efficiency

The deglycosylation efficiency of the employed enzyme mixture (PNGase F and neuraminidase 5:1 U) was followed over time. The bead-based procedure described above was used to capture AAT

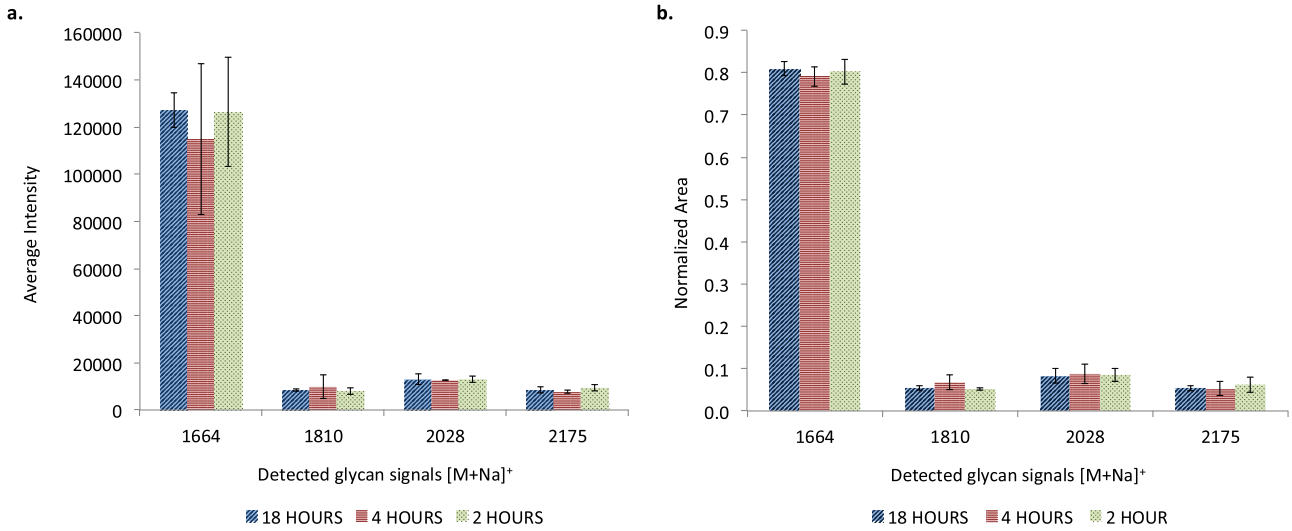


Fig. 2. Glycan species detected and their (a) average intensity and (b) normalized area after different deglycosylation times for AAT standard. The normalized area is calculated as the area for a specific glycan signal (for instance $m/z = 1810$) divided by the sum of the areas from all the glycan signals in that spectrum. Mean values were generated from two replicates analyzed in duplicate. Error bars indicate standard deviations.

from a standard solution (0.046 mg/mL). The captured glycoprotein was then deglycosylated for 18, 4, and 2 h using the enzyme mixture at 37 °C under agitation. For each time point tested, two replicates were analyzed in duplicate. The comparison of the results from each experiment showed no significance difference, in terms of the intensities (Fig. 2a) and ratios (Fig. 2b) of glycan species detected, when the deglycosylation was performed for a shorter time. The intensities (Fig. 2a) have a large relative variation, which is to be expected when comparing the absolute signal intensities from MALDI measurements but this is not reflected in the relative intensities between different signals within spectra (Fig. 2b).

3.4. Specificity and reproducibility of the bead-based approach

The cross-selectivity of the immunoaffinity binders used in this study was assessed by testing different standard protein mixtures. Protein mix A, containing both AAT and transferrin, was used as a positive control for both proteins, whereas protein mixes B and C, not containing the target protein AAT or transferrin respectively, were used as negative controls. Representative N-glycan spectra of the proteins extracted from protein mix A can be seen in Supporting information Fig. S2 (AAT) and S3 (transferrin).

To avoid contamination by other proteins adsorbed to the plastic, the beads (with the target protein) were transferred to new tubes before deglycosylation. A comparison between a protein mix containing the target protein (positive control) and a protein mix not containing the target protein (negative control) showed that the signal deriving from nonspecific binding of other proteins was less than 5%, i.e. the intensity from the protein mix not containing the target protein was less than 5% of the signal from the protein mix containing the target protein, see Supporting information Fig. S2 and S3 .

To assess the reproducibility of the method, standard AAT protein was analyzed on three different days. Mean values for each day were generated from two replicates analyzed in duplicate (Fig. S4). The RSD values from variations between days were found to be smaller than the variations within a day (Table S1). Thus, the method can be deemed to be reproducible.

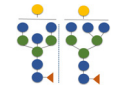
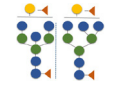
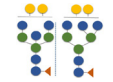
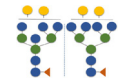
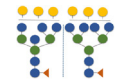
Proposed glycan structures, based on the detected *m/z* values and findings reported in the literature [9,28–30], can be seen in Table 1. The glycans are all detected as sodium adducts, but traces of potassium adducts can also be seen.

3.5. Glycosylation pattern of AAT and transferrin in biological samples

Representative N-glycan spectra from AAT and transferrin are shown in Fig. 3. The spectra shown are from experiments with standard protein solution (Fig. 3a), serum (Fig. 3b), and CSF (Fig. 3c). For both AAT and transferrin, the glycan patterns of human serum correspond to the glycan pattern seen for standard protein and they are in accordance with the literature [28,29]. Thus, the developed bead-based procedure can be considered accurate. The developed bead-based method was used to investigate the glycan pattern of AAT in both serum and CSF. Five healthy control samples were used for serum analysis and three for CSF analysis. Very similar AAT glycan patterns were obtained for the different individuals, both in serum and CSF (Fig. 4). The detected glycan pattern in CSF is however markedly different from that observed in serum. In serum, the major glycan for all individuals is H5N4, but all test subjects also have H5N4F, H6N5, and H6N5F glycans (Table 1, Fig. 4). H6N5 is the second largest glycan, followed by H6N5F, and finally H5N4F.

The most striking difference between CSF and serum is the large number of different glycans observed in CSF. In serum, four different glycans were detected, whereas 15 different glycans were observed in CSF. As in serum, the most abundant glycan in CSF is H5N4. However, in serum, H5N4 constitutes 60–75% of the total glycans, whereas in CSF, that proportion is only around 25–50% (Fig. 4). Two other glycan species, H5N4F and H6N5F, are found in proportions of around 10% or more and the rest of the detected glycans are in amounts under 10%. Several of the detected glycans are bisecting (e.g. H3N5F, H4N5F, and H5N6F) and one is a high mannose (H5N2) (Table 1, Fig. 4). To the best of our knowledge, this is the first time that the full glycan pattern of AAT in CSF has been analyzed.

Table 1
Identification of the different glycan species.
*Composition residues of glycans: H, hexose; N, N-acetylglucosamine; F, fucose
*Structure composition: blue circles, N-acetylglucosamine; green circles, mannose; yellow circles, galactose; orange triangles, fucose

Peak	$[M+Na]^+$	Identity	Structure
1	1257	H5N2	
2	1337	H3N4	
3	1485	H3N4F	
4	1502	H4N4	
5	1648	H4N4F	
6	1664	H5N4	
7	1688	H3N5F	
8	1810	H5N4F	
9	1851	H4N5F	
10	1891	H3N6F	
11	1996	H4N5F2	
12	2013	H5N5F	
13	2028	H6N5	
14	2053	H4N6F	
15	2175	H6N5F	
16	2216	H5N6F	
17	2378	H6N6F	
18	2540	H7N6F	

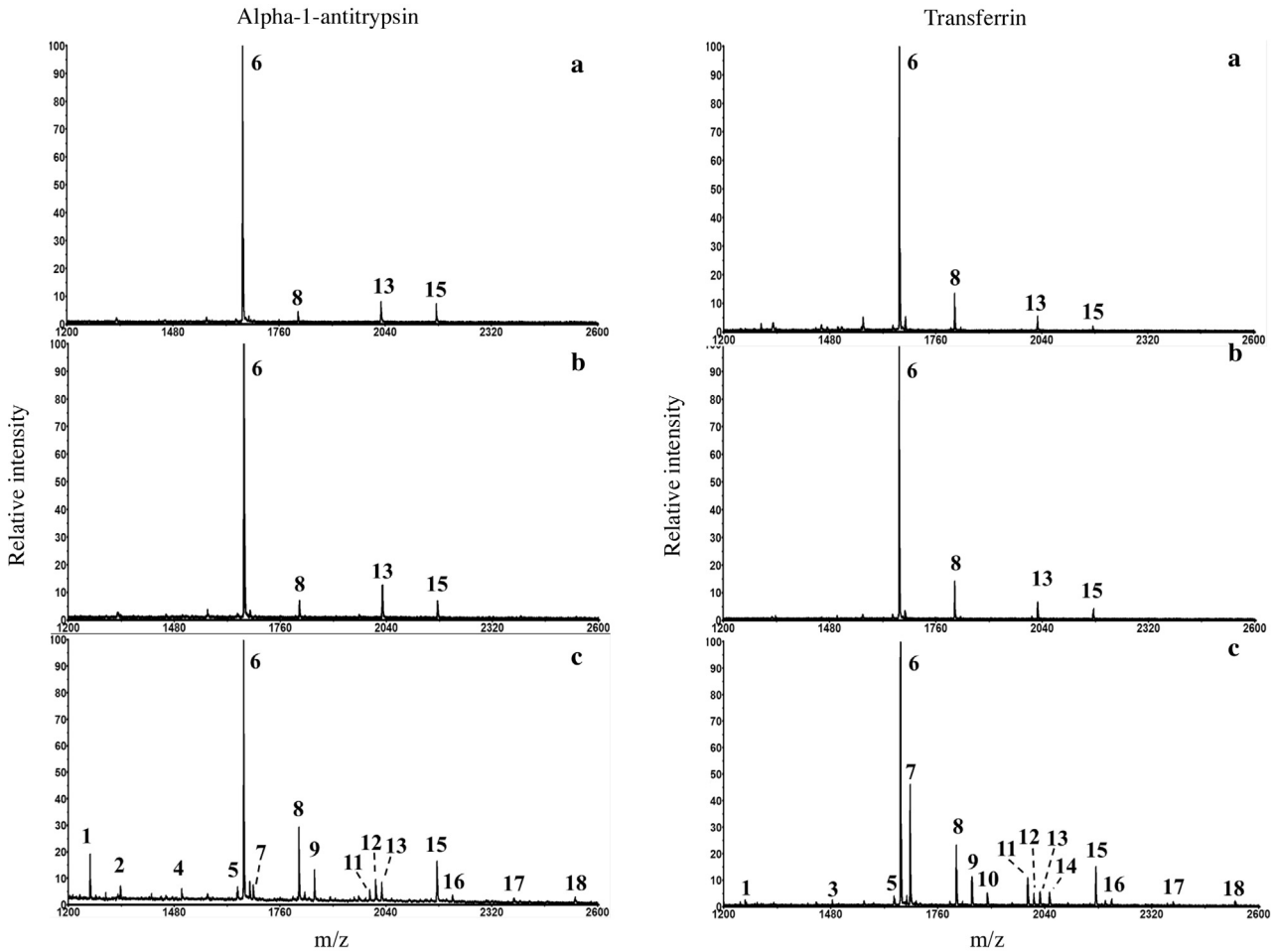


Fig. 3. Representative *N*-glycan spectra of alpha-1-antitrypsin and transferrin purified from (a) standard protein, (b) human serum, and (c) human cerebrospinal fluid. The suggested identity of each glycan signal is displayed in Table 1.

Only one CSF sample was analyzed regarding transferrin. However, one striking difference compared with AAT would appear to be the much higher abundance of H3N5F (Fig. 3). A probable reason for this observation is that CSF has been shown to contain two transferrin glycoforms, usually referred to as transferrin-1 and transferrin-2 [30]. Transferrin-2 has H5N4 as the most abundant glycan but transferrin-1 has H3N5F as the most abundant, which would thus explain the much higher level of H3N5F in transferrin compared to AAT.

3.6. General discussion

Glycosylation can be viewed as the final step in the cell's information stream – functioning as an on/off switch for protein function [31]. Associated with this, defects in protein glycosylation have been noted in many diseases including cancer, inflammation, and autoimmune disorders. We have developed a method capable of multiplexed analyses of specific proteins. The procedure is both fast and efficient and uses only commercially available materials and reagents. Thus, any laboratory with access to a MALDI-TOF-MS can adapt the described method to investigate changes in N-glycosylation of specific proteins in complex sample matrixes, such as serum and CSF.

Alterations in the glycan pattern of serum proteins have shown to have potential as biomarkers for both diagnostic purposes and disease progression [32]. The current method can be used to develop panels of particle assays targeting several proteins in which N-glycosylation has been suggested as a possible cancer biomarker, such as transferrin, haptoglobin, and alpha-fetoprotein for hepatocellular cancer. Panels may also be conceived for autoimmune and inflammatory disorders containing for example alfa-1-acid glycoprotein, AAT and IgG. The continued development of new affinity binders of the VHH type, lacking glycans, should benefit the future development of a wider range of assays.

In this study, the enzymatic release of glycans was achieved using the enzyme PNGase F in combination with neuraminidase. All N-linked glycans, except those that contain an α1-3-linked fucose at the reducing terminal GlcNAc, will be cleaved from the asparagine residue by PNGase F. Additionally, neuraminidase will cleave the glycosidic bonds of the terminal sialic acids. Although changes in the sialic acid content of different proteins have been reported as potential biomarkers in different types of cancer (breast, liver, and ovarian) [1], the heterogeneity of the obtained spectra is increased by the presence of those residues because different sialylation levels of glycans with the same core structure will give rise to different *m/z* values. By removing sialic acid residues, the complexity of the spectra will be reduced and the sensitivity enhanced, as glycans with a certain core structure will appear as only one signal [33]. For a number of different diseases, alterations to the core structure or branching of the glycans have been presented, which can be studied using the present method [1]. If differences in sialylation levels are to be measured, then the present method will probably need to be extended to include permethylation or esterification of the released glycans prior to MALDI-MS analysis.

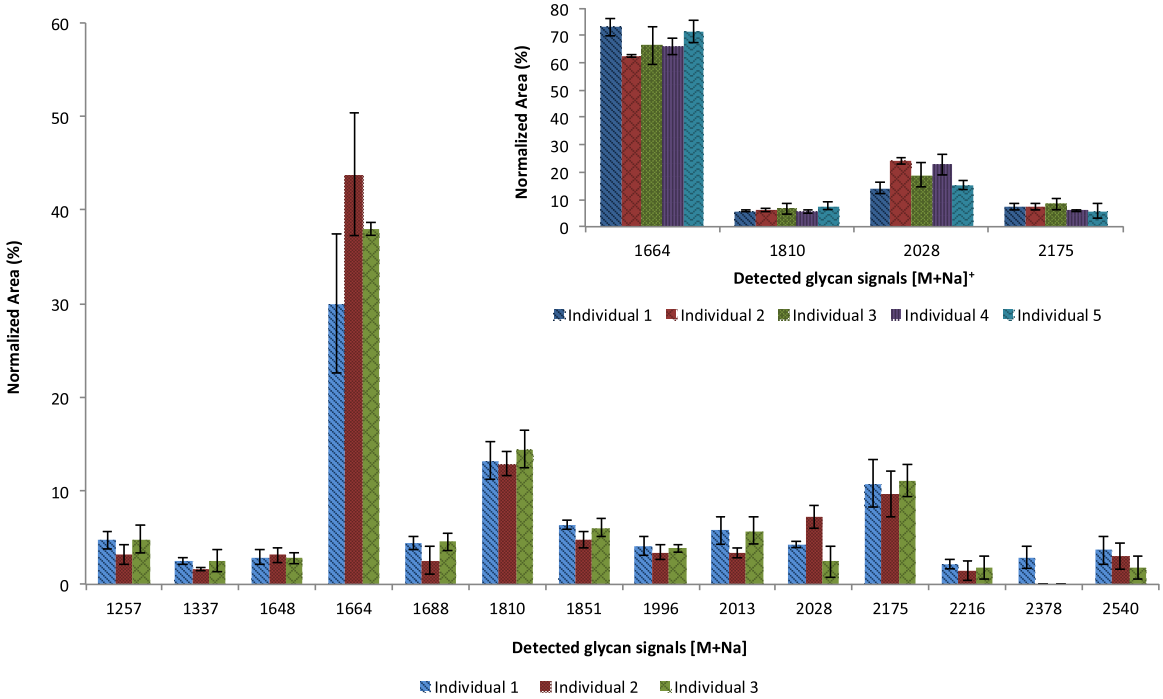


Fig. 4. Graphical comparison of the glycosylation pattern of alpha-1-antitrypsin in serum from five different healthy control individuals (chart inserted in the top right corner) and in cerebrospinal fluid from three different healthy control individuals (main chart). Mean values were generated from two replicates analyzed in duplicate. Error bars indicate standard deviations. The relative abundance of glycan signals is calculated as the area for a specific glycan signal (for instance $m/z = 1810$) divided by the sum of the areas from all the glycan signals in that spectrum, given as percent.

Analysis of glycan patterns of proteins from CSF is emerging as a promising new route for identifying biomarkers for neurodegenerative diseases. However, most studies so far have focused on changes to the total glycosylation pattern in CSF [3,9,34]. A problem with analyses of the entire glycoprotein content is that changes in the concentration of certain proteins may enhance or obscure the changes in glycosylation. Therefore, methods that can be used to analyze changes in the glycosylation profile of individual proteins known to be of importance for specific conditions have more potential when it comes to the diagnosis and monitoring of a disease. For example, differences in glycosylation of AAT have been noted in Parkinson's disease [11] and Alzheimer's disease [12]. For transferrin, two different glycoforms have been identified, transferrin-1 and transferrin-2. The ratio between these two different glycoforms in CSF has been suggested as a possible biomarker for Alzheimer's disease [35] and idiopathic normal pressure hydrocephalus [30]. Despite the potential for analyzing glycan patterns of specific proteins as biomarkers in neurological diseases, very few methods are available. This is probably owing to the more complex glycan patterns of proteins in CSF compared with those in serum. The particle-based assay described here has the potential to fill this void, as it can easily be expanded into an array-type method targeting several proteins in which N-glycosylation has been suggested as a possible biomarker.

4. Conclusions

A bead-based affinity purification method for profiling N-linked glycan species in serum and CSF has been developed. Streptavidin-coated magnetic microbeads and VHH based affinity binders were used as these confer the possibility of rapidly exchanging the affinity binders or easily using a number of different binders in a single experiment. This experimental setup shows a high degree of flexibility and a good possibility for automation. This, together with the ease of handling, makes this setup extremely promising for the development of array-type experiments for the simultaneous analysis of the glycosylation pattern of several target proteins. Moreover, due to the short time required for the on-beads deglycosylation and since no separation step is required, the entire procedure is performed in 6 h, allowing for the processing of a large number of samples in parallel during a single working day. To our knowledge the method proposed here is the only bead-based approach not requiring an overnight enzymatic reaction or a glycan separation step.

The applicability of this method has been exemplified by the analysis of two proteins, alpha-1-antitrypsin and transferrin, in both serum and CSF. These have been suggested as possible N-linked glycan biomarkers in a number of conditions, such as cancer, inflammation, and neurodegenerative diseases, and exhibit different glycan profiles in the tested sample types. To our knowledge, this is the first example of the use of magnetic microbeads and nonglycosylated immunoaffinity binders in combination with MS analysis for fast and efficient bead-based glycan analysis of individual proteins from serum and CSF in a protein array-type experiment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2016.09.029.

References

[1] H.J. An, S.R. Kronewitter, M.L. de Leoz, C.B. Lebrilla, Curr. Opin. Chem. Biol. 13 (2009) 601–607.
[2] E. Marklova, Z. Albahri, M. Valis, J. Neurol. Sci. 314 (2012) 20–25.
[3] R. Barone, L. Sturiale, A. Palmigiano, M. Zappia, D. Garozzo, J. Proteomics 75 (2012) 5123–5139.
[4] A. Mackiewicz, K. Mackiewicz, Glycoconjugate J. 12 (1995) 241–247.
[5] C. McCarthy, R. Saldova, M.R. Wormald, P.M. Rudd, N.G. McElvaney, E.P. Reeves, J. Proteome Res. 13 (2014) 3131–3143.
[6] D.H. Dube, C.R. Bertozzi, Nat. Rev. Drug Discov. 4 (2005) 477–488.
[7] M. Novokmet, E. Lukic, F. Vuckovic, Z. Ethuric, T. Keser, K. Rajsl, D. Remondini, G. Castellani, H. Gasparovic, O. Gornik, G. Lauc, Sci. Rep. 4 (2014) 4347.
[8] O. Gornik, G. Lauc, Dis. Markers 25 (2008) 267–278.
[9] A. Palmigiano, R. Barone, L. Sturiale, C. Sanfilippo, R.O. Bua, D.A. Romeo, A. Messina, M.L. Capuana, T. Maci, F. Le Pira, M. Zappia, D. Garozzo, J. Proteomics 131 (2016) 29–37.
[10] R. Barone, L. Sturiale, A. Fiumara, A. Palmigiano, R.O. Bua, R. Rizzo, M. Zappia, D. Garozzo, Autism Res. Off. J. Int. Soc. Autism Res. 9 (2016) 423–428.
[11] S. Jesse, S. Lehnert, O. Jahn, L. Parnetti, H. Soininen, S.K. Herukka, P. Steinacker, S. Tawfik, H. Tumani, C.A. von Arnim, M. Neumann, H.A. Kretzschmar, H. Kulaksiz, M. Lenter, J. Wiltfang, B. Ferger, B. Hengerer, M. Otto, PLoS One 7 (2012) e48783.
[12] C. Sihlbom, P. Davidsson, M. Sjogren, L.O. Wahlund, C.L. Nilsson, Neurochem. Res. 33 (2008) 1332–1340.
[13] A. Hoffmann, M. Nimtz, R. Getzlaff, H.S. Conradt, FEBS Lett. 359 (1995) 164–168.
[14] A. Hoffmann, M. Nimtz, U. Wurster, H.S. Conradt, J. Neurochem. 63 (1994) 2185–2196.
[15] A. Goyallon, S. Cholet, M. Chapelle, C. Junot, F. Fenaille, Rapid Commun. Mass Spectrom. RCM 29 (2015) 461–473.
[16] M. Pabst, F. Altmann, Proteomics 11 (2011) 631–643.
[17] M.V. Novotny, W.R. Alley Jr., Curr. Opin. Chem. Biol. 17 (2013) 832–840.
[18] J. Bones, S. Mittermayr, N. OíDonoghue, A. Guttman, P.M. Rudd, Anal. Chem. 82 (2010) 10208–10215.
[19] I.M. Lazar, J. Deng, F. Ikenishi, A.C. Lazar, Electrophoresis 36 (2015) 225–237.
[20] L.R. Ruhaak, C.A. Koeleman, H.W. Uh, J.C. Stam, D. van Heemst, A.B. Maier, J.J. Houwing-Duistermaat, P.J. Hensbergen, P.E. Slagboom, A.M. Deelder, M. Wuhrer, PLoS One 8 (2013) e73082.
[21] A.L. Lind, D. Wu, E. Freyhult, C. Bodolea, T. Ekegren, A. Larsson, M.G. Gustafsson, L. Katila, J. Bergquist, T. Gordh, U. Landegren, M. Kamali-Moghaddam, PLoS One 11 (2016) e0149821.
[22] E.A. Padlan, Mol. Immunol. 31 (1994) 169–217.
[23] C. Hamers-Casterman, T. Atarhouch, S. Muyldermans, G. Robinson, C. Hamers, E.B. Songa, N. Bendahman, R. Hamers, Nature 363 (1993) 446–448.
[24] M. Arbabi Ghahroudi, A. Desmyter, L. Wyns, R. Hamers, S. Muyldermans, FEBS Lett. 414 (1997) 521–526.
[25] M. Dumoulin, K. Conrath, A. Van Meirhaeghe, F. Meersman, K. Heremans, L.G. Frenken, S. Muyldermans, L. Wyns, A. Matagne, Protein Sci. Publ. Protein Soc. 11 (2002) 500–515.
[26] S.J. van Rensburg, P.A. Berman, F.C. Potocnik, J.J. Taljaard, Metab. Brain Dis. 15 (2000) 243–247.
[27] S.J. van Rensburg, P. Berman, F. Potocnik, P. MacGregor, D. Hon, N. de Villiers, Metab. Brain Dis. 19 (2004) 89–96.
[28] E. Landberg, E. Astrom, B. Kagedal, P. Pahlsson, Clin. Chim. Acta Int. J. Clin. Chem. 414 (2012) 58–64.
[29] F. Clerc, K.R. Reiding, B.C. Jansen, G.S. Kammeijer, A. Bondt, M. Wuhrer, Glycoconjugate J. (2015).
[30] S. Futakawa, K. Nara, M. Miyajima, A. Kuno, H. Ito, H. Kaji, K. Shirotani, T. Honda, Y. Tohyama, K. Hoshi, Y. Hanzawa, S. Kitazume, R. Imamaki, K. Furukawa, K. Tasaki, H. Arai, T. Yuasa, M. Abe, H. Arai, H. Narimatsu, Y. Hashimoto, Neurobiol. Aging 33 (2012) 1807–1815.
[31] E. Maverakis, K. Kim, M. Shimoda, M.E. Gershwin, F. Patel, R. Wilken, S. Raychaudhuri, L.R. Ruhaak, C.B. Lebrilla, J. Autoimmun. 57 (2015) 1–13.
[32] S.S. Pinho, C.A. Reis, Nat. Rev. Cancer 15 (2015) 540–555.
[33] Z. Lin, D.M. Simeone, M.A. Anderson, R.E. Brand, X. Xie, K.A. Shedden, M.T. Ruffin, D.M. Lubman, J. Proteome Res. 10 (2011) 2602–2611.
[34] S.T. Gizaw, T. Ohashi, M. Tanaka, H. Hinou, S.I. Nishimura, Biochim. Biophys. Acta (2016).
[35] K. Shirotani, S. Futakawa, K. Nara, K. Hoshi, T. Saito, Y. Tohyama, S. Kitazume, T. Yuasa, M. Miyajima, H. Arai, A. Kuno, H. Narimatsu, Y. Hashimoto, Int. J. Alzheimer's Dis. (2011) (2011) 352787.

Supporting Information for:

Particle-based N-linked glycan analysis of selected proteins from biological samples using nonglycosylated binders

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Figure S4. Reproducibility of the bead-based method 6

Table 1. Comparison of within day variation with between day variation 6

Binding Efficiency of Bead-based Approach

Capture Efficiency of the Protein by the Immunoaffinity Reagent was Studied using Capillary Electrophoresis

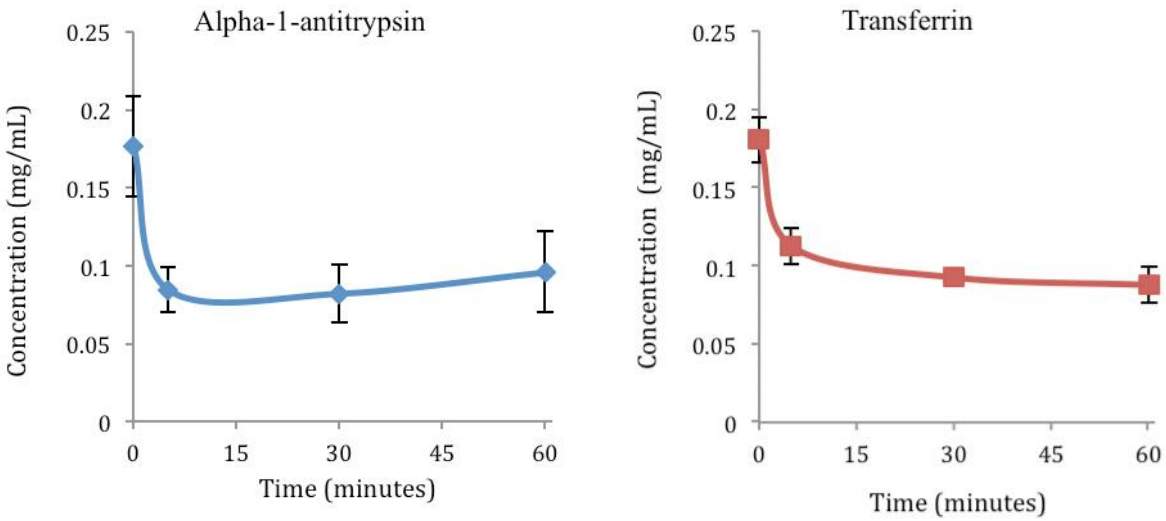

Figure S1. Capture Efficiency of the Bead-based Procedure
Concentration of standard protein in the diluted supernatant during the binding of the protein to the VHH antibody fragments for (**a**) alpha-1-antitrypsin and (**b**) transferrin.

Specificity of Bead-based Approach

Detected Amount of Unspecific Binding for Alpha-1-Antitrypsin VHH

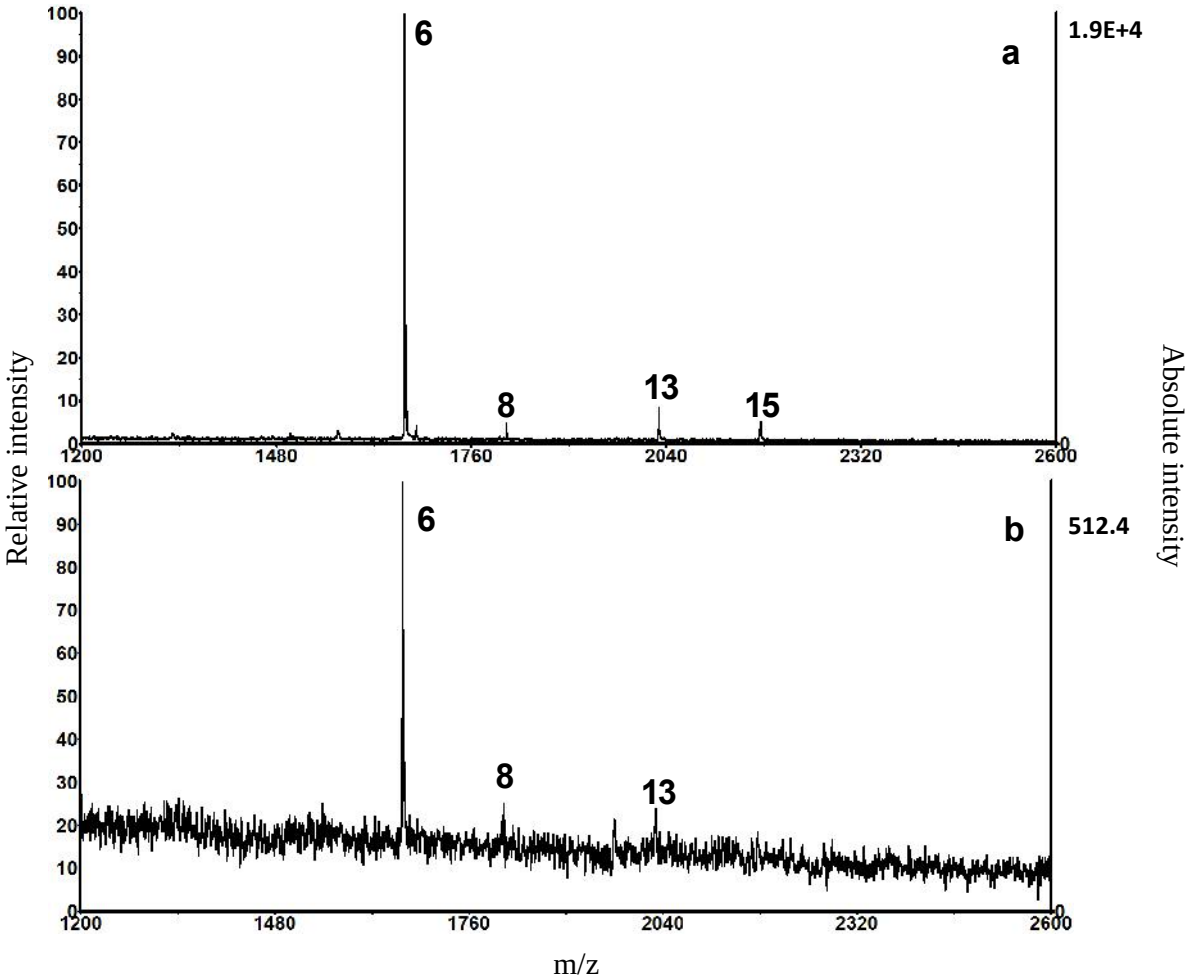

Figure S2. Representative N-glycan Spectra Using the Alpha-1-Antitrypsin VHH Antibody Fragment
(**a**) Analysis of standard mix A (positive control, contains alpha-1-antitrypsin). (**b**) Analysis of standard mix B (negative control, does not contain alpha-1-antitrypsin). The suggested identity of each glycan signal is displayed in Table 1.

Detected Amount of Unspecific Binding for Transferrin VHH

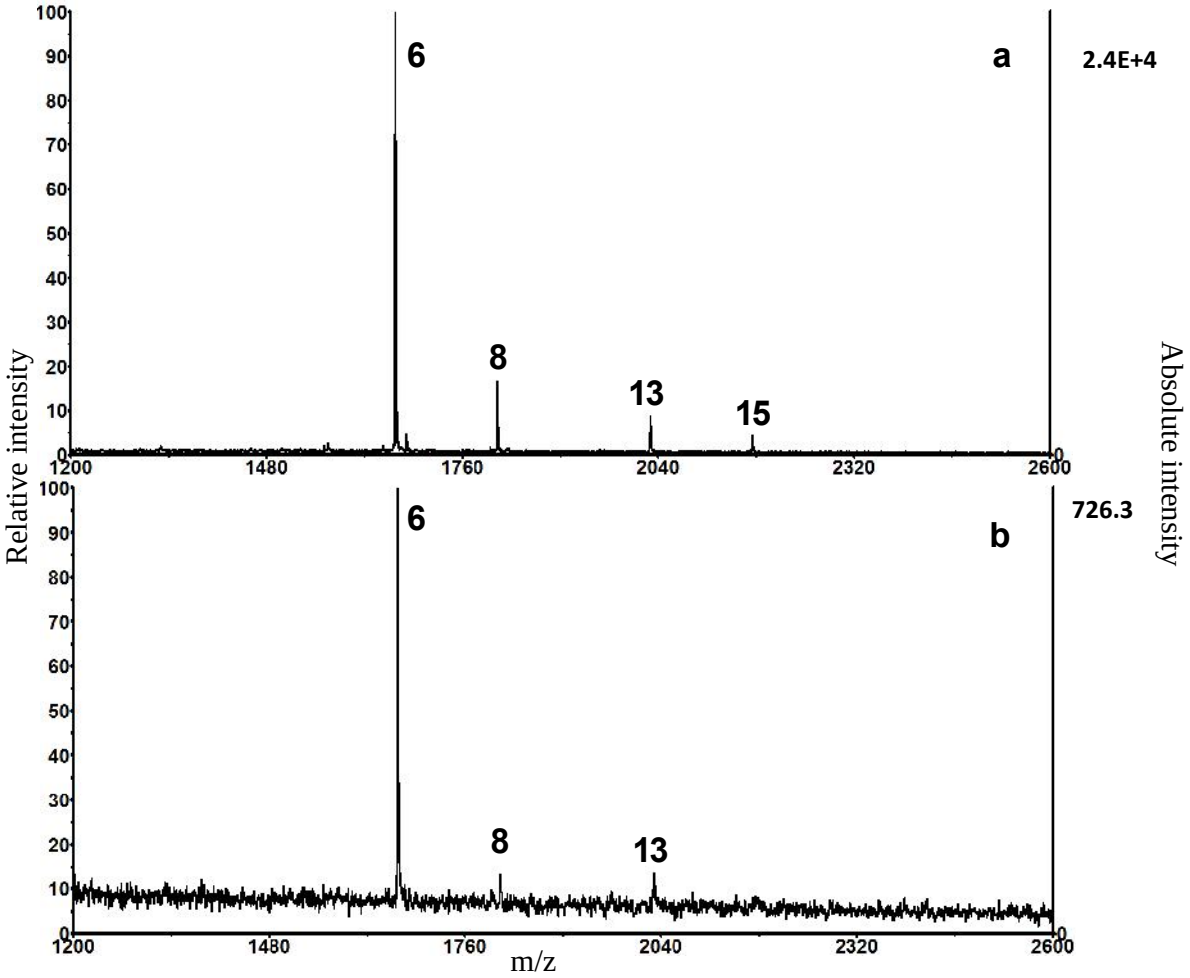

Figure S3. Representative N-glycan Spectra Using the Transferrin VHH Antibody Fragment (**a**) Analysis of standard mix A (positive control, contains transferrin). (**b**) Analysis of standard mix C (negative control, does not contain transferrin). The suggested identity of each glycan signal is displayed in Table 1.

Reproducibility of Bead-based Approach

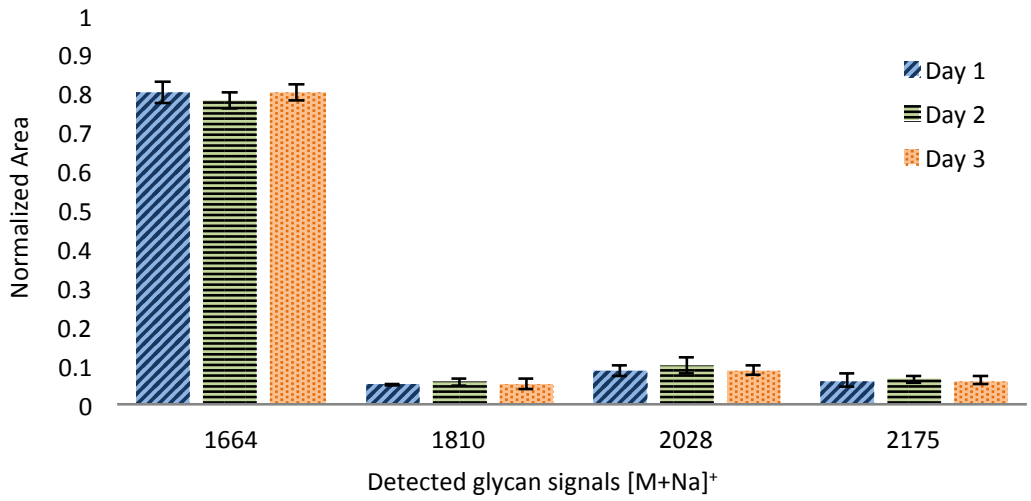

Figure S4. Reproducibility of the bead-based method
Normalized areas of the glycan species from standard AAT protein after performing the bead-based procedure on three different days. Mean values were generated from two replicates analyzed in duplicate. Error bars indicate standard deviations.

Table 1. Comparison of within day variation with between day variation

Glycan Signal $[M+Na]^+$	Within day %RSD Day 1	Within day %RSD Day 2	Within day %RSD Day 3	Between days %RSD
1664	3.7	3.0	2.7	1.5
1810	5.7	16.8	23.0	6.9
2028	17.3	21.7	12.7	7.7
2175	28.6	14.1	19.2	2.6