

Chapter 2

Analysis of Serum Protein Glycosylation with Antibody–Lectin Microarray for High-Throughput Biomarker Screening

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Abstract

The complexity of carbohydrate structures and their derivatives makes the study of the glycome a challenging subset of proteomic research. The microarray platform has become an essential tool to characterize glycan structure and to study glycosylation-related biological interactions, by using probes as a means to interrogate the spotted or captured glycosylated molecules on the arrays. The high-throughput and reproducible nature of microarray platforms have been highlighted by their extensive applications in the field of biomarker validation, where a large number of samples must be analyzed multiple times. This chapter presents an antibody–lectin microarray approach, which allows the efficient, multiplexed study of the glycosylation of multiple individual proteins from complex mixtures with both fluorescence labeling detection and label-free detection based on mass spectrometry.

Key words: Microarray, Antibody, Glycoprotein, Biomarker, Serum, Lectin, MALDI, Mass spectrometry

1. Introduction

Glycosylation is the most commonly occurring posttranslational modification on proteins involved in numerous biological processes, such as protein–protein interactions, protein folding, immune recognition, cell adhesion, and intercellular signaling. The function of glycoproteins is highly dependent on their carbohydrate structure. The alteration on the glycans is associated with multiple biological events and has been reported in a variety of diseases, especially cancer (1–4). In the search for effective glycosylated biomarkers for targeted diseases, there has been a great deal of effort invested in profiling and characterization of

glycoproteins in complex samples. Cell lines, tissue, and other types of biofluids have been studied by mass spectrometry, fractionation techniques, and microarrays (5–9). Although a microarray assay does not usually provide in-depth structural information on the glycans compared to mass spectrometry, it is able to identify and quantify numerous glycosylation patterns and simultaneously analyze hundreds of samples in a high-throughput manner with excellent reproducibility (9–13).

We herein describe an antibody–glycoprotein sandwich assay for high-throughput glycoprotein biomarker screening, where a fluorescent lectin and MALDI-MS are used to quantitatively measure glycosylation levels and identify analytes captured on the antibody arrays, respectively. The scheme for this procedure is illustrated in Fig. 1. The antibodies are first printed on nitrocellulose coated glass slides to generate identical arrays. Printed slides are processed to chemically block the glycans on the antibodies, which are otherwise reactive with lectins used for detection (10). After properly diluted human serum samples are deposited onto the separated antibody arrays, the captured antigens are probed with different lectins with a wide spectrum of binding specificity. The binding of the lectin is measured with a secondary fluorescent dye through a biotin–streptavidin reaction. To verify the effectiveness of previously discovered glycoprotein biomarkers, hundreds of serum samples collected from patients with different disease states are examined in parallel with healthy controls for altered glycosylation patterns. The technical error and bias in the analysis is minimized in several ways, including introducing a control slide to assess spatial variation on a slide and balancing samples from different groups on each slide to reduce experimental bias. MALDI-MS detection has only recently been used to detect peptides on a modified gold surface

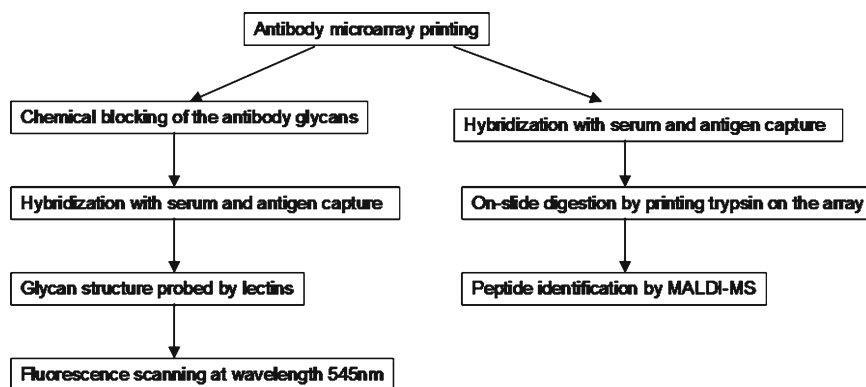


Fig. 1. Experimental scheme using lectin and MALDI-MS detection with antibody microarray to analyze glycosylation of serum glycoproteins.

coated with antibodies (14). An on-slide digestion method, developed in our previous work (15), exploited the utility of MALDI-MS to identify antibody-captured proteins. The whole digestion, including automatic trypsin spotting and incubation, requires less than 10 min.

While the antibody–lectin sandwich microarray provides a means to measure glycosylation changes on specific proteins captured from complex samples using lectin probes in a high-throughput array format, fluorescence-based detection provides limited structural information and cannot distinguish some glycoforms that have similar affinity with lectins, such as $(\text{GlcNAc})_2(\text{Man})_8$ and $(\text{GlcNAc})_2(\text{Man})_9$. Therefore, the MALDI-MS detection of the tryptic products of the captured protein on the antibody array serves as a complementary technique to verify the identity of the target of the antibody and a means to monitor the nonspecific binding so as to optimize the dilution fold for the experiment. As such, mass spectrometry is a powerful alternative to fluorescent detection, as it confirms the identity of the captured analyte and detects any undesired binding.

2. Materials

2.1. Antibody–Lectin Microarray with Fluorescence Detection

2.1.1. Printing

1. Monoclonal antibodies, for serum amyloid P component (SAP; Abcam), Alpha-1-beta glycoprotein (A1BG; Abnova), Antithrombin III (Abcam).
2. Nanoplotter 2.0 (GeSiM).
3. Nova nitrocellulose slides (GraceBio), PATH nitrocellulose slides (Gentel).
4. Printing buffer: 30% phosphate buffering saline (PBS), concentration of antibody diluted by water to 0.3 $\mu\text{g}/\mu\text{L}$.
5. 96-Well sample plate (BioRad).

2.1.2. Antibody Blocking

1. Washing buffer: PBS-T 0.1 (0.1% Tween-20).
2. Coupling buffer: 0.02 M sodium acetate, pH 5.5.
3. Oxidation buffer: 0.2 M sodium periodate in coupling buffer.
4. 4-(4-N-maleimidophenyl)butyric acid hydrazide hydrochloride (MPBH) (Pierce), Cys–Gly (Sigma).

2.1.3. Hybridization of Slides

1. Blocking buffer: 1% w/v BSA in PBS-T 0.5 (0.5% Tween-20).
2. Sample buffer: 0.1% Brij-45, 0.1% Tween-20 in PBS.
3. Primary detection solution: For *Aleuria aurentia* (AAL), *Maackia amurensis* (MAL), *Lens culinaris* agglutinin (LCA),

and Sambuccus Nigra (SNA) – 10 $\mu\text{g}/\text{mL}$ biotinylated lectin solution in PBS-T 0.1; and for Concanavalin A (ConA), 1 $\mu\text{g}/\text{mL}$ lectin in PBS-T 0.1. All biotinylated lectins were purchased from Vector Laboratories (Burlingame, CA).

4. Washing buffer: PBS-T 0.1 (0.1% Tween-20).
5. Secondary detection solution: 1:1,000 solution of 1 mg/mL Streptavidin conjugated to Alexafluor555 (Invitrogen) in PBS-T 0.1.
6. Speedvac.
7. SIMplex Multiplexing system (Gentel).

2.1.4. Slide Scanning

1. Axon 4000A scanner (Molecular Devices, Sunnyvale, CA).

2.1.5. On-Slide Digestion

1. Sequencing grade modified trypsin (Sigma).
2. Acetonitrile (ACN).
3. Ammonia bicarbonate.
4. Oven.
5. Nanoplotter 2.0 (GeSiM).
6. Wetted paper box.

2.1.6. MALDI-QIT-TOF

1. MALDI-QIT-TOF (Shimadzu Biotech, Manchester, UK).
2. Trifluoroacetic acid (TFA).
3. 2,5-Dihydroxybenzoic acid (DHB), prepare 10 $\mu\text{g}/\mu\text{L}$ solution in 50% ACN, 0.1% TFA.
4. Stainless steel plate adaptor.

3. Methods

3.1. Antibody Array Printing

The number of antibody arrays that can be printed on each slide is determined by the size of the arrays. The most popular format involves 16 coated pads on a standard 1 \times 3 in. slide. Each pad is able to contain more than 9 \times 9 spots with 0.6 mm spacing. For MALDI-MS detection, the sensitivity is much lower than fluorescence. Therefore to generate a spectrum with good S/N, additional sample needs to be printed on each spot.

1. Antibodies are diluted to 0.5 $\mu\text{g}/\mu\text{L}$ in printing buffer and transferred to a 96-well sample plate.
2. Edit the spot layout in the NanoPlotter program to produce a 2 \times 7 format of identical arrays with a 9 mm row and column distance from each other. The spacing between the spots is 0.6 mm. Each antibody is printed in triplicate. For MALDI-MS detection, the spacing between the spots is 1.5 mm.

3. Antibody solution is spotted onto nine ultrathin nitrocellulose coated slides. The first slide is discarded because of high variation of printing; the other eight are used for the experiment. Each spotting event results in 500 pL of sample being deposited and is programmed to occur 5 times/spot to ensure that 2.5 nL is being spotted per sample. The spot diameter is around 250 μm . For MALDI-MS detection, the amount of antibody on each of the spots in the antibody array is increased from 5 to 100 droplets. The spot diameter is around 700 μm (see Note 1).

3.2. Antibody–Lectin Array with Fluorescence Detection

3.2.1. Antibody Array Blocking

The IgG antibodies are usually glycosylated (15). The antibody glycans are reactive to detection lectins, thus need to be modified. To prevent the reaction between the antibody glycan and lectin, the antibodies on the slides are chemically derivatized with a modified method described in the previous work of Haab (10).

1. The printed slides are dried at room temperature overnight before gently being washed with PBS-T 0.1 and incubated in coupling buffer with 0.1% Tween 20 for 10 min. The slides are washed again with coupling buffer without Tween 20 before oxidation (see Note 2).
2. The slides are incubated in freshly made oxidation solution at 4°C in the dark. After 3 h the slides are removed from the oxidizing solution and rinsed with coupling buffer with 0.1% Tween 20 until the white precipitation disappears. The washing usually takes 30–60 min (see Note 3).
3. The slides are immersed in fresh 1 mM MPBH (in coupling buffer) at room temperature for 2 h to derivatize the carbonyl groups, then incubated with 1 mM Cys-Gly (in PBS-T 0.1) at 4°C overnight to stabilize the –SH group on MPBH. The slides are subsequently blocked with blocking buffer for 1 h and dried by spinning the slide at 1,000 rpm in a centrifuge (see Note 4).

3.2.2. Optimizing Conditions

Before screening a large number of samples, the optimum concentration of the serum is determined by a serial dilution test. In the dilution test, serum is diluted with sample buffer by 2–600 folds and incubated with different blocks of antibody arrays on a single slide (details of the experiment in Subheading 3.2.4). The signal is detected by lectin SNA (or any lectin) and plotted in Fig. 2. The figure depicts how the intensity of the signal changes for three antibodies (against Serum Amyloid P component, A1BG, and Antithrombin III) with decreasing dilution fold. A rising trend was noted from the 600 \times dilution to the 50 \times dilution for the three glycoproteins shown. In the 50 \times dilution to the 20 \times dilution, the signal was relatively unchanged

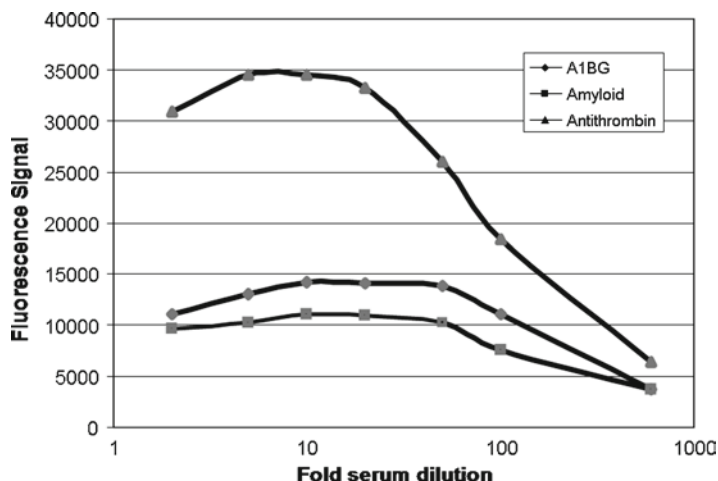


Fig. 2. Saturation curve showing how the antibodies (against serum Amyloid C component, A1BG, Antithrombin III) respond to different dilution of serum with SNA lectin detection. X-axis shows fold serum dilution before hybridization on the antibody array. The y-axis is the intensity of the signal. Reprinted with permission from Li et al. (15).

except for Antithrombin III, where the signal increased 20% from the 50× dilution to the 20×. The signal remained the same from the 20× dilution until it reached the 5× dilution, where a saturation of the signal has occurred. A decrease of signal for all three glycoproteins from the 5× dilution to the 2× dilution of serum sample can be seen in Fig. 3, likely due to competing nonspecific binding on the antibodies.

The result of the dilution test demonstrates that the antibodies were saturated by their target protein at 20× dilution or above in the process of hybridization. Below 50× dilution, the antibodies were not completely occupied so the signal decreased with additional dilution. The nonlinear relation between the concentration of the serum and the intensity of the signal could be attributed to various factors that may affect the antibody–antigen reaction, including accessibility of the antibodies, diffusion rate, and solubility of the antigen in the hybridization buffer. Nonspecific binding on the antibodies was also considered as a possibility, but was further investigated and excluded by on-target digestion and MALDI-MS analysis.

To analyze the difference of the glycosylation on potential biomarker proteins, protein expression levels must be normalized. Under saturation conditions, the amount of target biomarkers captured on the antibody spots was equal to the capacity of the printed antibody which should be the same in all the replicate blocks. As a result, protein assay is no longer needed and the intensity of the signal on the microarray directly represents the level of glycosylation.

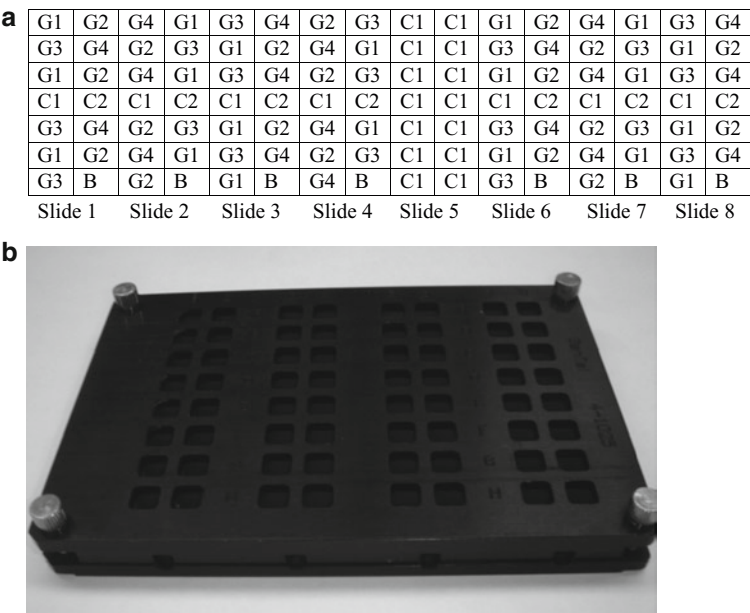


Fig. 3. Parallel processing of 77 samples on eight slides. **(a)** Sample arrangement on eight slides. G1, G2, G3, and G4 are four different groups of samples. Control samples are C1 and C2. B is blank. **(b)** A picture of SIMplex multiwell device.

3.2.3. Experimental Design

In the high-throughput biomarker screening, we usually parallel print and process eight slides which contain 112 identical blocks of antibody array. To minimize the technical error and bias on these blocks, serum samples are arranged to balance different disease/healthy groups and reference blocks are also introduced to adjust to signals of different blocks and slides. We provide an example of how to arrange samples on slides to minimize experimental biases in Fig. 3.

1. The slides are labeled from 1 to 8 in their printing order (see Note 2).
2. Slide 5 is used as a control slide; all the blocks on the control slide are incubated with a control serum sample C1.
3. Block 7 and block 8 on each slide except slide 5 are used as control blocks; they are incubated with control samples C1 and C2, respectively.
4. Block 14 is used as blank and incubated with sample buffer only.
5. The other 77 blocks are incubated with 19 samples from each of the four disease groups and 1 extra sample from a random group in a designated order to balance the number of samples from each group on any particular block (Fig. 3a).

3.2.4. Hybridization of Slides

1. The slides are placed into the SIMplex (Gentel) Multiplexing device which has 16 wells for each slide (the bottom two wells are not used) to separate the antibody arrays and prevent cross contamination between adjacent wells (Fig. 3b).
2. Serum samples are aliquoted into a volume of 10 μ L in each vial and diluted 10 \times with 90 μ L sample buffer. Diluted samples are added into the wells of the SIMplex Multiplexing device and incubated for 1 h with gentle shaking at room temperature. The wells must be sealed to prevent evaporation of samples (see Notes 5 and 6).
3. After completion of serum hybridization, slides are rinsed with PBS-T 0.1 three times to remove unbound proteins. The slides are incubated with biotinylated lectin solution in a plastic box with gentle shaking for an hour at room temperature.
4. The slides are washed 3 times with PBS-T 0.1 and incubated with secondary detection solution with gentle shaking for an hour at room temperature.
5. The slides are again washed 3 times with PBS-T 0.1, dried by centrifuge and kept at 4°C before scanning.

3.2.5. Slide Scanning

1. The dried slides are scanned with an Axon 4000A scanner.
2. Alexa555 labeled slides are scanned in the green channel (wavelength 545 nm). The photomultiplier tube (PMT) gain should be adjusted to obtain the best S/N without saturation. The size of the pixel of the image is 10 μ m.
3. The program Genepix Pro 6.0 is used to extract the numerical data.

3.2.6. Data Analysis

The nonbiological variation between blocks on the same slide is termed as on-slide variation. This variation is mainly generated by antibody printing and slide scanning and its feature is that every slide follows the same pattern (i.e., the blocks at the top of the slides are brighter than the bottom ones). The blocks on the control slide incubated with the same control sample are thus used to estimate the on-slide variation and calculate adjustment index for all the blocks. The slide-to-slide variation is considered as specific changes of the signal that effect all the blocks on a single slide. This variation is estimated by control blocks on each of the slides. The data is adjusted by a second index calculated by comparing the signal of the control blocks to exclude the slide-to-slide variation.

An example of assaying glycosylation expression of AIBG is shown in Fig. 4. Lectin SNA is used to probe the sialic acid present at the termini of the glycans of this protein. As shown in this figure, the mean value of the cancer samples is significantly higher than the other three groups ($p < 0.05$).

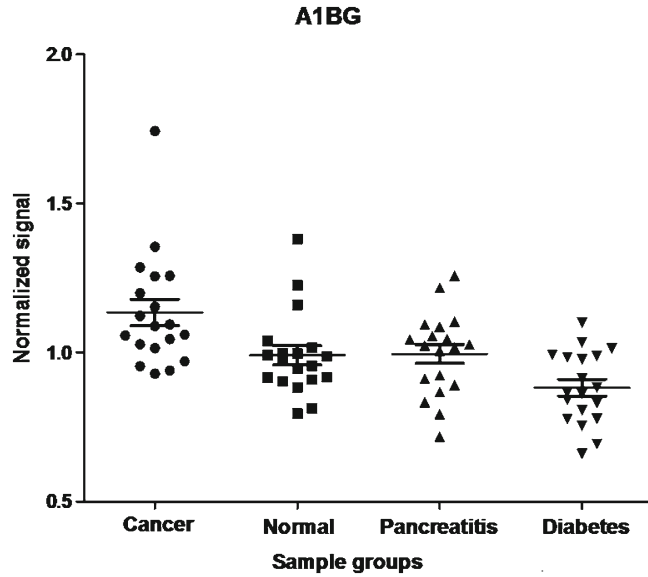


Fig. 4. Distribution of sialylation levels detected by lectin SNA on A1BG. The spots present the signal of the glycan on captured antigen for individual samples from different classes. The *long* and *short* lines give the mean value and the standard error of the mean, respectively.

1. A threshold of signal-to-background ratio is set at 3 and spots that are under this threshold are excluded.
2. The background-subtracted median of the intensity for the triplicates of each antibody is averaged and taken as a single data point into analysis.
3. On-slide variation index for antibody 1 in block 1 equals to the average signal of antibody 1 over all the blocks on the control slide divided by the signal of antibody 1 in block 1.

$$I_{Ab1.B1} = Avg_{Ab1.CS} / S_{Ab1.B1}$$

4. Slide-to-slide variation index for antibody 1 on slide 1 is calculated as follows: $Avg_{Ab1.S1}$ is the average signal of antibody 1 on slide 1. $Avg_{Ab1.AS}$ is the average signal of antibody 1 on all the slides.

$$I_{Ab1.S1S} = Avg_{Ab1.A} / Avg_{Ab1.S1}$$

5. The final adjusted signal is calculated by the following formula: $S_{Ab1.B1.S1}$ is the raw signal, $S_{Ab1.B1.S1.ad}$ is the adjusted signal.

$$S_{Ab1.B1.S1.ad} = S_{Ab1.B1.S1} * I_{Ab1.B1} * I_{Ab1.S1}$$

6. For each antibody the signal can be normalized to one for easy comparison, $S_{Ab1.B1.S1.n}$ is the normalized signal.

$$S_{Ab1.B1.S1.n} = S_{Ab1.B1.S1.ad} / Avg_{Ab1}$$

3.3. On-Slide Digestion and MALDI Sample Preparation

Nonspecific binding on antibodies may occur when the microarray is exposed to a concentrated and complex protein mixture such as serum. A commonly used method to study the specificity of an antibody is to digest and identify the protein released from antibody-conjugated medium, whereas eluting the captured protein is not very efficient and the procedure includes four or more steps. Thin layers of a conductive metal oxide and nitrocellulose make the surface of PATH slide perfect for MALDI. We developed an on-slide digestion and MALDI sample preparation protocol using the NanoPlotter to precisely spot enzyme and matrix to antibody arrays on the slide after the serum hybridization. Antibody arrays exposed to differently diluted sera are analyzed by this method to see if nonspecific binding occurs. Trypsin spotted on the antibody array usually simultaneously digests both the captured protein and the antibody; hence the tryptic peptides of the antibody must be excluded from the mass spectra for us to choose the peaks of interest. In an example, we prepared three identical spots of SAP antibody in separated blocks, which were then incubated with sample buffer (as control), 10× diluted serum, and 2× diluted serum and subjected to on-slide digestion and MALDI-MS. The MALDI-MS spectra of the three spots are shown in Fig. 5. The peaks that appear in the spectrum of the control spot are considered to be peptides of the antibody. The three highest peaks between 1,150 and 1,250 were identified by MS/MS as peptides from the Fc region of mouse IgG. In spectrum b where the antibody spot was hybridized with 10× diluted serum, the peaks at 1,166 and 1,407 m/z, are identified by MS/MS as the peptides digested from the target antigen, and the peak 993 matches the mass of a tryptic peptide of SAP. In the spectrum c there are two additional peaks. One of these was identified as human albumin, while the other one could not be identified or matched with a peptide mass of the target antigen. The additional peaks indicate that nonspecific binding might have occurred to the antibody spot. The serum was further diluted to assess the detection limit of the MALDI-MS technique. At 500× dilution (data not shown), the peak at 1,166 m/z disappeared while the 1,407 m/z still showed a signal-to-noise ratio of 2–3. Thus, the 500× dilution is considered as the detection limit of SAP, which is present in human serum with a concentration of around 30 µg/mL (16).

The introduction of mass spectrometry based label-free detection has the potential to further characterize the glycan structure. However, due to the presence of the tryptic peptides of the antibody and the lack of a glycopeptide enrichment step, only a limited number of the nonglycosylated peptides of the antigen could be seen in the spectra. To improve the MALDI-MS detection of the targeted antigen and its glycopeptides, we are searching for other chemical strategies to block the tryptic digestion of the antibody and enrichment methods to selectively ionize the glycopeptides.

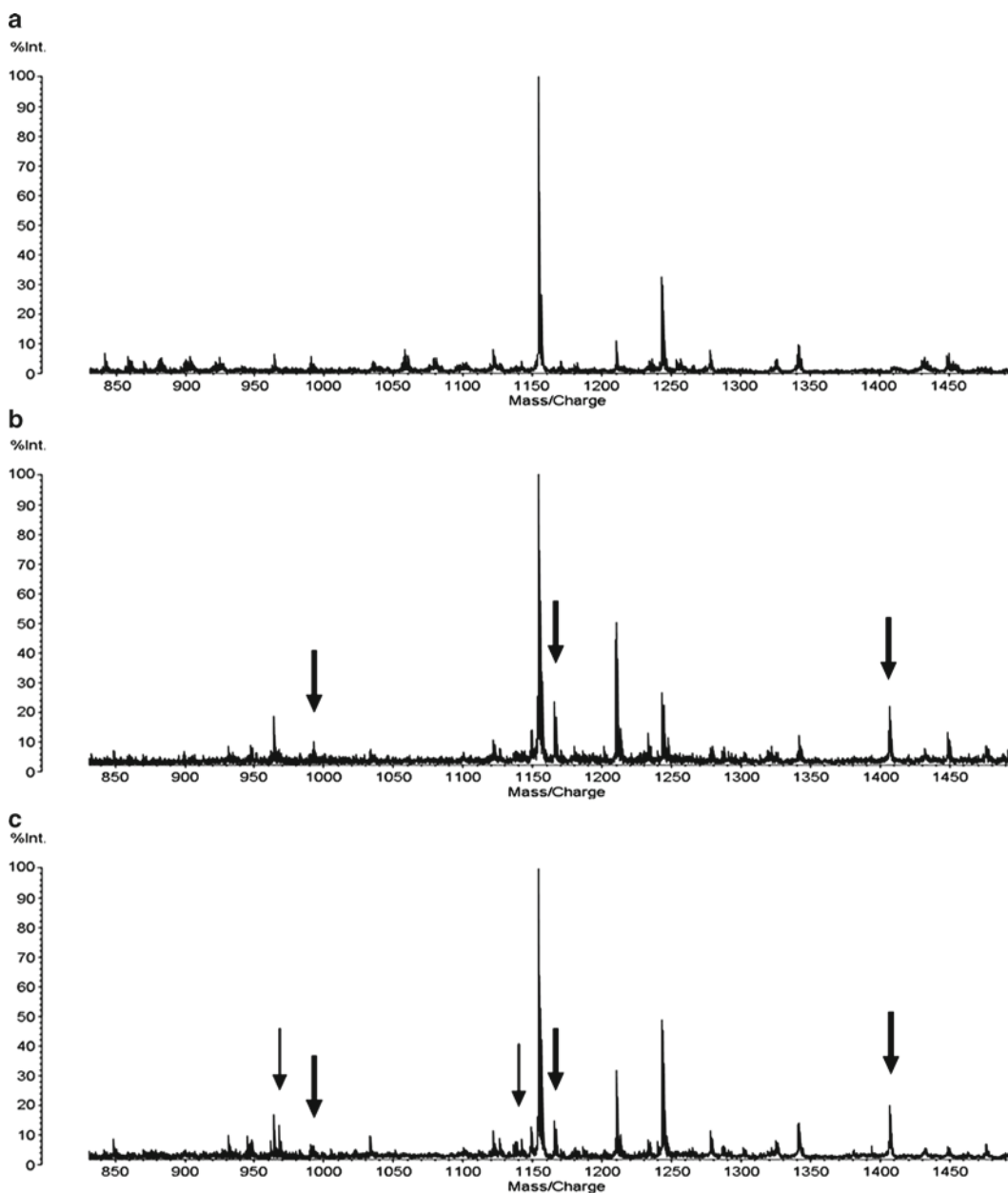


Fig. 5. The MALDI-MS spectra generated on the microarray spots of Amyloid p component antibody after on-target digestion. The peaks identified as Amyloid p component were marked with *bold arrows* where the extra peaks appearing in (c) were marked with *regular arrows*. (a) Control spot, without incubation of serum; (b) incubated with 10× diluted serum; (c) incubated with 2× diluted serum. Reprinted with permission from Li et al. (15).

1. Antibody slide is printed and hybridized with diluted serum as described above.
2. Trypsin is diluted with 50 mM ammonium bicarbonate in 20% ACN and kept on ice before use.
3. Keep the humidity of the Nanoplotter chamber higher than 70% (use a humidifier or lay a wet paper towel on the deck).

4. In the program, set the same spot layout on the slide, print 100 droplets (0.5 nL per droplet) of trypsin on each spot (see Note 7).
5. Move the printed slide to a wet paper box and incubate them in an oven at 37°C for 5 min. Make sure the trypsin solution does not dry out on the spots.
6. Take the slide out from the oven, print the DHB solution on the slide with the same spot layout (50 droplets per spot).

3.4. MALDI-MS

1. Tape the slide onto a stainless steel MALDI plate adaptor, insert it into the MALDI-MS instrument.
2. Mass spectrometric analysis of the microarray slides was performed using the Axima quadrupole ion trap-TOF. Acquisition and data processing were controlled by Launchpad software (Kratos, Manchester, UK). A pulsed N₂ 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.
3. TOF was externally calibrated using 500 fmol/μL of 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-Aldrich). The mass accuracy of the measurement under these conditions was 50 ppm.
4. The power of the laser is set at 80 to ionize the spots on the microarray. The focus of the laser can be moved from spot to spot manually under the camera or by using the Raster function to set up an automatic scan for all the spots.

4. Notes

1. When the pin on the Nanoplotter is in poor condition or the instrument is not set up correctly, the quality of antibody printing may fluctuate or gradually worsen as the printing continues. Sticky components, such as glycerol, in the antibody printing solution may also cause unstable printing. A simple test can be done in advance to assess the performance of the pin. Print 1,000 spots with a random antibody on a transparent slide. Observe the residue after the spots are dried. If the residues are in an intact round shape and their sizes and colors do not vary significantly, then the printing is acceptable, otherwise the printer needs to be checked or the printing solution must be changed.

2. Many types of chemicals can contaminate the nitrocellulose coating on the slide, resulting in increased background. The slides should not be labeled with any kind of marker. A disposable plastic box is a very good container for slide washing.
3. In the glycan blocking procedure, after the antibody is oxidized by NaIO_4 , white precipitation forms on the slides. This precipitation must be completely washed away before moving on to the next step.
4. Blocked slides should not be kept in solution for too long, while dried ones can be stored at 4°C for a long period of time.
5. Serum sample must be aliquoted immediately upon arrival and stored at -80°C . Serum frozen and thawed more than twice should not be used. When the sample set consists of multiple groups, all the samples must be in the same frozen and thaw cycle for bias-free comparison.
6. All the incubation should be done with gentle shaking to prevent uneven binding.
7. The higher number of droplets of antibody solution printed on the slides does not result in a higher density of antibody on the spot because the coating of the PATH slides is so thin that a few droplets are able to saturate the surface. The concern for the minimum amount of antibody solution printed on each spot is position variation, i.e., repeated printings on the same spot do not perfectly overlap. Printing 100 droplets of antibody solution produces a larger spot size which guarantees a certain area of overlap between the antibody spot and the printing of trypsin and matrix.

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