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Biochips that Sequentially Capture and Focus Antigens for Immunoaffinity MALDI-TOF MS: A New Tool for Biomarker Verification

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Abstract

A novel approach to immunoaffinity MS is described wherein antibodies are appended to a patterned gold Biochip surface. The Biochip surface is patterned with an array of concentric immunocapture zones comprised of highly hydrophilic central zones surrounded by moderately hydrophilic zones that reside on a non-wetting background, with protein attachment via electrochemically cleavable linkers. After linker cleavage, matrix application forms a discrete spot suitable for MALDI-TOF-MS. Use of the Biochip to purify Transthyretin from human serum allowed distinct resolution of four disulfide conjugates and one truncated form isoforms with good mass resolution and sensitivity.

Keywords

Antibody immobilization; Biochip; biomarker; mass spectrometry; proteomics

The use of discovery-based proteomics has greatly increased our ability to obtain candidate proteins potentially important in a wide range of biological settings. However, the confirmation and validation of these candidates is a more daunting task that tends to be more biochemically driven[1]. Often proteins are confirmed and validated through immunological assays with ELISA being the “gold standard”[2]. Mass Spectrometry (MS) technology has been used for the discovery of blood borne biomarkers of cancers of the ovary[3–5],

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SUPPORTING INFORMATION AVAILABLE

Methods for Biochip production

Supporting Figure 1. Biochip surface modification reagents.

Supporting Figure 2. UV-photopatterning of surface-modified substrates.

Supporting Figure 3. Electrochemical cleavage of Biochips.

Supporting Figure 4. Deconvolution of mass spectra for determination of TTR isoforms.

prostate[6–8], pancreas[9,10], breast[11], and skin[12] as well as biomarkers for other disease. A variety of MS technologies for proteomic discovery have been used including “shotgun” MS, MALDI-TOF-MS, and numerous variations on these and other platforms. MALDI can resolve signals from hundreds of proteins in complex mixtures, such as human serum, but abundant proteins and signal suppression can mask low abundance proteins of potential interest. On the other hand, immunological assays such as ELISAs most often do not distinguish between different isoforms of the protein that may arise from protein modifications and cleavages.

Here we introduce a novel approach that combines immunoaffinity capture, antigen-focusing and MALDI-TOF-MS to afford the sensitivity and selectivity required to differentiate post-translational modifications including isoforms. To this end, antibody is appended to the surface of a MALDI Biochip through a novel electrochemically cleavable linker. The biochip surface is patterned with an array of concentric immunocapture zones comprised of highly hydrophilic central zones surrounded by moderately hydrophilic zones that reside on a non-wetting background (see Supporting Information for methods of Biochip fabrication). After sample application, immunocapture, and washing, the linker associated with the immunocapture zones is cleaved just prior to application of matrix solution, and antigen is focused to central zones on the patterned Biochip surface during drying due to surface tension considerations.

Recently, patterned self-assembled monolayers on gold have been utilized to prepare a variety of biochips designed to facilitate aspects of MALDI-TOF-MS sample preparation[13] including concentration, fractionation, and immunoaffinity capture[14,15]. Very recently, cellular adhesion has been modulated via dynamic self-assembled monolayers that release immobilized ligands in response to an applied potential [16–18]. These surfaces exploit appended electrochemically active moieties that may result in nonspecific adsorption when present in self-assembled monolayers at concentrations exceeding 1–2%[19]. The approach we have developed is novel as it is the first to exploit a traceless cleavable linker to afford, after cleavage, zones that differ sufficiently with respect to surface tension to effect analyte-focusing. Aspects of the Biochip surface chemistry are described elsewhere[20]. This approach involving immunoaffinity capture and analyte-focusing combines attractive elements of the previously described mass spectrometric immunoassay[21] and prestructured MALDI-MS sample supports (AnchorChip™)[22].

We decided to apply this approach to the detection of the isoforms of Transthyretin (TTR, also known as prealbumin). TTR levels have previously been shown to change in individuals with breast or ovarian cancer[23] and some preliminary data we had collected indicated that the glutathionylated isoform of TTR might be increased in breast cancer but the TTR signals were often too low to measure. To solve this problem, anti-TTR antibodies were immobilized on the surface of MALDI Biochips for selective retention of TTR from serum. After processing, we obtained high quality spectra with clearly discernible peaks that showed essentially only TTR present at 5 different masses corresponding to a known cleavage product, native TTR and three disulfide adducts including cysteinylated, cystenylglycylated and glutathionylated products.

Immunoaffinity capture on the Biochip surface involves: 1) immobilization of antibody via capture on protein G, 2) incubation of the serum sample containing antigen, 3) washing to remove unbound protein, and 4) electrochemical cleavage to release immobilized protein G, antibody, and antigen. Each immunoaffinity capture zone is comprised of a highly hydrophilic central zone ($\theta < 15^\circ$, 0.5 mm O.D.) surrounded by a moderately hydrophilic zone ($\theta < 40^\circ$, 3.0 mm O.D.). Upon application of matrix solution, protein G, antibody, and antigen are concentrated and co-crystallized with matrix in the central zone due to the

surface tension differential (Figure 1). Eight serum samples were analyzed on patterned biochips with and without antigen-focusing features and afforded average integrated ion currents of 138,400 $\pm$ 12,180 and 9,610 $\pm$ 1,500, respectively, indicating that antigen focusing yielded a 14.4-fold average increase in signal. Similar proportional increases in signal were observed for cytochrome c, angiotensin and insulin B-chain. Additionally, when replicate samples (n=5) were investigated on antigen-focusing biochips, the internal standard (cytochrome c) was detected with better precision after analyte-focusing (CV reduced from 0.15 to 0.09) due to reduced sample heterogeneity.

Preliminary data from our laboratory using ACN-precipitated serum samples indicated that specific isoforms of TTR might be biomarkers of breast cancer. Full-length TTR has a mass of 13,758 Da, with the truncated form of N-10 AA found at 12,210 Da. There are also three modified TTR isoforms: cys-TTR at 13,876, cysgly-TTR at 13,924 and glutathionylated-TTR at 14,062 Da. However, the resolving power of conventional MALDI-TOF of partially purified serum was not sufficient to be confident of the peak assignment or signal resolution in these samples – a sample showing relatively high levels of TTR is shown in Figure 2A. Therefore, we explored the ability of the Biochip to concentrate TTR and differentiate the isoforms. When we compared peptides in the 10,000–20,000 m/z range, TTR was by far the predominant species in the spectra from the Biochip preparation (Figure 2B). All of the different isoforms of TTR are clearly discernable and they could be readily deconvolved by PeakFit software as shown in the left inset of Figure 2B. These results indicate that the Biochip extensively purifies TTR from sera. Evaluation of TTR isoforms at different concentrations by comparing MALDI-TOF MS signals from 2.5, 5 and 10 μ l of serum (Figure 2B) showed that Biochips with an antigen-focusing zone (thick lines) had much higher TTR-related ion current than those that were unfocused (thin lines) (Figure 2B plus middle and right side insets, note different signal intensity scales). When examined at the 2.5 μ l level, focused antigen could still readily differentiate isoforms of TTR, but unfocused antigen lacked sufficient signal to detect different isoforms.

Historically, there has been some controversy regarding the ability of MALDI-TOF-MS and SELDI-TOF-MS (Surface Enhanced Laser Desorption/Ionization-TOF-MS) to resolve the isoforms of TTR[24]. For a practical demonstration the Biochip utility, we chose to examine TTR levels and isoforms using the Biochip with plasma from a set of 400 Japanese women that made a clinic visit to the Aichi Cancer Center, Japan and were either ultimately diagnosed with breast cancer (200) or had no disease (200 controls). The study age range was 30–79 years, and control selection used frequency-matching based on age. We also examined plasma from 200 controls collected in the US as part of a different study at the Fred Hutchinson Cancer Research Center (FHCRC) from mostly Caucasian individuals. Informed consent and human subjects review were conducted in Japan and at the FHCRC. Eligible subjects participated by providing blood samples and answering a series of demographic, family history, lifestyle and other health-related questions for molecular-epidemiologic research purposes.

Our analysis revealed no significant difference in the levels of the glutathionylated isoform (our putative candidate) nor any other isoforms of TTR for the Japanese samples (Figure 3). In fact, the levels of all isoforms from both control and diseased Japanese individuals were amazingly similar. In addition, the total levels of all isoforms of TTR between the cancer and control Japanese population showed less than a 1% difference. However, when the US and the Japanese control samples were compared significant differences were observed. Most notably, there was a 4.1% increase in cysgly-TTR among the Japanese subjects and a 6.6% decrease in unmodified TTR. The differences observed between Japanese and US control subjects were significant for the truncated TTR form of N-10AA ($P<0.0001$), full-length TTR ($P=0.0003$), the cysgly-TTR isoform ($P=0.024$) and glutathionylated-TTR

($P=0.0059$). The cys-TTR isoform difference was not significant between the two control groups ($P=0.25$). Each sample was spiked with equal amounts of an internal standard, cytochrome C, to allow comparison. This data shows the ability to differentiate subjects from two different groups *via* distribution of different TTR isoforms.

In this study, we demonstrated the utility of Biochips to sequentially capture and focus antigens for the confirmation and validation of protein biomarkers. This technology combines the power of both immunological assays and the ability to detect different isoforms *via* the mass spectrometer. It harnesses the ability of antibodies to purify and potentially concentrate antigen (*via* multiple applications of fresh analyte solution) and the ability to concentrate analyte *via* differences in surface tension of the Biochip surface. Without prior purification, raw serum or plasma yields no TTR signal in our mass spectrometer. ACN precipitation yielded discernible TTR signal in ~50% of our samples. Use of the Biochip for single step purification directly on the MALDI target yielded clearly discernible signal for all isoforms in all samples in a highly consistent manner.

TTR levels have previously been reported to change in individuals with breast or ovarian cancer[25]. We had also obtained some preliminary data that indicated that the glutathionylated isoform of TTR in particular might be higher in human breast cancer. However, the low level of the signal from the glutathionylated isoform of TTR and the high density of other peaks in that mass range in our spectra made resolution and quantification difficult. Therefore, we produced Biochips with bound TTR antibody. The performance of these Biochips was excellent with very clean TTR signals for all of the isoforms. However, we did not observe any difference in the distribution or overall levels of TTR in the Japanese samples. From these results, we would conclude that TTR is not a good biomarker for breast cancer at least in a Japanese population. We did observe a difference between the samples collected in Japan compared to Caucasian controls from the United States. Obviously, many reasons could explain these differences including race, diet, minor collection protocol differences and other miscellaneous differences. Therefore, no clear explanations for these differences can be offered. We can conclude, however, that this technology can clearly differentiate TTR total and isoform levels between two populations.

In summary, our data suggest that these Biochips could be very valuable for biomarker validation involving antigens present in multiple isoforms that are biologically interesting. Furthermore, it requires only a single, capture antibody and could potentially be readily multiplexed for multiple species of different m/z values. In essence, the MS replaces the second detection antibody normally needed for a “sandwich” ELISA with the added ability to detect different post-translational modifications or isoforms. Anti-peptide and stable isotopic standards could be exploited allowing for absolute quantitation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ABBREVIATIONS

TTR	Transthyretin
EDC	<i>N</i> -(3-Dimethylaminopropyl)- <i>N'</i> -ethyl-carbodiimide hydrochloride
NHS	<i>N</i> -hydroxysuccinimide
ACN	Acetonitrile

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Figure 1.

Experimental protocol for immunoaffinity MS. (A) Sample is added to a virtual well on the Biochip surface and incubated to allow antigen binding. (B) Unbound proteins are removed by washing. (C,D) Electrolyte solution is added and a potentiostat connected with the positive pole on the Biochip surface, and potential is applied effecting linker cleavage and release of immobilized antibody and captured antigen from both the highly hydrophilic central zone and moderately hydrophilic surrounding zone. The antigen-containing solution is allowed to evaporate. Finally, a droplet of matrix solution is applied to the surface of the Biochip dissolving the antigen. As the matrix solution evaporates, the antigen and matrix co-crystallize over the area corresponding to the highly hydrophilic central zone.

Figure 2.

MALDI of Transthyretin and Biochip Immunoaffinity Capture. Spectra resulting from acetonitrile (ACN) precipitated serum (A) and from 2.5 to 10 μ L of serum purified on a Biochip (B). In A, a reference spectrum produced from serum depleted of abundant proteins via ACN-precipitation is shown. For the Biochip in B, the absence of other peaks and optimal signal-to-noise ratio for TTR allows clarity in the identification of the full length form of TTR (13 758 m/z); the truncated form of TTR, N-10AAs (12 210 m/z) and three isoforms: cystTTR (13 876 m/z), cystglycTTR (13 924 m/z) and glutathionylated-TTR (14 062 m/z). Mass spectra of TTR affinity captured from 10.0 μ L (B), 5.0 μ L (B, middle inset), 2.5 μ L (B, right inset) of human serum. Thin lines represent antigen that was obtained from a Biochip with only the central affinity capture zone whereas the thick lines are from a Biochip with both the central antigen-focusing and surrounding immunoaffinity capture zones. The PeakFit graph (Systat Software, San Jose, CA) resulting from analysis of a representative TTR mass spectrum with AutoFit III Gaussian deconvolution option is illustrated with the component peaks that result from the deconvolution (B, left inset). The R -squared value of 0.991648, determined at the 95% confidence level, indicated the high quality of the overall fit.

Figure 3.

Distribution of TTR isoforms by breast cancer status and nationality. Essentially no difference was observed in the distribution of TTR isoforms for Japanese breast (upper row) cancer cases (right) and controls (left). There are no significant findings that breast cancer status changes the distribution of TTR. There are significant differences in the distribution of TTR and TTR + cysteinylglycine between US (lower row) controls and Japanese controls (upper row, left).