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Mass-Tag Enhanced Immuno-Laser Desorption/Ionization Mass Spectrometry for Sensitive Detection of Intact Protein Antigens

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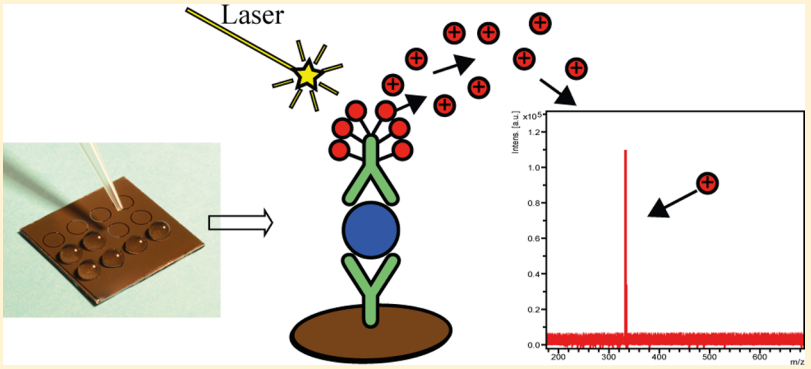
Supporting Information



ABSTRACT: A new read-out method for antibody arrays using laser desorption/ionization-mass spectrometry (LDI-MS) is presented. Small, photocleavable reporter molecules with a defined mass called "mass-tags" are used for detection of immunocaptured proteins from human plasma. Using prostate specific antigen (PSA), a biomarker for prostate cancer, as a model antigen, a high sensitivity generic detection methodology based immunocapture with a primary antibody and with a biotin labeled secondary antibody coupled to mass-tagged avidin is demonstrated. As each secondary antibody can bind several avidin molecules, each having a large number of mass-tags, signal amplification can be achieved. The developed PSA sandwich mass-tag analysis method provided a limit of detection below 200 pg/mL (6 pM) for a 10 μL plasma sample, well below the clinically relevant cutoff value of 3−4 ng/mL. This brings the limit of detection (LOD) for detection of intact antigens with matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) down to levels comparable to capture by anti-peptide antibodies selected reaction monitoring (SISCAPA SRM) and enzyme linked immunosorbent assay (ELISA), as 6 pM corresponds to a maximal amount of 60 amol PSA captured on-spot. We propose the potential use of LDI (laser desorption/ionization) with mass-tag read-out implemented in a sandwich assay format for low abundant and/or early disease biomarker detection.

The use of photocleavable mass-tags as probes in matrix-assisted laser desorption/ionization-mass spectrometry (LDI-MS) based applications has recently gained momentum. Most successfully, the mass-tags have been applied in matrix-assisted laser desorption/ionization-imaging mass spectrometry (MALDI-MSI)[1−6] but also in single nucleotide polymorphism detection,[7−9] combinatorial synthesis,[10,11] and as ionization enhancers.[12] Two different types of mass-tag molecules have been reported in the literature, either peptide tags connected to a binder via a photocleavable linker group[3] or triphenylmethyl-(trityl) based tags.[1,2,5,6,10−12] The trityl tags are photolabile trityl thioesters connected to the probe or carrier via its free amino groups, and upon radiation, they become ionized and result in a very stable cation which is easily detectable by LDI-MS (Figure 1).

The major advantages of mass-tags as a means of secondary detection are that there is no size limitation of the targeted biomolecule, compared to MALDI where there is a strong

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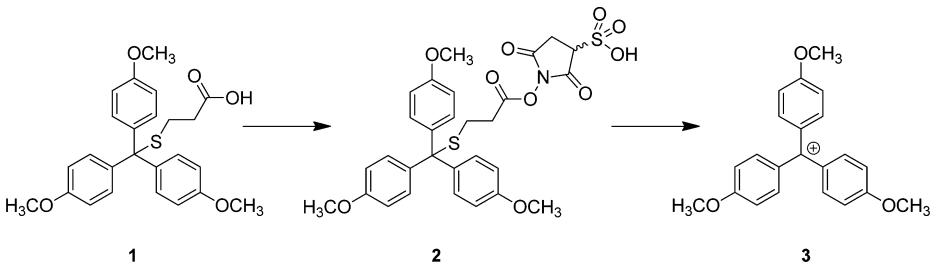

Figure 1. Synthesis of the mass-tag, its activation, and the resulting carbocation. Carboxyl group of the mass-tag, i.e., 3-[tris(4-methoxyphenyl)methylsulfanyl]propanoic acid (**1**), is activated by using 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) and *N*-hydroxysulfosuccinimide (Sulfo-NHS) to yield 2,5-dioxo-1-[[3-[tris(4-methoxyphenyl)methylsulfanyl]propanoyl]oxy]pyrrolidine-3-sulfonic acid (**2**), which is coupled to the free amino groups of any biomolecule with which it is incubated. Upon laser irradiation, the labile carbon−sulfur bond breaks yielding the very stable carbocation (**3**). For more information see the Supporting Information.

mass-dependent sensitivity; furthermore, no digestion of the targeted protein is needed, and the targets abundance compared to other molecules in the sample plays a minor role. Also, the typical use of a matrix compound in a MALDI experiment is avoided allowing easy detection of the mass-tag without contaminating background signals from the matrix itself, and as very small mass differences can be resolved by MS analysis, multiplex experiments with a panel of different mass-tags in the same sample are possible.[1,2]

Antibody microarrays with optical read-out have been shown to allow for detection of low abundant markers in small sample volumes and have become important diagnostic tools,[13] for example, in clinical cancer diagnostics.[14,15] Many different methods using MALDI/LDI MS for analysis of immunocaptured peptides and proteins have been presented in the literature over the years.[16] The main benefit of using MS instead of optical read-out is that a mass and sequence confirmation of capturing the correct antigen is obtained, which also can be used to pinpoint structural variants.[14]

Current state of the art immunocapture-MS approaches use LC-MS selected reaction monitoring; the immunocapture is done either on peptide (stable isotope standards and capture by anti-peptide antibodies, SISCAPA)[17] or at the intact protein level.[18] These LC-MS based methods employ a digestion step either prior to or after the immunocapture and allow for multiplexing and detection of biomarkers in ng/mL down to the low pg/mL range.[15] While MALDI/LDI approaches have a much higher potential for throughput, sensitivity is limited by the lack of separation, lower dynamic range, and possibility for multiplexing.[17] It is therefore of interest to investigate possibilities to circumvent the sensitivity issues of immuno-MALDI/LDI.

In this paper, we demonstrate the use of trityl tags as mass-tags, which are easily synthesized at low cost and do not necessitate the use of a matrix for efficient laser desorption ionization[12] as reporter ions for detecting immunocaptured intact proteins. Porous silicon arrays are used for the antibody immobilization, which has an enlarged surface area and hence higher capture capacity.[19−22] The behavior of porous silicon is hydrophobic due to the surface nanomorphology although the actual surface at the molecular level is hydrophilic, which minimizes denaturation at the same time as it favors printing a denser array of antibodies.

The porous silicon is hydrophilic (SiO_2) and will retain the antibodies in their original active conformations on the porous silicon surface, but the nanoporous surface morphology makes the surface behave hydrophobically, which facilitates robotic liquid handling.[23] A protocol for direct capture and read-out of mass-tagged transferrin in plasma using porous silicon antibody arrays is demonstrated. In addition, a highly sensitive sandwich mass-tag assay was subsequently realized to capture prostate specific antigen (PSA) in blood plasma at clinically relevant concentrations ranging from 2 μg/mL to 200 pg/mL. Analogous to other tag based systems,[22] this work also applied several tags per molecule to provide signal amplification. This allowed for a sensitivity increase 2−3 orders of magnitude compared to the corresponding intact protein detection with MALDI MS. The mass-tag system applied to antibody microarrays using mass spectrometry read-out could add great advantage to the microarray field in terms of sensitivity, multiplexing, and speed of detection.

EXPERIMENTAL SECTION

Chemicals and Materials. Unless stated otherwise, all chemicals and proteins were purchased from Sigma-Aldrich Co. (St. Louis, MO) and used without prior purification. Buffers were lab-made phosphate buffered saline (PBS; 10 mM, pH 7.4) and phosphate buffer (PB; 0.1 M, pH 7.2). Antibodies H117 and 2E9 were generously provided by Professor Ulf-Håkan Stenman, Dr. Hannu Koistinen (University of Helsinki), Dr. Mari Peltola and Professor Kim Petterson (University of Turku).[24,25] All water was Millipore purified. MALDI-MS measurements were performed on the Bruker Ultraflextreme (Bruker Daltonics, Bremen, Germany) and unless stated otherwise, without adding matrix, in linear mode over a mass range of 160−840 accumulating 10 000 spectra for each sample. HCCA matrix and LeuEnk peptide peaks were used for calibration.

Tagging of Proteins. The mass-tag compound **1** (3-[tris(4-methoxyphenyl)methylsulfanyl]propanoic acid, Figure 1) was synthesized by a nucleophilic substitution of 4,4′,4″-trimethoxytrityl chloride and thioacetic acid in dry dichloromethane. Compound **1** was purified by liquid chromatography (Biotage Isolera Flash Chromatography, Uppsala, Sweden).[26] Synthetic details and associated structural data are available in the Supporting Information.

Tagging of transferrin, avidin, and PSA was performed in the same way. The tag **1** was activated by solubilizing it in dimethyl sulfoxide (DMSO) and incubating it overnight with 1.3 equiv of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) and *N*-hydroxysulfosuccinimide (Sulfo-NHS) in water[27] to yield the activated mass-tag **2**, (i.e., 2,5-dioxo-1-[[3-[tris(4-methoxyphenyl)methylsulfanyl]propanoyl]oxy]pyrrolidine-3-sulfonic acid, Figure 1). The protein was resuspended in PBS and incubated for 2 h on ice with a 100−1000 molar excess (100 molar excess tag for transferrin, 150 molar excess tag for

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Anal. Chem. XXXX, XXX, XXX−XXX

avidin, or 1000 molar excess tag for PSA) of **2**. Unbound tag was removed by Micro Bio-Spin chromatography columns (Bio-Rad, Hercules, CA), and the success of the tagging was evaluated by loading 1 μL of purified tagged protein on a MALDI target plate (Bruker, Billerica, MA) and analyzing the height of the resulting mass peak. The concentration of tagged protein was measured by NanoDrop 8000 (Thermo Fisher Scientific, Waltham, MA) at the wavelength 280 nm.

Tagging of plasma was performed by adding 7.5 mg (12.5 μmol) of activated tag to 500 μL of female human plasma and incubating for 6−12 h on ice. Due to the vast excess of protein (55−70 mg/mL) in the plasma, no size-exclusion purification to remove unbound tag was performed to keep the plasma undiluted.

Biotinylation of H117 Antibody. The antibody was concentrated using Amicon Ultra-0.5 mL Centrifugal Filters 10 kDa cutoff (Merck Millipore, Billerica, MA) to a final concentration of 2 mg/mL. The antibody was incubated with 50 mol equiv of biotin *N*-hydroxysulfosuccinimide ester overnight at 4 °C. Unbound biotin *N*-hydroxysulfosuccinimide ester was removed by Micro Bio-Spin chromatography columns (Bio-Rad, Hercules, CA). Biotinylation was verified (not quantitatively) by observation of a mass shift for biotinylated and unbiotinylated antibody using MALDI-MS with sinapinic acid matrix on a Bruker standard steel target plate (data not shown).

Porous Silicon Surface. The silicon wafers, 5−10 Ωcm, ⟨100⟩, P type and boron-doped, were from Addison Engineering Inc. (San Jose, CA). The chips were patterned using the standard UV-lithography protocol with silicon dioxide as a mask. The circular porous spot on the chip was 3 mm in diameter with a surrounding silicon dioxide ring of 200 μm in width, and the pitch between positions was 4.5 mm which conforms to standard Society for Biomolecular Sciences (SBS) measurements of a 384 micro plate and facilitates robotic liquid handling. The detailed electrochemical porosification protocol has been reported previously.[20,21] The porosified silicon wafer was diced into 2 cm × 2 cm chips holding 3 × 4 circle array positions. The antibody immobilization on the porous silicon was performed by surface adsorption.[20,21,28] All washings and incubations were performed manually per spot to avoid cross-contamination (Figure 2).

Immunocapture of Mass-Tagged Transferrin. Antihuman transferrin antibodies (10 μL of ∼0.1 mg/mL in PBS) were deposited on the planar silicon surfaces and allowed to immobilize for 30 min. On one spot, goat-antirabbit detection antibody was immobilized as a negative control, and subsequent washing was performed. The spots were blocked with 5% milk powder in PBS for 30 min to avoid unspecific binding. After washing, the spots were incubated with 10-fold dilutions of tagged transferrin in PBS for 30 min; unbound protein was removed, and the spots were analyzed without adding matrix.

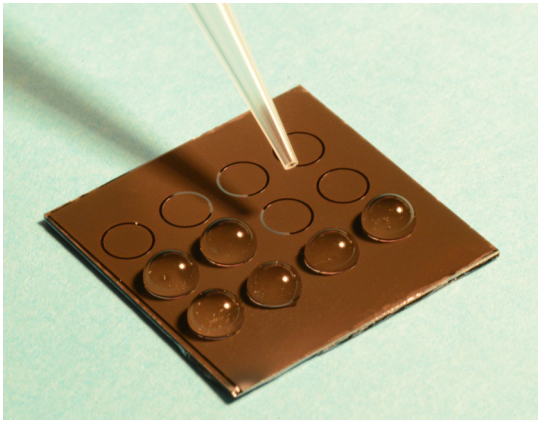

Figure 2. Photograph of the porous silicon chip with the 12 circular porous spots measuring 3 mm in diameter.

Immunocapture of Mass-Tagged Transferrin from Plasma. Antihuman transferrin antibodies (10 μL of ∼0.1 mg/mL in PBS) were deposited on the porous silicon surface and allowed to immobilize for 30 min. As negative controls, one spot was treated equally, just without immobilizing any antibody, and on one spot, antirabbit detection antibody was immobilized; subsequent washing was performed. The spots were blocked with 5% nonfat milk powder in PBS for 30 min to avoid unspecific binding. After washing, the spots were incubated with tagged plasma for 30 min; unbound protein was removed, and the spots were analyzed without adding matrix.

Detection of Mass-Tagged PSA. For each spot, 10 μL (∼0.1 mg/mL in PBS) of anti-PSA 2E9 capture antibody and antirabbit detection antibody (negative control) were allowed to adsorb on a porous silicon surface on different spots for 30 min. Unbound antibody was removed by washing 3 times with 10 μL of PBS. Mass-tagged PSA was spiked in female human plasma to a final concentration of 5.1 μg/mL and incubated on the spots for 30 min. Unbound PSA and plasma was removed by washing twice with 10 μL of PBS and once with 10 μL of PB, to reduce the amount of salt on the surface. The chip was dried at room temperature and introduced into the mass spectrometer and analyzed.

Sandwich Assay PSA. For each spot, 10 μL (∼0.1 mg/mL in PBS) of capture antibody 2E9 was deposited on the porous silicon surface and allowed to bind for 30 min. Unbound antibody was removed by washing 3 times with 10 μL of PBS. One μL of PSA in solutions of 10-fold serial dilutions, resulting in concentrations from 0.18 mg/mL to 18.6 ng/mL, was spiked into 9 μL of female plasma and incubated on the capture antibody for 30 min. Unbound PSA and plasma were removed by washing 3 times with 10 μL of PBS. Tagged avidin was premixed with biotinylated H117 antibody for 20 min on ice to build an immunocomplex and incubated with the captured PSA for 30 min. Unbound immunocomplex was removed by washing twice with 10 μL of PBS and once with 10 μL of PB, to reduce the amount of salt on the surface. The chip was dried at room temperature and analyzed by MS.

RESULTS AND DISCUSSION

Trityl mass-tags have strong advantages; their UV-labile nature allows MS analysis without using a matrix and the fact that they can be used to detect virtually any molecule for which a specific binder can be raised makes them a highly versatile tool. Unlike fluorescence tags, mass-tags do not exhibit strong mutual quenching effects and thus allow true multiplex experiments with a library of mass-tags, as the resolution of MS allows one to distinguish between masses that differ just a few Daltons from each other.[1,2,12] In contrast, wavelength peaks in optical methods tend to be much broader and thus limit the amount of different tags that can be used in one sample simultaneously.

Porous silicon has previously been successfully applied as a substrate for antibody arrays using fluorescence detec-

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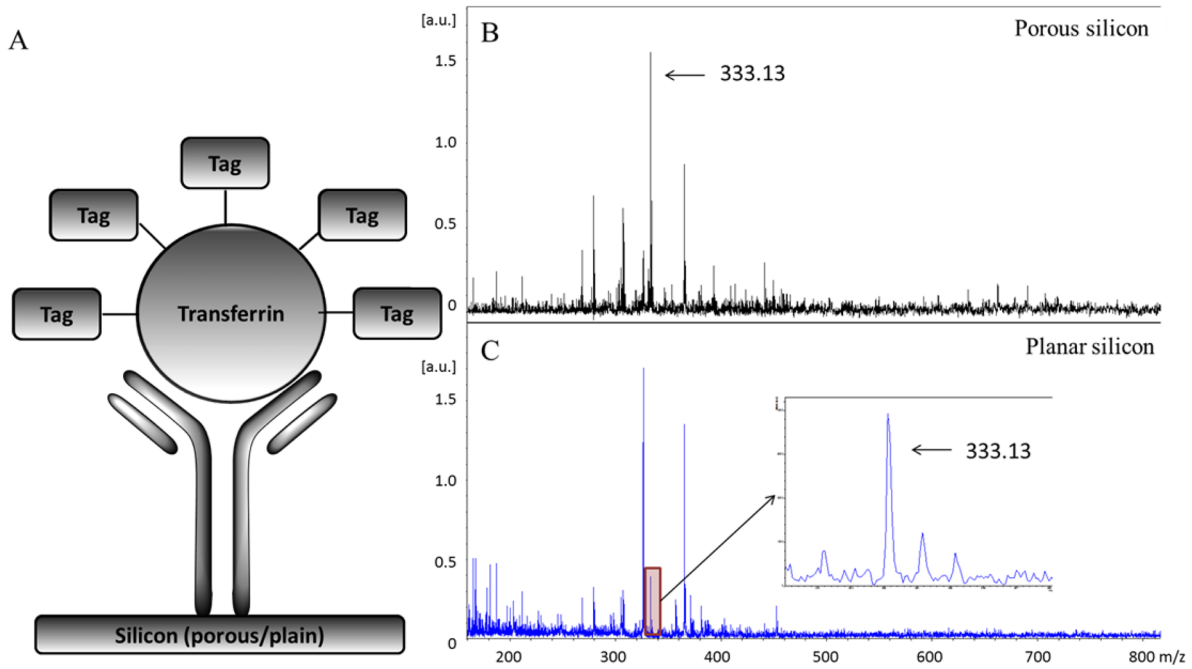

Figure 3. Immunocapture of tagged human transferrin on porous and planar silicon. Panel A illustrates the assay. Capture antibody antihuman transferrin was immobilized on porous silicon and planar silicon. Panel B shows the MS spectrum from 0.53 ng/mL mass-tagged transferrin on the porous silicon surface. Panel C shows the same mass-tagged transferrin on a planar silicon surface.

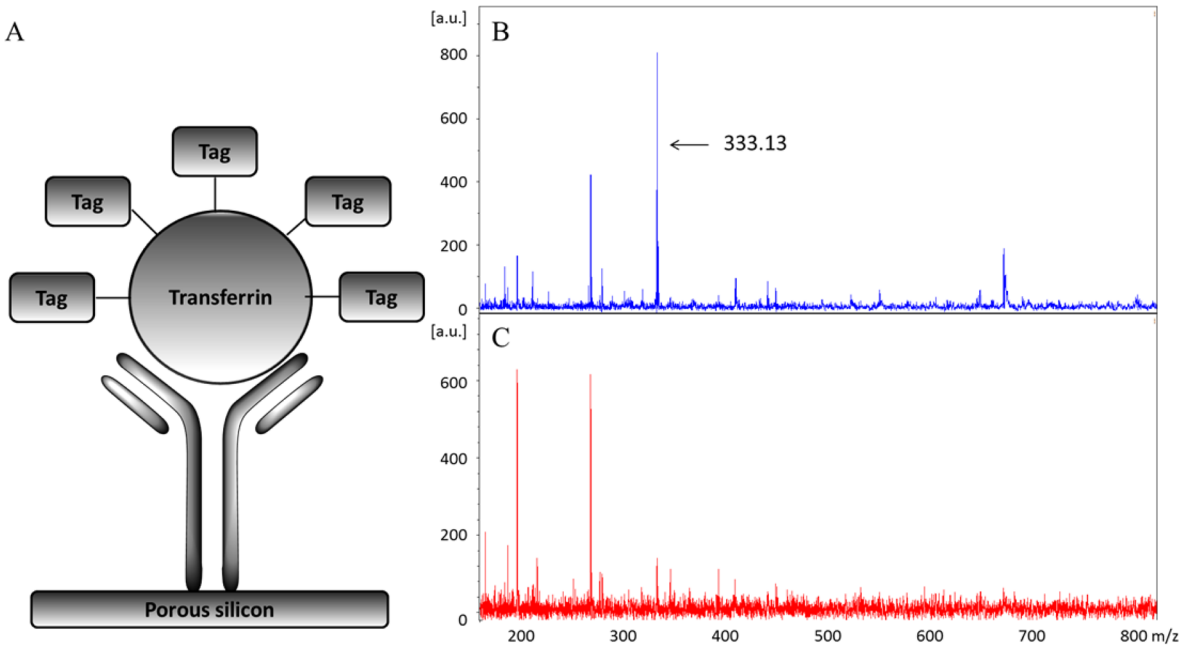

Figure 4. Immunocapture of human transferrin from a mass-tagged plasma sample. Panel A illustrates the assay. Panel B shows the MS spectrum resulting from specific capture of mass-tagged transferrin from human plasma with the antitransferrin antibody positioned on porous silicon array. Panel C shows the read-out from the negative control, where the antitransferrin was substituted with a secondary goat-antirabbit antibody.

tion.[19−21,29] It has also been used as a surface for peptide antibody capture of angiotensin 1 at 1 nM from 10 μL of diluted plasma, although an elution and SPE of the captured antigen was required for successful MALDI-MS read-out.[23] As mass-tags would facilitate direct read-out of the immunocapture with a high sensitivity, an initial investigation was performed to evaluate if the porous silicon would provide any benefits compared to a planar silicon surface. This assay was done using a capture antibody and a mass-tagged antigen.

Human transferrin was mass-tagged and immunocaptured with a goat antihuman transferrin capture antibody on planar and porous silicon (Figure 3A). The antibody was immobilized on the different surfaces using saturating conditions, after which the surfaces were blocked to prevent unspecific binding and incubated with mass-tagged human transferrin in serial 10-fold dilutions from 0.53 mg/mL down to 50 pg/mL in PBS. Compared to planar silicon, the porous silicon provided increased sensitivity and the mass-tag peak at 333.13 Da was

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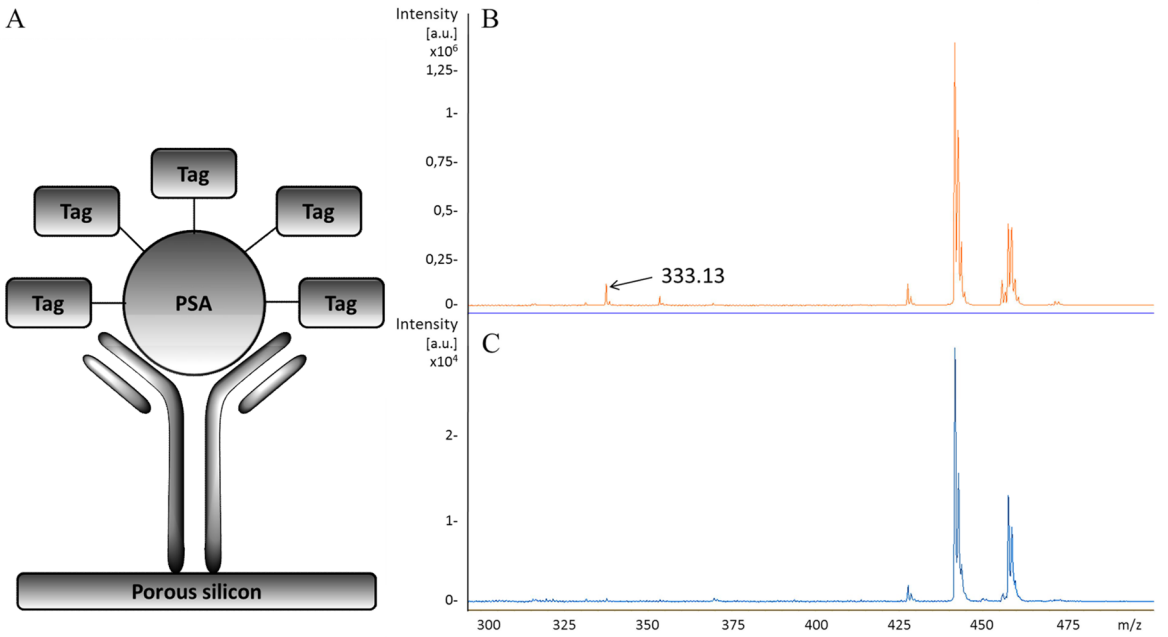

Figure 5. Mass-tagged PSA was spiked into female human plasma and detected on the porous silicon immuno-MALDI antibody array. Panel A illustrates the assay. Capture antibody 2E9 was immobilized on porous silicon and mass-tagged PSA captured by the antibody. Panel B shows the MS spectrum after capture of mass-tagged PSA at a concentration of 5.1 μg/mL in human plasma. Panel C shows the negative control with immobilized antirabbit-antibody, where no mass-tag was visible.

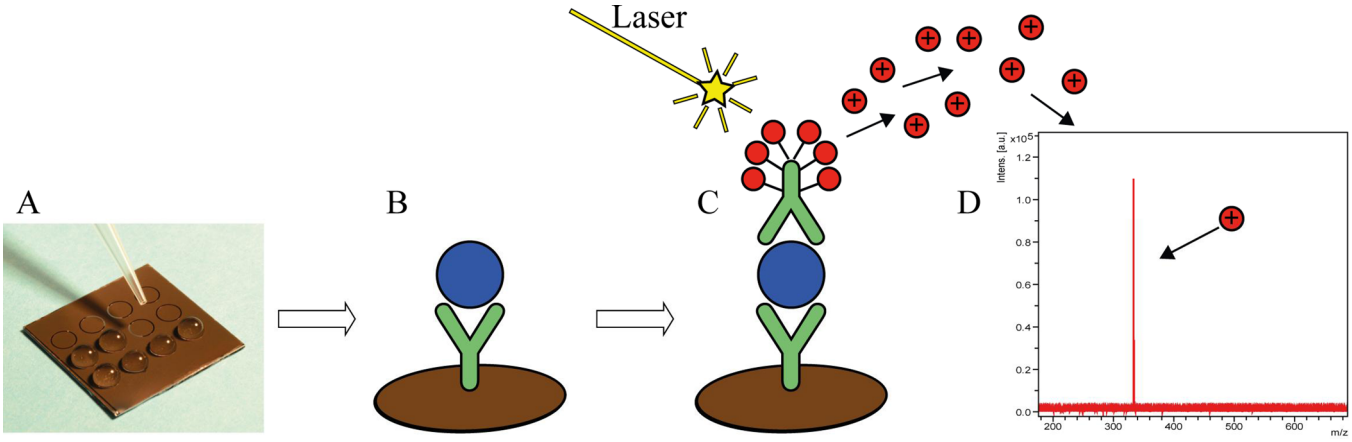

Figure 6. Workflow of the PSA-sandwich assay with mass-tag read-out. Panel A illustrates the porous silicon chip. In panel B, capture antibody is immobilized on the porous silicon surface and incubated with the antigen in human plasma. In panel C, biotinylated detection antibody is incubated with mass-tagged avidin to form an immunocomplex and then incubated on the captured antigen. When subjected to laser irradiation, the mass-tags are ionized, released, and recorded by the mass detector in panel D.

detectable down to 50 pg/mL, while the LOD (limit of detection) for planar silicon was above 0.5 ng/mL (Figure 3B,C). The increased sensitivity of the porous silicon compared to planar silicon is probably due to the fact that the 3D surface of the porous silicon allows for a higher surface density of antibodies/mass-tags. A serious problem with the planar silicon was that wetting on the hydrophilic surface led to spreading of the antibody capture spots during liquid handling. In contrast, the hydrophobicity provided by the nanostructured porous silicon array allowed for both secure confinement and easy removal of 10 μL liquid volumes on each of the 3 mm^2 circular arrays positions.

After concluding that antibody immobilization on the porous silicon array provided the highest sensitivity, the compatibility of trityl tags in a complex biomatrix was tested. Blood plasma was selected as it provides a very complex background and also is the most common sample for which biomarker detection is performed in clinical diagnostic assays. Human plasma was tagged using 15 mg of mass-tag per mL of plasma, containing 50−70 mg of proteins per mL, and incubated on antitransferrin antibodies immobilized on a porous silicon array. The immobilized antibody selectively pulled out the tagged transferrin, which has a natural abundance of ∼2.5 mg/mL in plasma[30,31] and was subsequently detected by direct laser desorption ionization mass spectrometry (LDI-MS) (Figure 4). As a negative control, antirabbit-antibody was immobilized on some of the array positions of porous silicon chip and subjected to the same plasma sample. A very low mass-tag peak with an intensity of ∼350 au due to unspecific binding was detected in

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Anal. Chem. XXXX, XXX, XXX−XXX

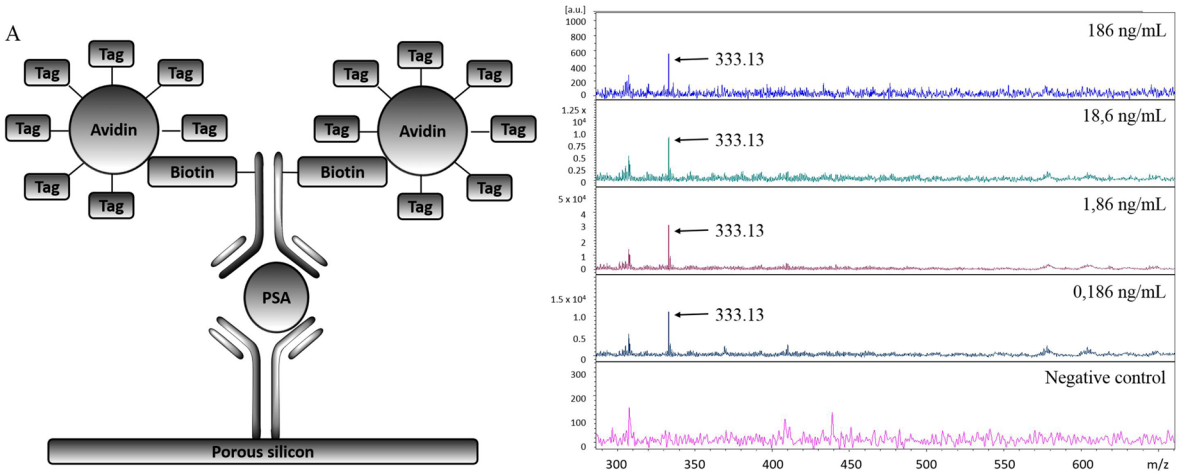

Figure 7. Scheme of PSA-sandwich assay with mass-tag read-out. Panel A illustrates the assay. Capture anti-PSA antibody 2E9 was immobilized on a porous silicon surface and incubated with PSA spiked in known concentrations into female human plasma. Biotinylated detection antibody H117 was incubated with the mass-tagged avidin to form an immunocomplex and then incubated on the captured PSA. The mass-tags were ionized and released by laser irradiation in the MALDI instrument and were recorded by the mass detector. Panels B–E show PSA in final concentrations of 0.186 μg/mL, 18.6 ng/mL, 1.86 ng/mL, and 0.186 ng/mL, respectively, detected with the assay performed in PSA spiked female human plasma. Panel F shows the negative control, where the capture antibody was replaced with antirabbit antibody and PSA was spiked in 18.6 μg/mL, the highest concentration of PSA used in this assay.

the control compared to >8000 au for the specific capture (Figure 4).

As the mass-tag read-out worked well for immunocapture of a high abundant intact antigen like transferrin in plasma, a model for a low abundant and disease linked protein expression was investigated. The clinically relevant cancer biomarker prostate specific antigen (PSA) was selected as it is very well characterized and extensively used in prostate cancer diagnostics and disease monitoring.[24,32,33] The immunocapture experiments were conducted by spiking PSA into undiluted female blood plasma, which is devoid of endogenous PSA. Initially, a direct assay setup was tested where mass-tagged PSA was captured with 2E9 anti-PSA antibody immobilized on the porous silicon array (Figure 5A). The limit of detection for PSA spiked in plasma was found to be ~5000 ng/mL (Figure 5B), which is several orders of magnitude higher than the clinical cutoff value of 3–4 ng/mL. The most probable reason for the low sensitivity in this case is that the mass-tag can bind to the epitope region of PSA which in turn leads to impaired antigen recognition. Another possible explanation could be conformational changes of PSA due to the large amount of tags per protein.

To improve the sensitivity of the direct PSA capture assay and to further investigate the PSA detection limit of mass-tag read-out for immunoassays, the corresponding sandwich assay format used in clinical diagnostics[24,25,32−34] was utilized (Figure 6). In this assay, PSA was spiked in various known concentrations (186 ng/mL, 18.6 ng/mL, 1.86 ng/mL, and 186 pg/mL) into female human plasma and detected with a biotinylated detection-antibody in complex with mass-tagged avidin (Figure 7). The mass-tag was not attached to the antigen or detection antibody directly where it could potentially block the binding sites, but to avidin. The detection antibody carried several biotin groups. The detection limit of the sandwich assay was determined to be 0.186 ng/mL, which is well below the relevant clinical cutoff value of 3–4 ng/mL indicating increased prostate cancer risk. It should be noted that at a concentration of 0.186 ng/mL and using 10 μL of sample the maximum amount of PSA available on the 3 mm^2 spot is below 60 amol. The theoretical maximum binding capacity of one porous silicon spot is approximately 350 fmol;[23] in reality, the capacity has been found to be closer to 50 fmol during antibody capture (data not shown). Thus, even with a porous silicon spot saturated with intact PSA antigen (26–28 kDa) after immunocapture, detection using direct MALDI MS would be very difficult, where sensitivity rapidly decreases with increasing mass. Applying a digestion step in order to generate peptides that could be used for more sensitive detection of the antigen would require capture amounts in the 10–100 fmol range for successful analysis.[35] Thus, the mass-tag approach provides a clear improvement of 2–3 orders of magnitude in the detection sensitivity as compared to direct MALDI MS of PSA to a direct protein digest assay.

The very low LOD of the antibody-mass-tag system is due to a double amplification effect. Every avidin binds several tag molecules and every antibody has several biotin residues to bind tagged avidin, which results in a signal amplification for the mass-tag. Also the high concentration of antibodies on the porous silicon surface and thus high concentration of antigen per surface area contributes to the low LOD.

Depending on the efficiency of the tagging reaction, fairly large experiment-to-experiment variations of the LODs were observed and further work to gain absolute control over this step is needed to reach a better coefficient of variation (CV), linear dynamic range, and quantitation. We observed two major causes for variability between measurements; first, the tagging procedure does not lead to the same amount of tags/avidin every time. The second issue is the variability of signal intensity on the porous surface spots. The surface appears homogeneous in SEM. but there might be effects of the porosity that lead to uneven distribution in the drying steps. For better reproducible quantitation, spiked-in internal standard peptides or proteins could be employed by immobilizing an adequate antibody against the standard-peptide or protein on one spot of the chip.

A big advantage of the mass-tag method as described here is that biotinylated antibodies are widely commercially available and only the avidin needs to be tagged prior to the assay, instead of having to perform chemical tagging of plasma

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Anal. Chem. XXXX, XXX, XXX−XXX

samples. With the optimized protocol for the PSA sandwich assay in this paper, the assay is easy to implement for all antigens for which a sandwich format exists. Due to the low LOD, it would be possible to apply this method for early detection of disease biomarkers and expand it to a disease-specific multiplex format. Platforms like the MALDI Biotyper from Bruker to identify microorganisms and MassARRAY MALDI-TOF mass spectrometer from Agena Biosciences providing precise, rapid, and cost-effective analysis of bacteria, single-nucleotide polymorphisms, insertion or deletion of bases in the DNA (INDEL), copy-number variation, and DNA methylation are just two examples for existing MALDI platforms in clinical healthcare. Further developing the mass-tag approach would allow one to use these platforms to implement more analysis methods.

CONCLUSIONS

The application of mass-tags as a read-out method in immuno-MALDI assays has been demonstrated. As proof of principle, mass-tagged transferrin has been captured in human plasma on a porous silicon substrate and detected by MALDI mass spectrometry. The potential for this method for high sensitivity biomarker analysis has been demonstrated in the form of a sandwich assay detecting PSA, a biomarker used to diagnose prostate cancer in the clinic. The assay reached a LOD of 186 pg/mL PSA in human plasma, which is well below the clinical cutoff value of 3−4 ng/mL. The current limit of detection was obtained with a sample spot size of 3 mm^2. Ongoing work is underway to optimize the sample loading of the arrays, further improving the sensitivity. Finally, the full strength of the mass-tag technology needs to be further explored as the mass-tags do not have the typical quenching effects of colored labels, making them ideal for multiplex assays to simultaneously detect many different markers in the same sample.

ASSOCIATED CONTENT

Supporting Information

Synthetic details and associated structural data of the mass-tag. This material is available free of charge via the Internet at http://pubs.acs.org/.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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Anal. Chem. XXXX, XXX, XXX−XXX