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MALDI-target integrated platform for affinity-captured protein digestion

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Abstract

To address immunocapture of proteins in large cohorts of clinical samples high throughput sample processing is required. Here a method using the proteomic sample platform, ISET (Integrated Selective Enrichment Target) that integrates highly specific immunoaffinity capture of protein biomarker, digestion and sample cleanup with a direct interface to mass spectrometry is presented. The robustness of the on-ISET protein digestion protocol was validated by MALDI MS analysis of model proteins, ranging from 40 fmol - 1 pmol per nanovial. On-ISET digestion and MALDI MS/MS analysis of immunoaffinity captured disease-associated biomarker PSA (prostate specific antigen) from human seminal plasma are presented.

Keywords

ISET; MALDI; proteomics; PSA; immunoaffinity; digestion

1. Introduction

Protein biomarkers have a crucial role not only in the early diagnosis of a disease, but for prognosis and therapy monitoring as well. Thus, for many years, extensive effort has been

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Conflict of interest

ISET AB holds the patent application for the ISET technology. Thomas Laurell is one of the inventors and is founder and shareholder of ISET AB. Dr. Lilja holds patents for free PSA, intact PSA, and hK2 assays. Dr. Lilja is named as co-inventor on a patent application for a statistical method to predict the result of prostate biopsy.

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put in the search for disease correlated biomarkers [1]. In addition to the search for new and reliable biomarkers, current research trends are also directed to re-evaluation of existing protein biomarkers, particularly the protein isoforms or structural variants that might have the potential of enhancing diagnostic capabilities [2].

Mass spectrometric (MS) detection can offer simultaneous, high throughput identification of these protein biomarkers. However, the detection and identification of important protein biomarkers and their linked isoforms are often difficult due to laborious protein sample preparation prior to mass spectrometry detection. Also, many protein biomarker forms may occur at very low abundance in complex biological samples with high background [3], making robust and standardized detection method highly challenging. In MS-based plasma proteomics, various affinity pre-fractionation techniques are used [4–6]. Such protein or peptide affinity enrichment methods have been employed to reach the analytical depth of clinically relevant protein biomarkers.

Another approach is immunoaffinity separation of the protein biomarker with subsequent mass spectrometric detection, this presents a simplified quantitative analysis path as demonstrated for several established protein biomarkers [7–10]. It has been shown that a strategy of combining protein immunoaffinity separation and mass spectrometric detection using an multiple reaction monitoring (MRM) approach can enrich targeted proteins and quantify their concentrations at low ng mL⁻¹ in plasma samples [11]. In addition to intact protein immunoaffinity, peptide antibody extraction coupled to mass spectrometry has also extensively been interfaced to LC/MS/MS systems utilizing stable isotope standards for quantification of protein biomarkers in plasma and serum [12, 13].

While an affinity separation reduces the complexity of biological samples, [14–17] the proteins isolated typically needs to undergo a digestion process prior to identification and/or quantitation with mass spectrometry. Studies focusing on improving tryptic digestion of proteins with higher throughput have been widely reported [18–22]. Many studies on the development of high throughput protein digestion have focused on reducing the digestion time by improved proteolysis and automation of sample handling for analysis of large number of samples [23–26]. In this perspective, developing an integrated platform for highly specific affinity capture, protein digestion and sample cleanup with a direct interface to mass spectrometry, could provide route to accomplish the throughput required to study immunocaptured biomarkers in large cohorts.

The use of matrix-assisted laser desorption/ionization (MALDI) MS in combination with affinity capture and intact protein digest ion has previously been shown to have large potential [27] and examples include both on-chip [28–30] based techniques as well as surface plasmon resonance (SPR) chip [31] and tip-based approaches [32, 33]. The microfabricated proteomic sample handling platform, Integrated Selective Enrichment Target (ISET), previously described by our group has been demonstrated to provide improved performance in solid-phase extraction (SPE) sample preparation [34, 35]. The benefits of the ISET are high sensitivity in the purification/concentration step of obtained peptides and low absorptive sample losses as all sample preparation steps are performed on the same platform. The ISET comprises an array of 96 perforated nanovials and is used as a direct interface for MALDI MS. The platform has previously been successfully applied to purify peptides captured by antibodies [36] and automated digestion of HIS-tag purified recombinant proteins [37].

Prostate specific antigen (PSA) testing has been widely used in the diagnosis and monitoring of treatment of prostate cancer [38, 39], although the value of PSA-testing for early detection of prostate cancer is questioned [40] and remains controversial [41]. Efforts have

been put to improve conventional PSA tests, by investigating different PSA forms/ derivatives [42–45], using PSA dynamics [46], along with other potential prostate cancer biomarkers, such as hK2 and MSP/MSMB [47–49]. The PSA levels are also used in infertility diagnosis as a marker of prostatitis. The PSA concentration in seminal fluid ($0.3 - 3 \text{ mg mL}^{-1}$) is about a million-fold higher than PSA in blood [50], but the complexity of the human seminal plasma which contains a diverse array of lipids, carbohydrates, peptides and proteins makes it a very challenging sample [51, 52].

In this paper we report on a protocol for efficient on-bead enzymatic digestion of affinity-captured full length proteins in the ISET nanovials. The on-ISET digestion protocol was validated by MALDI MS analysis of three model proteins absorbed to reverse-phase (RP) beads, at levels ranging from 40 fmol- 1 pmol/nanovial. To demonstrate the capability of the ISET as an integrated proteomic sample processing platform for immunocaptured intact proteins, we also report on immunoaffinity capture of PSA from pooled seminal plasma obtained from healthy donors followed by on-ISET digestion.

2. Materials and methods

2.1 Proteins and reagents

The carbonic anhydrase, myoglobin and amyloglucosidase, used as model proteins in this study were from Sigma-Aldrich (St Louis, MO, USA). The 95% pure Prostate Specific Antigen, PSA, isolated from human semen was also purchased from Sigma-Aldrich (St Louis, MO, USA). Pooled human seminal plasma samples for the immunocapture were obtained from healthy volunteers. The proteolytic enzyme, trypsin, from Promega (Madison, WI, USA) was used in the digestion step of all proteins. The monoclonal mouse anti-PSA antibody H117 used for the capturing of PSA was produced and characterized as previously described [53, 54]. The reverse phase beads, POROS 20 R2 ($20 \mu\text{m}$), filled in the perforated ISET nanovials, were obtained from Applied Biosystems (Framingham, MA, USA). Dynabeads M-270 carboxylic acid beads were purchased from Invitrogen (DynaL Biotech ASA, Oslo, Norway). All other chemicals and reagents used were from Sigma-Aldrich (St Louis, MO, USA).

2.2 ISET

The ISET functions as a combined sample processing and presentation device to MALDI mass spectrometry. The ISET devices were manufactured by silicon microfabrication, which provides excellent control of dimensions and a surface that is suitable for MALDI MS with regard to surface flatness and conductivity. To prevent sample cross-talk and improve liquid handling, during the application of washing solution and digestion buffer, the ISET surface was made hydrophobic via silanization in toluene with octadecyltrimethoxysilane (OTMS) using *n*-butylamine as the catalyst [55].

2.2.1 ISET work flow—The work scheme for on-ISET digestion of proteins and the inherent SPE of the obtained peptides is described in figure 1. The on-ISET protein digestion protocol begins with the binding of protein on the reverse phase beads or by pre-incubating the protein sample with a small amount of reverse phase beads prior to loading on-ISET by applying vacuum to the backside of the platform. Reduction and alkylation of the protein is followed by tryptic digestion and subsequent elution of the generated peptides from the RP beads. After elution, the analyte formed a crystallized spot surrounding the outlet on the ISET back-side. The ISET was then turned upside down making the analyte positions accessible for laser desorption/ionization in the MALDI MS. The ISET was loaded in an adapted target holder and inserted into the MALDI. The detailed protocol for on-ISET digestion was as follows:

- 1 Fill ISET nanovial with R2 POROS reverse phase beads (50 – 300 nL bead volume) and load protein sample at –800 mbar. Or add pre-incubated sample beads to the nanovials at –800 mbar
- 3 Turn off vacuum; reduction of protein with 1 μ L 10 mM dithiothreitol for 5 min
- 4 Alkylate the reduced protein with 1 μ L 27 mM iodoacetamide for 5 min
- 5 Wash with $2 \times 10 \mu$ L mass spectrometry grade water
- 6 Add 1 pmol trypsin at –200 mbar, turn off vacuum and add $3 \times 2 \mu$ L 20% acetonitrile (ACN) in 25 mM tris. Keep the beads wetted with 20% ACN in water throughout the 15 min – 2 hour digestion time
- 7 Wash with $2 \times 10 \mu$ L 0.1% TFA (trifluoroacetic acid) at –800 mbar
- 8 Elute with matrix 5 mg mL⁻¹ CHCA (alpha-cyanohydroxycinnamic acid) in 50% ACN/0.1 – 1% TFA, $2 \times 0.3 \mu$ L through the bead volume (–50 mbar)
- 9 Flip over the ISET and insert in holder for MALDI MS

2.3 On-ISET digestion of model proteins

In the study comparing on-ISET digest and in-solution digest, 200 fmol myoglobin in 50 mM ammonium bicarbonate buffer was used, subsequent digested on-ISET according to the protocol described above (2.2.1 ISET work flow) sans the reduction and alkylation.

To test the robustness of the on-ISET protein processing protocol myoglobin, carbonic anhydrase and amyloglucosidase in 50 mM ammonium bicarbonate buffer to final amounts of 40 fmol - 1 pmol were loaded to each ISET nanovials as follows:

1. myoglobin, at 50 fmol, 200 fmol and 1 pmol
2. amyloglucosidase, at 40 fmol, 400 fmol and 1 pmol
3. carbonic anhydrase, at 60 pmol, 600 fmol and 1 pmol

Simultaneously, we performed replicate studies of 1 hour digested proteins on the ISET platform (N=12) and subjected them to MALDI MS readout.

In order to predict which PSA peaks would be generated from the immunocapture, 400 fmol pure PSA in 50 mM ammonium bicarbonate buffer was used with and without reduction and alkylation.

2.4 Immunoaffinity captured PSA from pooled seminal plasma

The mouse monoclonal anti-PSA antibody H117 was covalently immobilized onto superparamagnetic Dynabeads M-270 carboxylic acid beads, according to the recommended protocol by Invitrogen (DynaL Biotech ASA, Oslo, Norway). The antibody was applied in excess in order to ensure maximum binding of the antibody. H117 antibody-coated Dynabeads were used to immunoaffinity capture PSA via magnetic separation. In these experiments, 20 μ L of pooled human seminal plasma was incubated with H117 antibody-coated Dynabeads (30 μ L slurry) in a microtube, with slow shaking for 2 hours at 2 – 8°C. The tube was then placed on a magnet for 8 minutes to collect the PSA-bound antibody beads. The supernatant was removed; the beads were washed twice with 60 μ L 25 mM tris and subsequently transferred to a new microtube to reduce unspecific binding. The washed, PSA-bound antibody beads were resuspended in 30 μ L of 25 mM tris and deposited into ISET nanovial positions pre-filled with RP beads for a 2 hour trypsin digestion, as described in the ISET digestion protocol. Direct on-ISET MALDI MS/MS analysis was then carried out for identification of the obtained peptides. In order to find the most frequently observed

peptides resulting from successful immunoaffinity capture of PSA, a direct 1 hour on-ISET digestion of 20 μ L 28 nM PSA in 25 mM ammonium bicarbonate buffer was performed. The PSA peaks of 1407, 1871 and the 1887 Da methionine oxidation peak were identified as the best choice (figure S1, supplementary data).

2.5 MALDI Mass spectrometry

MALDI analysis was made using a Waters M@ldi ToF MS (Milford, MA, USA) or a MALDI-Orbitrap XL from Thermo Scientific (Waltham, MA, USA). The MALDI-TOF instrument was used in reflector mode, MCP 1950 V and reflector voltage of 2000 V. All the spectra were acquired manually and approximately 100 shots were averaged. The instrument was calibrated using ProteoMass Normal Mass Calibration Mix (Sigma, St Louis, MO, USA). MALDI-Orbitrap XL mass spectra were obtained in positive mode mass range of 800 – 3000 Da, using 60,000 resolution (determined at 400 m/z). For each spot, 20 FT-MS mass scans (2 microscans/scan) were collected. IT MS/MS data was performed on known PSA masses using a collision energy of 50% during an activation time of 30 ms and activation Q of 0.250. Resulting spectra were processed by Xcalibur software v2.0.7 (Thermo Scientific, Waltham, MA, USA) and MS/MS was analyzed with the SEQUEST search engine for confirmation of identity.

3. Results and Discussion

3.1 ISET as a MALDI-target integrated protein digestion platform

A strategy for rapid, on-target digestion of proteins and simultaneous sample clean up prior to MALDI mass spectrometry readout was developed. Each of the ISET nanovials can be filled with approximately a 600 nL bead volume. The capacity of the reverse phase beads is specified to 5 mg lysozyme mL^{-1} of bed volume. In our ISET digestion experiments, the amount of beads in the ISET nanovial was typically 1/3 of a vial, corresponding to a capacity of approximately 25 pmol lysozyme. This capacity is sufficient for the purposes of digesting affinity captured proteins, where one can expect to capture protein amounts in low femtomol amounts.

Standard protein digestion parameters for the on-ISET digestion protocol were optimized, i.e. pH, time and enzyme-to-substrate ratio. More specific parameters such as size of reverse phase beads for sample binding, digestion buffer and solvent concentration were also investigated in the earlier studies (data not shown). On-ISET trypsin digestion of proteins can be carried out from 15 minutes to 2 hours by continuous addition of digestion buffer and solvent to the packed bed. The continuous supply of digestion buffer containing 20% organic solvent ensures the pH to be maintained and that bound trypsin is made available for the digestion process. Since the ISET has the same pitch as a standard 384 microplate, automated sample handling is feasible [37]. The hydrophobicity of the ISET surface achieved by silanization provided a good confinement of 5 – 10 μ L fluid volume on each of the ISET nanovial positions.

Inherent with the on-ISET digestion follows that generated peptides are enriched on the solid phase in the drying step prior to the wash and elution. Optimized washes and final elution of the obtained peptides with matrix resulted in a crystallized matrix spot. The combination of on-target trypsinization of proteins and enrichment of generated peptides on a MALDI instrument compatible platform provides a unique proteomic sample processing platform.

3.2 On-ISET digestion compared to in solution digestion

To compare on-ISET protein digestion and the conventional in-solution digestion, myoglobin, known to be relatively resistant to tryptic digestion [56], was used. Since the volume of the ISET nanovial is very small the tryptic reaction is faster compared to an in-solution digestion which depends on the diffusion rate of the molecules. The resulting mass spectrum show comparable number of detected tryptic peptides for 1 hour on-ISET and 24 hour in-solution digestion (figure 2). Peptide fragments produced from on-ISET digestion of myoglobin, as assigned by the MASCOT search engine can be seen in supplementary data, table S1. On the ISET 11 tryptic peptides, compared to the 10 peptides obtained by in-solution digestion, were identified providing a sequence coverage of 85% and a Mowse score ($P < 0.05$) of 106. The 1 h on-ISET digestion effectiveness in this case is comparable to overnight in-solution digestion. Although, the surface-bound protein digestion may result in different digestion products i.e. a wider range of peptide fragments with increased missed cleavages and even unique peptides [57], when compared to those obtained from an in-solution digest using the same enzyme.

3.3 Robustness of on-ISET sample processing

To demonstrate the robustness of the on-ISET digestion protocol, three model proteins: myoglobin, amyloglucosidase and carbonic anhydrase were digested at low fmol to pmol levels per ISET nanovial. For each of the different protein amounts, on-ISET digestion ($N=12$) were performed simultaneously. A 3D MALDI mass spectra representation of carbonic anhydrase, after on-ISET digestion showed reproducible spectra obtained for the on-ISET digestion replicates (supplementary data, figure S2). We further analyzed all digestion replicates for the number of occurrences for a number of selected tryptic peptides. Number of occurrences for four frequently observed tryptic peptides of carbonic anhydrase at varying amounts of processed sample can be seen in figure 3. Myoglobin and amyloglucosidase on-ISET digest replicates were similarly analyzed (figure S3–S6, supplementary data). From the data it is evident that on-ISET digestion of proteins can be performed down to a level of 60 fmol total protein loading, with a success rate of 88%, depending on which peptides an assay would be based on (figure 3). This is well within in the range of the immunocapture capacity provided by the beads put in an ISET nanovial.

3.4 On-ISET reduction and alkylation of pure PSA

Reduction and alkylation of proteins prior to tryptic digestion increases the digestion efficiency by breaking the disulfide bonds present in the protein and prevent reformation of the disulfide bridges, making enzymatic cleavage sites more accessible. To demonstrate the effectiveness of the on-ISET reduction and alkylation steps, pure PSA, a 33 kDa protein biomarker with five conserved disulfide bonds [58] was on-ISET reduced and alkylated prior to trypsin digestion.

A higher number of detected PSA peptides, eight were observed after on-ISET reduction and alkylation treated sample, as compared three without reduction and alkylation (figure 4). Table S2 lists the observed peptides from the on-ISET reduction, alkylation and digestion of PSA. Thus, as the reduction and alkylation generates more peptides it is best used when high sequence coverage is important, i.e. if tracking isoforms or mutation shifts. If an assay can be based on peptides generated without these steps it can be omitted in order to save time.

3.5 PSA immunoaffinity on-ISET detection from seminal plasma, targeted MS screening

PSA immunoaffinity capture from human seminal plasma was carried out using H117 monoclonal antibody-coupled Dynabeads as described earlier (section 2.4). The washed,

PSA-bound antibody beads were then transferred to the nanovials for on-ISET digestion and MALDI analysis.

Figure 5 shows the MALDI-orbitrap mass spectrum resulting from a direct in-solution digestion of 20 μ L pooled seminal plasma sample, i.e. without using PSA immunoaffinity capture. Due to the complexity of the seminal plasma contributed by other seminal plasma proteins, no PSA peaks could be detected in this spectrum. With the use of PSA-immunoaffinity capture followed by ISET-digestion, PSA peaks at 1407, 1871 and 1887 Da were successfully detected with MALDI MS/MS (figure 6). This verified the specific enrichment of PSA via the antibody capture. The interference in the resulting mass spectrum is mainly a result of the monoclonal antibody peaks arising due to digesting the antigen while bound to the antibody and also background signal from unspecific binding. This result is comparable to standard protocols but here the final analysis read-out can be made much faster as compared to performing an in-solution digestion of the eluted antigen. In a standard protocol the eluted antigen has to be subjected to a buffer change that results in sample losses prior to digestion, these are completely avoided by the suggested protocol.

A major part of the interference can theoretically be reduced by inclusion of an elution step of the captured antigen from the antibody-beads if the antibody is covalently immobilized. It should be noted that standard immunocapture protocols are based on protein A/G beads and display the same background interference due to leakage of antibody during elution.

In a clinical setting, the PSA immunoaffinity on-ISET digestion would be using MS/MS for identification and quantification. In figure 6, an MS/MS analysis was directly performed on-ISET for 1407 and 1871 PSA parent peptides resulting in MS/MS data of the fragment ions enabling a sequence confirmation of the PSA (inserts of figure 6). As a specificity control for the PSA immunoaffinity on-ISET digestion, an assay comparing non-relevant antibody-beads and antiPSA-beads was performed. No unspecific binding of PSA was observed, as shown in figure 7. Based on the capability of ISET as a MALDI target-integrated sample processing platform, a direct MS/MS detection of immunoaffinity captured PSA from complex biological samples has been demonstrated.

The benefits of ISET in targeted MS screening as such are that on-target trypsinization and SPE minimized sample transfers while a direct interface to the MALDI mass spectrometry is enabled. Protein-immunoaffinity separation coupled to mass spectrometric detection offers high specificity since it provides selection at both the peptide and fragment level, which is especially beneficial in screening and validation work for potential biomarkers.

4. Conclusion

A MALDI-target integrated platform for antibody affinity-capture, combining on-bead trypsinization of captured antigen with SPE sample clean-up and concentration of the obtained peptides in the ISET nanovials was realized. The proposed platform allows for a minimum of samples transfers and is highly amendable for automation.

Prior to the application of ISET as a digestion platform for immunoaffinity captured PSA, we developed the on-ISET digestion protocol using three model proteins to ensure optimized performance of the digestion platform. A methodology of first digesting the antigen on-ISET to find the most stable peptides of identification is suggested. Using PSA as a model we have demonstrated the potential of the ISET protocol for targeted MS screening via protein immunoaffinity capturing in a complex biological sample, i.e. seminal plasma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MS	mass spectrometry
MRM	multiple reaction monitoring
MALDI	matrix-assisted laser desorption/ionization
SPR	surface plasmon resonance
ISET	integrated selective enrichment target
SPE	solid-phase extraction
RP	reverse phase
PSA	prostate specific antigen

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Highlights

- We report a new methodology on the proteomic sample processing platform ISET
- on-ISET tryptic digestion of femtomole amounts of full length proteins
- We demonstrate an application where we immunocaptured the clinically relevant PSA

Figure 1.

Workflow of the on-ISET protein digestion. The protocol begins with the binding of protein on the reverse phase beads present in the ISET nanovials, followed by 5 min reduction and alkylation of the protein respectively. On-ISET trypsin digestion was subsequently performed for 15 min – 2 hours. With several washes in between the steps, sample cleanup was efficiently carried out. The final elution of the analyte resulted in a confined crystallized matrix spot surrounding the ISET nanovial outlet. The ISET was then turned upside down making the analyte positions accessible for laser desorption/ionization in the MALDI MS.

Figure 2.

Myoglobin at 200 fmol. In-solution vs. on-ISET digestion of myoglobin resulted in comparable number of detected peptides (as indicated with red arrows). Top spectrum: myoglobin at 200 fmol was digested using the conventional in-solution trypsin digestion for 24 hours. Spectrum below: 200 fmol of myoglobin was digested in the ISET nanovial for 1 hour.

Figure 3.

Number of occurrences for selected peptides of carbonic anhydrase on-ISET digestion replicates at 60 fmol, 600 fmol and 1 pmol per nanovial. 12 on-ISET digestion replicates were performed simultaneously for each varying amount of carbonic anhydrase (N=12).

Figure 4.

On-ISET digestion of pure PSA at 400 fmol. Red arrows indicate PSA peptides. Top spectrum: PSA, 1 hour on-ISET digested, without reduction and alkylation. Spectrum below: PSA, on-ISET reduced and alkylated prior to 1 hour on-ISET digestion. Spectrum below shows a higher number of detected peptides with the incorporation of on-ISET reduction and alkylation.

Figure 5. MALDI MS spectrum resulting from in-solution trypsin digestion of a pooled seminal plasma sample obtained from healthy donors. Without PSA immunoaffinity on-ISET digestion, no PSA peaks could be seen due to the complexity of the seminal plasma digestion spectrum. Inserts are zoomed in images of mass range 1407 and 1871. Red arrows indicate where the PSA peptides should have been seen.

Figure 6.

On-ISET detection of PSA from human seminal plasma sample. The above FTMS spectrum was obtained on a Maldi Orbitrap XL showing PSA peaks after PSA immunoaffinity on-ISET digestion. PSA was immunoaffinity captured using PSA antibody-coated magnetic beads, washed and transferred into ISET nanovials for on-bead trypsin digestion and direct readout by MALDI mass spectrometry. 1407, 1871 and 1887 Da were observed (as indicated by the red arrows). Further MS/MS spectra of 1407 and 1871 PSA peptides can be seen in the inserts: MALDI Orbitrap CID of 1407 and 1871, both resulting in fragment ions to confirm detection of PSA peptides from the seminal plasma.

Figure 7.

Specificity control. PSA immunoaffinity on-ISET for PSA detection using non-relevant antibody-coated magnetic beads. The MALDI mass spectrum shows no unspecific binding of PSA. Other resulting peptide peaks were induced from the digested antibody-coated magnetic beads (anti-amyloid precursor protein- coated Dynabeads), also present in the ISET nanovials. Inserts are zoomed in images of mass range 1407 and 1871Da.