Development of a polymeric planar microwell device (pMALDI chip) for enhancing protein analysis in combination with MALDI-TOF/MS instrumentation

Dissertation

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von M.Sc.
Alfredo Jesús Ibáñez Gabilondo
Geboren am 23.08.1977 in Lima, Perú

Referees (Gutachtern):

1. Prof. Dr. Hans-Peter Saluz
   Leibniz Institute for Natural Product Research and Infection Biology - Hans-Knöll-Institute
   Cell and Molecular Biology department head
   Beutenbergstr. 11a, 07745 Jena
   Thueringen, Germany
2. Prof. Dr. Karl Otto Greulich
   Leibniz Institute for Age Research - Fritz Lipmann Institute (FLI)
   Single cell and single molecule techniques research group head
   Beutenbergstr. 11, 07745 Jena
   Thueringen, Germany
3. Prof. Dr. Michael Koehler
   Technical University Ilmenau
   Faculty of Mathematics and Natural Sciences
   Department of Physical Chemistry and Microreaction Technology
   Weimarer Str. 32, 98693 Ilmenau
   Thueringen, Germany

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Table of Contents

Preface

The ability to perform proteomic analysis quickly and easily is becoming increasingly important in the fields of bioanalytical chemistry. The success of the high-throughput proteomic technique known as matrix-assisted laser/desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) is related to the sample preparation prior to the analysis. Unfortunately, state-of-the-art sample preparation techniques such as Zip-Tips® and dialysis-based methods cannot be performed on-line, hence reducing the efficiency of the MALDI-TOF/MS analysis.

The work presented in this thesis provides a general overview of my contribution to the field of disposable plastic MALDI-MS chips.

The goal of this thesis is to further develop the original concept of plastic MALDI (pMALDI) chips by addressing their drawbacks as well as by finding relevant applications for these chips in the fields of genomics and proteomics.

1. Introduction

1.1 Proteomic research: Past & Present

During the mid-nineties, a milestone was achieved in molecular biology: scientists unraveled the code of life embedded in the DNA biomolecule of an entire self-replicating organism, *Haemophilus influenzae*; this event heralded the sequencing of the human genome and the beginning of the "genomic era".[1,2] This achievement was possible due to the development of new analytical techniques for high-throughput DNA sequencing in combination with progress in the field of bioinformatics. In following years, further bacterial genomes (the complete set of genes of an organism) and higher organism genomes (including the human genome) were sequenced. The scientific community was confident of its ability to complete the sequence of large numbers of genomes and were optimistic about its ability to use this knowledge and similar analytical techniques for the field of protein analysis (i.e. long chains – biopolymer – of amino acids).[1]

Protein analysis focusing on biochemical functions is almost one century old (e.g. Krebs and Kurt Henseleit in 1932 studied the proteins involved in the urea cycle). Although many researchers during the 50s, 60s, and 70s of the last century laid the foundations of our knowledge of how protein functions are related,[2] it was not until 1982 that the idea of mapping all proteins and studying their biological function was officially pursued by scientists.[3] Nevertheless, the beginning of the "genomic era" concomitantly catalyzed the increase of research of "post-genome" research fields. Hence, also in 1995, the term "proteome" (the study of the proteins produced by a cell's genome) was coined by Wassinger *et al*.[4] The proteomic field (the study of the organism's proteome) was formed as a research field analogous to genomics.[4] Proteomics is more complicated than genomics, mostly because while an organism's genome is rather constant, a proteome differs from cell to cell and constantly changes in-time and through its biochemical interactions with the genome and the environment.

The proteomic field encompasses different key research branches that are required to catalog the proteins produced by a living organism during its life cycle. These key branches are:[5]

- Sequence & structural proteomics. Prior to the advances in genomics, protein sequence studies were carried out using Edman degradation in combination with endoproteases and HPLC separation. Meanwhile, structural studies were carried out using NMR and X-ray crystallography. The overall accuracy of the Edman degradation for protein sequence generally declines as the length of the amino acid sequence increases; hence, the protein must be cleaved using endoproteases to create shorter sequences. Although Edman degradation can be carried out by a sequenator (i.e. a robot which runs an Edman degradation reaction), new technologies are now available that permit the sequencing of similar short sequences in a matter of minutes, such as mass spectrometry. The elucidation of protein structure has also seen great progress due to the use of bioinformatics to predict structurally conserved motifs (i.e. sequences of amino acids), which are related to a specific protein activity.
- Expression proteomics. This field is devoted to the analysis of protein abundances; it involves the separation of complex protein mixtures and the identification of individual components and their systematic quantitative analysis.

- Interaction & functional proteomics. These two branches are closely related. The first refers to the study of protein interactions with other substances (i.e. DNA, RNA, small molecules, and other proteins), focusing on the spatial localization of such events in order to elucidate biological pathways or networks of protein activity (macro view). Meanwhile, the second focus is the in-depth study of the activity of individual recombinant proteins (micro view).
- Bioinformatics. Today, the increasing number of new technologies in the field of genomics and in the different fields of proteomics research have centered on increasing the throughput of information and the organization of such information in databases using bioinformatic tools. The creation of dedicated nucleic acid and protein sequence databases (e.g. the Protein Data Bank, SWISS-PROT, TrEMBL, etc.) reflect the increasing needs of scientists to store, present, compare and predict protein structures, which will allow them to recognize functional related proteins by relating structurally conserved motifs in the protein sequence.

The complete characterization of a proteome is a formidable task. Prior to 1995, scientists believed that mapping the proteins in an organism will consist of a unique technique that would examine a whole proteome with a single, integrated set of analytical operations. Today, researchers understand that this may not be possible. The proteome is exceedingly dynamic and extremely complex, because even with the DNA sequence information provided by a known genome, scientists cannot predict (see Figure 1)

- when and where the proteins will be produced;[2]
- the relative concentration in which the protein will be synthesized;[2]
- the influence of internal cell factors, such as splicing variants, genetic variation between individuals, and post-translational modifications (PTM); and external factors, such as drug administration, disease, environmental stress, etc., which may increase heterogeneity between proteomes in cell populations.[2,6]

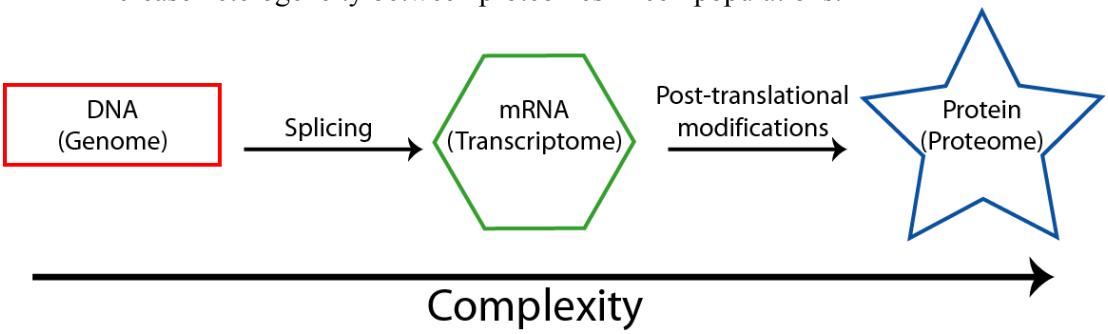


Figure 1. Dogma of molecular Biology

In humans, for example, the variability of the biosynthesis of different immonuglobulins is based on the random rearrangements of the gene segments in germ-line DNA. These rearrangements are carefully regulated so that the immunoglobulin DNA of only one parental chromosome is rearranged to form a functional gene. This allelic exclusion is necessary to assure that only single antigenic specificity is expressed per cell. Similarly, other proteins can be synthesized in different ways due to rearrangements during the transcription + translation steps, and their activity can be regulated *via* post-translational modifications. Therefore, the field of proteomics is indeed extremely challenging if for ~300 different identified genes for the recognition sites in human IgG antibodies, these code for more than >15000000 possible IgG proteins.[7]

The identification of proteins must be carried out using indirect means of detection because of size, abundance, and stability constraints. To overcome this challenge, proteomic research today includes a rapidly expanding set of technologies that are being used to provide protein identification and exhaustive proteome characterization (such as protein function determination).[8,9] The following list encompasses the major techniques in proteomic research. All of them are based on highly efficient methods of separation in combination with extremely sensitive detectors:

- Liquid chromatography (LC) separation is achieved through a partitioning equilibrium between a mobile and stationary phases. The variability of the mobile phase is what allows the equilibrium to shift and thereby directly influences the selectivity of the separation.[9] Although this technique presents several advantages, it is not highly robust when dealing with complex samples (for which tedious sample preparation protocols are required prior to running the sample). The detection systems associated to this technique are indeed numerous, for example: optical and electrochemical detectors, mass spectrometry (MS), nuclear magnetic resonance (NMR), etc.
- Capillary electrophoresis (CE) separation is based on taking advantage of the different mobility of analytes in a conductive liquid medium under the influence of an electric field. CE in combination with fluorescent detection (laser-induced fluorescence, LIF) has played a major role in sequencing a number of genomes, providing extensive information about gene sequence.[10] Today, CE in combination with MS, EC, and LIF detection, in both capillary and microchannel format, continues to provide an impressive number of applications at the forefront of proteomic and metabolomic research.[9,10] However, an important parameter to consider is the non-specific adsorption of peptides (i.e. short sequence of amino acids) and/or proteins to the surface of the capillaries, which results in loss of resolution and robustness due to variations in the electroosmotic flow (EOF).[11]
- One or two dimensional polyacrylamide gel electrophoresis (1D- 2D-PAGE) in combination with optical (coomassie or silver staining) and LIF detection[12,13] is the method of choice for the analysis of complex protein mixtures, due to its resolving power and robustness. It enables the separation of proteins according to apparent molecular mass or isoelectric point in 1D, and in 2D first according to their isoelectric point and then according to their molecular mass.[14,15] After protein purification, the proteins are digested by a sequence-specific endoprotease and converted to a set of peptides. The obtained peptides can subsequently be analyzed by LC or CE techniques with hybrid mass analyzer instruments, which are able to fragment and sequence the composition of amino acids. A computer can use the overlaps in the peptide sequences to reconstruct the whole protein; this process is known as *de novo* sequencing. In case the protein sequence is already present in a database, the peptides produced by the protein digestion can be quickly analyzed using matrix-assisted laser desorption/ionization mass spectrometer (MALDI-MS). An overview of the capabilities of these two approaches is presented later in this introduction.
- Ion mobility separation (IMS) is based on the different mobilities shown by gas-phase ions in the presence of an electric field and a countercurrent gas flow. When two gas-phase ion species with similar mass-to-charge ratio experience different frictional forces, they will separate from one another as they migrate through the

countercurrent gas flow. The frictional forces experienced by an analyte ion depend on the viscosity (η) of the medium and the size and shape of the ion. This separation method, capable of differentiating isomaric ion species with different special arrangements, is currently included as part of a new generation of mass spectrometers.

- Protein arrays are based on the detection of proteins which present affinity toward an immobilized biomolecule (e.g. antibodies, oligonucleotides, small organic molecules, etc.) on the surface of a glass slide or silicon microwell.[16,17] Detection methods usually associated with protein arrays are mass spectrometry (MS), laser-induced fluorescence (LIF) and surface plasmon resonance (SPR).

1.2 Laser desorption/ionization (LDI) techniques for proteomic research

1.2.1 Matrix-assisted laser desorption/ionization (MALDI)

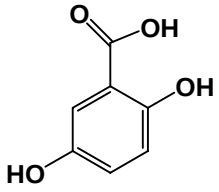
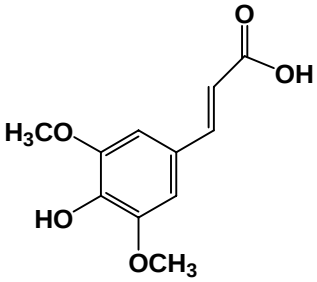
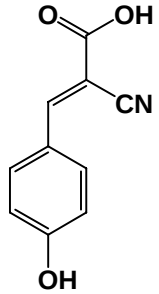
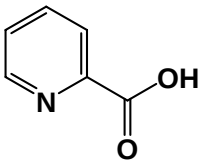
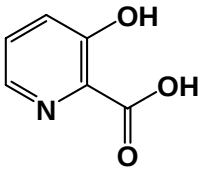
The desired end of proteomic research is to develop a complete database for each individual organism containing the identity of each protein that the organism can produce and to characterize these proteins in terms of their abundance, modification, localization and function. Such a comprehensive database of proteins could be useful for investigating cell responses during a drug trial, the prognosis of diseases, the response of a plant to insect attack, etc.

The recent increase in throughput for protein identification is related to progress in the field of mass spectrometry (MS), in combination with the development of comprehensive protein databases and advances in bioinformatics.[18,19] Mass spectrometry is a group of techniques that can be used to detect low amounts of proteins (picomoles to femtomoles) without the use of labeling tags, by directly determining their masses. In addition, MS techniques can provide the amino acid composition of proteins with or without the use of (bio)chemical reactions, and is easily adaptable to high-throughput formats.[18,19]

In 1988, matrix-assisted laser desorption/ionization (MALDI) mass spectrometry burst on the scene and revolutionized the field of protein measurement in mass spectrometry.[20,21] The term, coined by Franz Hillenkamp *et al*. in 1985,[22] refers to the ionization source/mechanism utilized to introduce the sample into the mass analyzer.

Although the details of the desorption/ionization mechanism in MALDI are not completely understood at the molecular level, several models try to explain it.[23] Briefly, the ionization mechanism is based on mixing (i.e. co-crystallize) the analyte with matrix molecules that absorb laser radiation. Most common MALDI matrixes adsorb ultraviolet, UV, radiation and are mixed with an excess (~10^4-fold) to the analyte, see Table 1.[24, 25]

Table 1. MALDI matrixes for UV lasers (argon; argon/fluor; nitrogen or neodymium/YAG)

Compound	Other Names	Solvent	Abs. max. (nm)	Applications
 **2,5-dihydroxy benzoic acid**	DHB	acetonitrile, water, methanol, acetone, chloroform	337, 355, 266	peptides, nucleotides, oligosaccharides, polar polymers
 **3,5-dimethoxy-4-hydroxycinnamic acid**	sinapic acid; SA	acetonitrile, water, acetone, chloroform	337, 355, 266	proteins
 **α-cyano-4-hydroxycinnamic acid**	CHCA; α-matrix	acetonitrile, water, methanol, ethanol, acetone	337, 355	peptides
 **2-picolinic acid**	PA	ethanol	266	oligonucleotides
 **3-hydroxy-2-picolinic acid**	HPA	ethanol	337, 355	oligonucleotides

During drying on the MALDI probe, the analyte molecules are intercalated into the matrix crystals (see Figure 2). The matrix is clearly the single most important factor in the success of the laser/desorption technique: It serves to (a) isolate the analyte molecules from each other; (b) absorb the energy of the laser; and (c) vaporize/propel the analyte molecules into the gas phase. In the gas phase, the analyte can be subsequently ionized by the molecules in the plume of the excited-state matrix to form gas-phase ion molecules $[M+H]^+$, if they were not previously ionized by an acid-base reaction prior to their co-crystallization with the matrix. It is also possible to create $[M-H]^-$ gas-phase ions as well as adducts with salts (all depending on the matrix used and the crystallization conditions). In this thesis, MALDI results will refer mostly to singly charged gas ions, written as $[M+H]^+$ for convenience. This does not imply that the negative mode measurements at any time were less successful, or that $[M+Na]^+$, $[M+K]^+$, $[M+NH_4]^+$, protein dimers or multiple charged proteins were not observed.

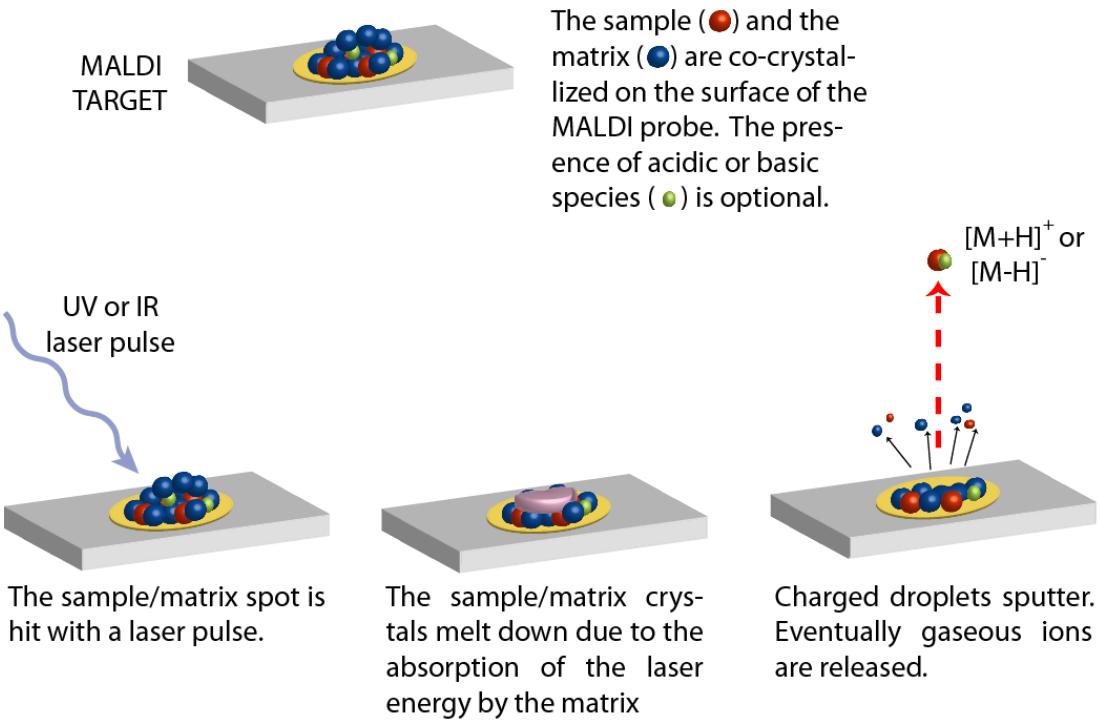


Figure 2. Matrix-assisted laser desorption/ionization (MALDI) process.

The mass-to-charge ratio (*m/z*) of each gas ion formed during the MALDI process can be subsequently analyzed using a time-of-flight (TOF) mass analyzer (see Figure 3). TOF mass analyzers measure the time taken for the gas-phase ion to travel through a field-free flight tube after being accelerated by an electrical potential. The equation for determining the time required to arrive to the detector is:

$$t = d * \left\{ 1 \Big/ \left[\sqrt{2q(V + Es)/m} + v_0 \right] \right\} \qquad \text{(equation 1)}$$

where t = time; m = mass; v_0 = native speed of ions in the plume; E = electric field generated near the laser spot; s = length of electric field generated near the laser spot; q =

Ze (electronic charge); V = accelerating potential; d = length of field-free region. The TOF analysers are built so the term $v_0 \approx 0$ and due to the conductivity of the target substrate $Es \approx 0$, therefore the equation can be re-written as:

$$m/z = \left(2eVt^2\right)/d^2 \qquad \text{(equation 2)}$$

Hence, the mass of a singly charged ion – if z = 1 then *m/z* for an specific $[M+H]^+$ is equal to its mass plus the mass of a proton – can be determined from the length of the tube, the electric potential difference applied (voltage), and the time taken to reach the detector at the end of the tube.

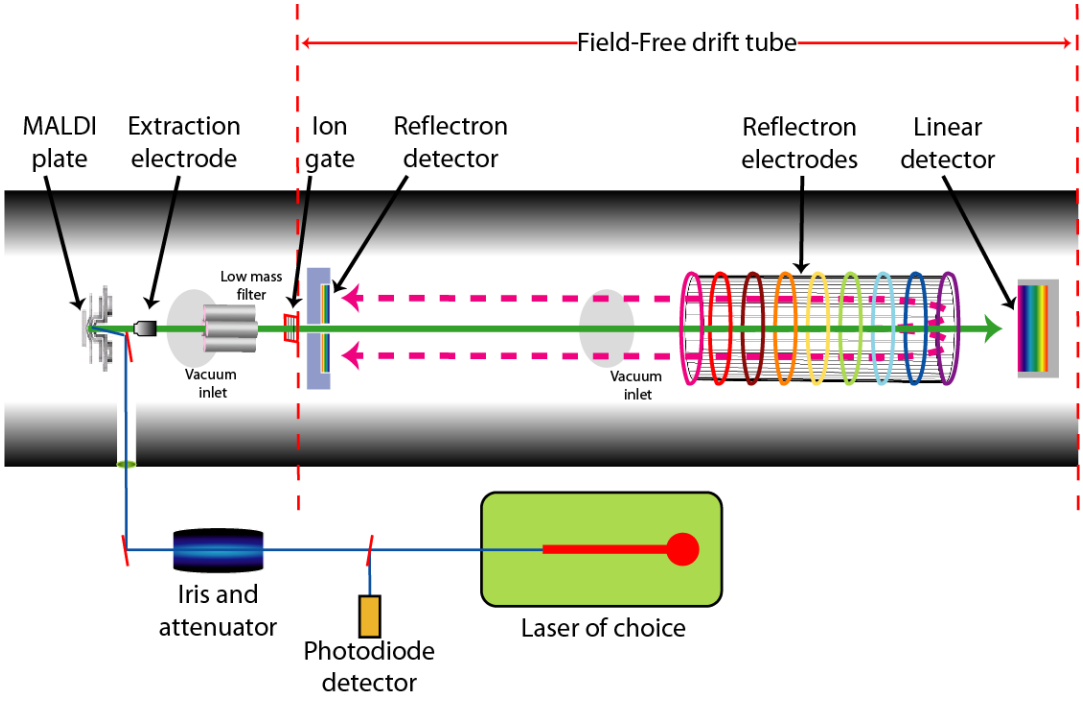


Figure 3. A schematic representation of a MALDI-TOF/MS system.

Thus, the simplicity, speed and performance of MALDI-MS make it a powerful mass analyzer for proteomic research (see Figure 4). Moreover, it permits the characterization of compounds previously intractable by other mass spectrometric analyzers, such as large biomolecules,[26] synthetic polymers,[27] coal products,[28] inorganic[29] and organic compounds.[30] For example, one of the standardized methods for identifying proteins in proteomic research is a technique called peptide mass fingerprinting (PMF), which combines protein separation, (bio)chemical reactions and MALDI-TOF/MS.[31-34] The PMF technique involves the separation of a protein either by 2D-PAGE, liquid chromatography, or capillary zone electrophoresis. Subsequently, the intact protein is identified by enzymatic hydrolysis with a sequence-specific endoprotease (better results are obtained when two or more endoproteases are used, e.g. trypsin, Lys-C, Arg-C, etc.), and the obtained peptide fragments are compared by MALDI-TOF mass spectra to the pattern in a database (called *in silico* digestion, which is the theoretical cleavage of the protein by the same enzyme). The advantage of PMF is that only the masses of the peptides have to be known; thus, *de novo* sequencing of the protein is avoided. *De novo* sequencing is a MS

approach that requires fragmenting isolated gas-phase ions to determine their amino acid sequence; hence for this approach tandem MS (MS/MS) mass analyzers are preferred.

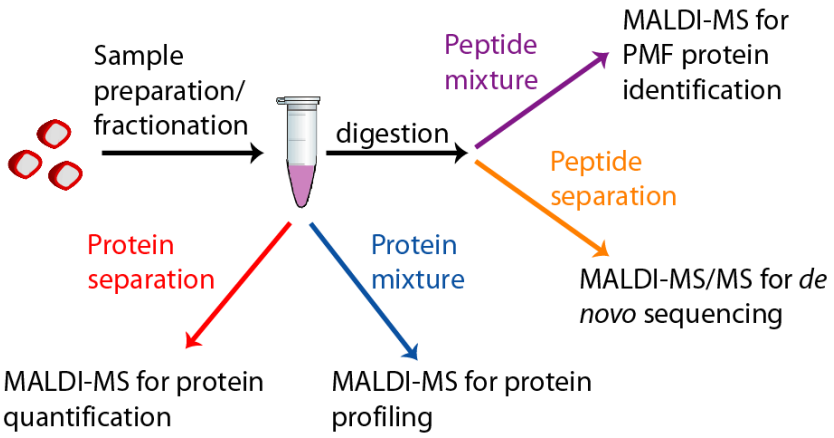


Figure 4. Workflow representation of a proteomic experiment using MALDI-MS and MALDI-MS/MS (tandem MS) systems.

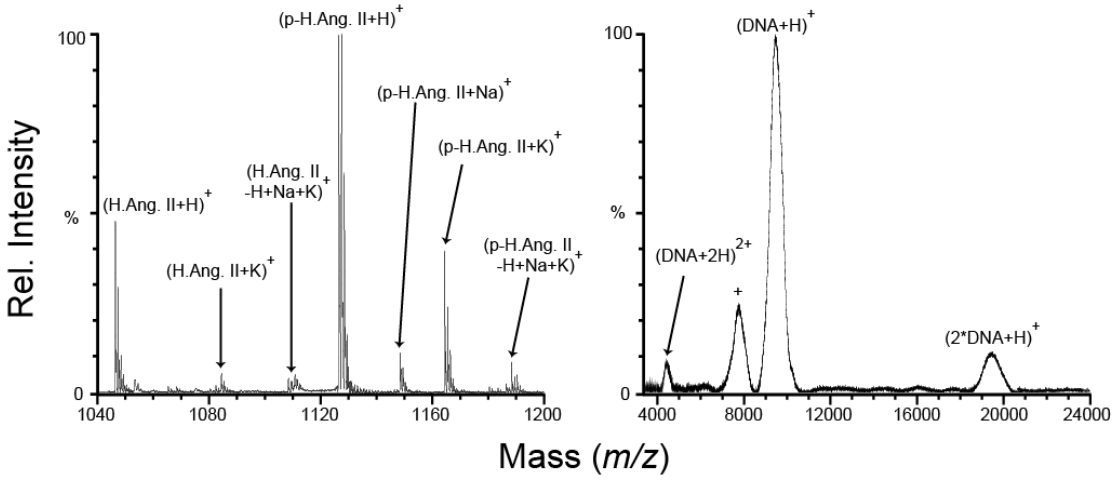


Figure 5. Phosphorylated Human angiotensin II (DRVpYIHPF, 1126.5721 Th) and oligonucleotide (30-mer; 5'-CAT AGA ATC AAG GAG CAC ATG CTG AAA AAA-3', 9269.629 Th) MALDI-MS spectra (positive mode, reflectron and linear mode, respectively). "+" represents [M+H-5A]+ fragment ions of the DNA.

1.2.2 Surface-enhanced neat desorption and surface-enhanced affinity capture

Although MALDI-TOF/MS is quite a robust technique for analyzing peptides and proteins, the presence of high amounts of inorganic salts, surfactants, and/or interference from other peptides/proteins can cause signal suppression effects.[35,36] Signal suppression occurs when the intensity of the $[M+H]^+$ ion signal is decreased or is totally hidden by the background noise of the measurement (poor signal-to-noise ratio). Currently, there are two strategies for dealing with such effects:

- The first is to find an effective matrix such as an organic salt compound (e.g. ionic liquid) instead of an organic acid. Nevertheless, this strategy may not be sufficiently sensitive compared to the traditional organic matrix, despite its ability to overcome the ion suppression effect.[37-39]
- The other strategy is to develop an efficient sample preparation protocol that will be suitable for measuring with an organic matrix.

The inherent complexity of samples makes their efficient preparation prior to analysis a constant challenge in the field of bioanalytical chemistry. Separation methods for target biomolecules or for groups of them that involve removing the signal-suppressing components from plant cells, blood serum, etc., are well known. Separation methods carried out prior to MALDI-MS can be referred to as "off-probe" (and consecutively subdivided as on-line or off-line) and "on-probe" (see Figure 6).

Whereas "off-probe" techniques refer to traditional pre-fractionation or clean-up steps such as 2D gel electrophoresis, multidimensional liquid chromatography, capillary electrophoresis, dialysis, and/or solid-phase extraction techniques that are carried out prior to the MALDI-TOF/MS measurement, "on-probe" techniques, of which surface-enhanced laser desorption/ionization (SELDI) is perhaps the most recognizable, refer to the fractionation of the sample on the MALDI plate itself.

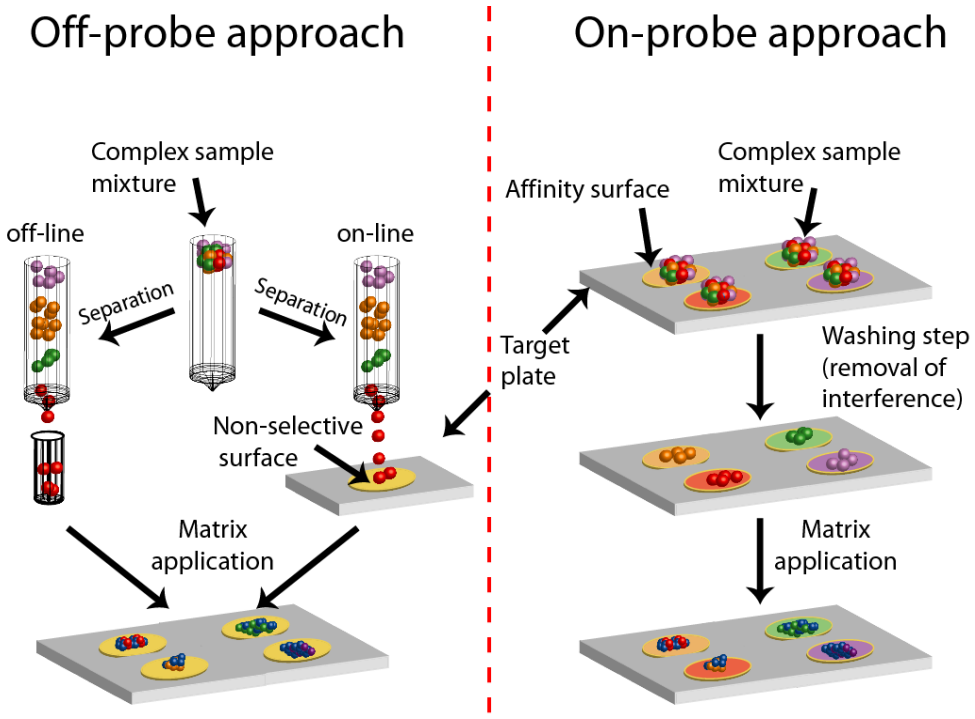


Figure 6. Workflow comparison between "on-probe" and "off-probe" approaches

In 1993, two new laser desorption/ionization strategies for the mass spectrometric analysis of macromolecules appeared as a modified version of MALDI-TOF/MS analysis.[35,40] These new laser desorption/ionization strategies are based on the molecular design and construction that encompasses two major subsets of MS technology:

- Matrix-analyte co-crystallization. In MALDI, the sample is mixed with the matrix either in solution and subsequently applied to the MALDI probe, or on the surface of the MALDI probe, using the sandwich technique. On the other hand, in surface-enhanced neat desorption (SEND), a molecule with matrix-type properties is incorporated onto the LDI probe surface. In that way, analytes deposited on the LDI probe do not require the addition of matrix, facilitating the analysis and reducing variation between measurements.[35,40] Moreover, sample surface contaminants can be removed by immersing the sample support into a liquid solution for 5 to 10 seconds (since the peptide/proteins are usually bound to the matrix/probe surface).[41]
- Surface-analyte interactions. In surface-enhanced affinity capture (SEAC), the sample is spotted first on a surface-modified MALDI target with specific chemical properties. The analytes of interest are fractionated and concentrated *in situ* on the surface of the SEAC probe by (a) the molecular interactions between the capture surface and the analyte, and (b) the conditions used to elute the interference. Hence, the advantage of SEAC compared to other separation techniques combined with MALDI-MS is that the "off-probe" sample-handling steps are eliminated. Such steps can lead to sample losses during the isolation and transfer steps, resulting in poorer limits of detection.[35,40]

The analyte prefactionated on SEAC targets can be ionized by laser irradiation and later analyzed with time-of-flight mass analyzers (TOF/MS) by subsequently applying an energy-absorbing molecule similar to the MALDI matrix after the adsorption/clean-up of the sample on the surface. The SEND and SEAC ionization mechanism is similar to that explained in MALDI (section 1.2.1, p.4): a laser desorbs/ionizes the sample/matrix mixture, creating singly charged gas-phase ion molecules $[M+H]^+$. The TOF/MS calculates the *m/z* ratio for the time taken for the gas-phase ion to travel from the ionization source to the detector.

Since 1997, SEAC-TOF/MS analysis technology has been commercially available as SELDI or ProteinChip® Arrays by following different approaches. For example since 2006, Bio-Rad Laboratories (original patent from Ciphergen Biosystems) has used commercial (hydrophobic/ionic) polymers deposit using plasma or thin-film casting for generating their SEAC probes; meanwhile Qiagen uses affinity elements immobilized onto self-assembled monolayers on gold surfaces for fabricating them.

1.3 State-of-the-art for LDI techniques

1.3.1 Miniaturization and its influence on MALDI & SEAC targets

The miniaturization of analytical systems is a concept which first appeared in 1990,[42] and it refers to the integration of several analytical steps (e.g. separation, reaction and detection) into a single device. The use of microfabricated devices with MALDI mass analyzers showed great potential in the field of proteomics.[43,44] For example, Laurell et al. presented in 2001 the "microfabricated toolbox",[45,46] which contains a microfabricated enzyme reactor for protein digestion, a piezoelectric microdispenser for spotting samples, and a silicon nanovial MALDI target plate.[45,46] Nevertheless, with the increasing number

of protein analyses done in laboratories and in clinical practice, the effectiveness of the pretreatment of samples and their associated costs are valid issues.

In protein identification laboratories with a medium or high sample turn-over, costly MALDI target plates need to be re-circulated to reduce costs per sample.[47-50] Unfortunately, a large number of samples (each of them originating from a vast number of biological origins and present at different abundances) can negatively influence the proteomic data generated by MALDI-MS. Memory effects (i.e. an observed signal produced by a substance from a previous analysis) as well as damage to the target surface from repeated use and washing (to remove previously measured samples from the plates). Thus, despite the advantages provided by the microfabrication techniques, it is evident that microfabricated silicon arrays do not offer much economic benefit. Material and fabrication techniques are costly when compared to conventional target plates.[47-50]

However, modern microfabrication technologies now provide polymer microstructures that are equal in quality to silicon microstructures. Disposable plastic MALDI probes are now seen as a way to produce lower-cost disposable targets.[47-50]

The use of polymers as coatings for MALDI and SEAC-MS measurements is not a new concept. One of the first experiments published in 1994 reported the increase of the protein $[M+H]^+$ signal proportional to the specificity of the polymeric membrane to which they were electroblotted.[52] Further in-depth studies were carried out,[53-56] where proteins/peptides were measured on metallic targets coated with different plastics such as polyethylene (PE), polyvinylidene difluoride (PVDF), nylon, polyurethane (PU), and polymethylmethacrylate (PMMA). In these studies, it was proved that the complete drying of the proteins on the polymeric surface or the extreme affinity of the protein to specific chemical groups on the surface can result in the conformational change of the protein. This change in the protein structure causes it to aggregate and strongly adsorb to the surface, preventing it from co-crystallizing with the matrix, hence reducing the intensity of the protein $[M+H]^+$ signal (the co-crystallization of the sample with the matrix is the key step for MALDI ionization).

Years later (2003) contradictory results appeared: proteins/peptides showed increased signal intensities on polymeric coated targets rather than on metallic ones. The increase was attributed to two different effects:

- the lower wettability of most polymers compared to metals,[57] that is, the reduction of the spot size due to the inherent hydrophobicity of these substrates;
- the increase of photo-electron yields emitted from the plastic target during laser irradiation, which decreases the formation of multiply charged species and indirectly enhances the sensitivity of mono-charged $[M+H]^+$ species.[58]

These effects in combination with the possibility of non-covalent binding interactions occurring between plastic surfaces and peptides/proteins proved to be useful for the SEAC-MS approach, according to which non-strongly bound interferences can be flushed away, increasing the signal-to-noise ratio of the $[M+H]^+$ signal.

Thus, we can conclude that prior to finding a low-cost plastic for the fabrication of disposable MALDI/SEAC target, which provides reproducible results using a chosen

sample preparation technique, it is particularly critical to find an agreement between the properties of the polymer and the analytical approach. It was not until 2001 that the first disposable polymeric high-density array for MALDI-MS was built from a PMMA and polycarbonate (PC) using cold embossing or injection molding.[47,48] The MALDI data measured on these materials displayed mass resolution, accuracy and signal intensity for peptide standards similar to those obtained on high-density target arrays of different materials such as silicon, stainless steel, and gold; and consequently, the search for low-cost SEAC-MS probes followed.

In 2003, a PMMA MALDI target, which was produce by computer numerical control (CNC) milling technique, was chemically and biochemically functionalized (i.e. an enzyme was bound to its surface via chemical reactions).[49,50] The procedure consisted of replacing the chemically inert group ($-OCH_3$) on the surface with a chemically active group ($-NH_2$), and subsequently cross-linking these groups to a protein.[50]

The functionalization procedure (by which chemically active groups are added or changed) was previously published for the modification of microfluidic planar devices by Henry *et al.*[59] Although successful, this chemical modification procedure is rather complicated and time consuming. To modernize and simplify the modification procedure and to avoid the described problems, a quicker method to obtain reactive groups on the surface of plastic MALDI/SEAC probes involves the simple co-polymerization of a monomer containing the reactive group of interest with a backbone polymer that provides mechanical rigidity to the overall system.[60]

1.3.2 Plastic/polymeric matrix-assisted laser desorption/ionization targets (pMALDI)

An appropriate protocol for prototyping disposable functionalized polyacrylate/ methacrylate probes for MALDI and SEAC-MS was introduced in 2005,[61] using an established method for fabricating microfluidic planar devices.[62] Briefly, the MALDI or SEAC probes were fabricated by injecting the molding mixture (containing the functionalized and backbone acrylate or methacrylate solution) into a sandwich mold made of a micro-machined silicon master, an aluminum spacer, and glass cover plates. The mold containing the molding mixture was subsequently exposed to UV light to initiate the radical polymerization (see Figure 7).

This fabrication procedure enabled diverse, ready-to-spot, disposable polymer arrays to be fabricated and modified, because functional groups (monomers) could be tailored during the polymerization reaction. This thesis focuses on the use of plastic matrix-assisted laser/desorption ionization (pMALDI) chips, such as MALDI- and SEAC-MS probes. The goal of the thesis is to develop technological platforms and analytical protocols based on the novel pMALDI targets in order to assist different proteomic studies.

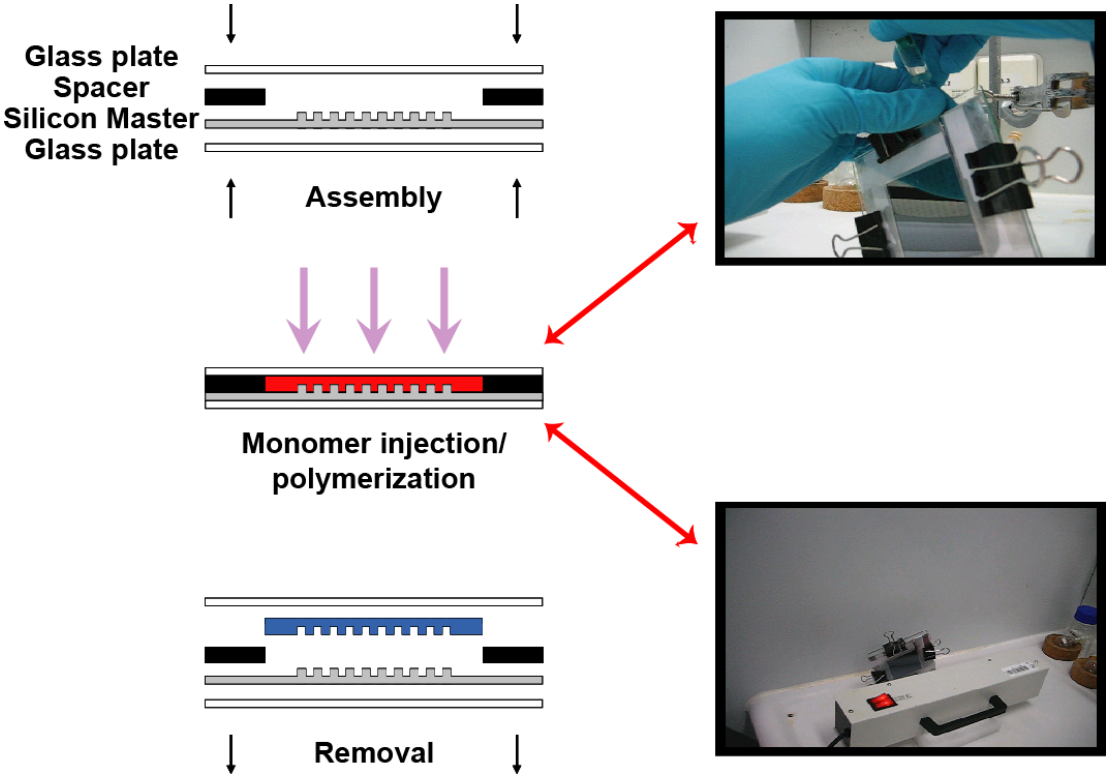


Figure 7. Scheme of the molding protocol workflow for the fabrication of plastic MALDI substrates.

1.4 Plan of the thesis

As previously mention, in order to elucidate the biochemical processes underlying the survival strategies of living organisms in nature, the ability to perform proteomic analysis quickly and easily is becoming increasingly important. The microfabrication of low-cost, ready-to-spot, disposable MALDI- and SEAC-MS target plates allows these challenges to be tackled. Nevertheless, several limitations of the pMALDI chips such as fabrication time, compatibility with MALDI-MS instruments, low mass resolution due to charging effects, and the fabrication of new plastic surfaces to improve selectivity must be addressed, before pMALDI chips can be used for proteomic routine studies.

This thesis presents several studies in which various aspects of the fabrication of pMALDI chips have been optimized, with the aim of overcoming the limitations listed, and to be able to use the novel pMALDI targets to realize different proteomic studies such as subproteome profiling, protein identification, and protein activity studies.

In Chapter 2, I present my contribution and research, which was carried out in the Mass Spectrometry research group at the Max Planck institute for chemical ecology under the supervision of Dr. Aleš Svatoš and Prof. Dr. Hans-Peter Saluz, in the field of co-polymeric plastic chips.

- Manuscript I focuses on the characterization of the charging effect.
- Manuscripts II & III focus on the development of new surfaces (ionic exchanger system, and ion metal affinity systems).
- Manuscript IV focuses on the development of a trypsin-pMALDI chip for fast peptide mass fingerprinting (PMF) identification of proteins.
- Manuscript V focuses on the development of a DNA microarray for the detection of a human *cytomegalovirus* (herpes virus 5).
- Manuscript VI focuses on the development of pMALDI chips as platforms for studying protein inhibition assays.

In Chapter 3, I present the results and conclusions of my research.

- In sections 3.1 & 3.2, the characterization of the physical events that occurs during the use of pMALDI chips are presented. For the detection of low-abundance compounds, pico- to femtomoles range, it is necessary to know how the pMALDI chip material properties affects the MALDI ionization mechanism, and which mechanisms contribute to the increase of signal intensity and/or to the decrease of signal intensity (the charging effect and surface protein/peptide interaction, respectively).
- In section 3.3, the detailed evaluation of new polymeric surfaces is presented. Traditionally SEAC-MS platforms depend on chromatographic-like stationary phases for the preconcentration and prefractionation of target analytes on their surface. Different methacrylate and acrylate monomers containing hydrophobic, ionic and chemically reactive moieties were used to create tailor-made surfaces for subproteome profiling.
- In section 3.4, a novel set-up for the enhancement of enzymatic reactions is presented based on the immobilization of enzymes on the pMALDI chip.
- In sections 3.5 & 3.6, the use of pMALDI chips for protein function determination is explored. The possibility of using DNA microarrays for homology-based searches has enabled the identification of proteins, and point toward their possible function in non-sequenced organisms. As a result of the multiple read-out capabilities of the pMALDI chips, they can also be used as protein microarrays for confirming protein activity by carrying out biochemical assays on their surface.
- In section 3.7, I present the conclusions of this thesis and offers an outlook for the further development of the pMALDI-MS platform for proteomic and genomic studies.

In Chapter 4, offers a summary of my work carried out in the field of disposable microfabricated pMALDI chips.

In Chapter 5, offers a summary in German language of my work carried out in the field of disposable microfabricated pMALDI chips.

In Chapter 6, offers the bibliography to support the content of this thesis.

Finally in Chapters 7, 8 & 9, offer the acknowledgments to those who contribute in my research during the development of my thesis, the declaration of independent assignment, and my curriculum vitae (with a complete list of my publications and oral/poster presentations), respectively.

2. Manuscripts

2.1 Manuscript I

Dissipation of charge on MALDI-TOF polymeric chips using an electron-acceptor: analysis of proteins

Alfredo J. Ibáñez, Alexander Muck and Aleš Svatoš

Journal of Mass Spectrometry **2007**, *42*, 634–640

The increasing number of analyses carried out in proteomic facilities suggests the need for low-cost, if possible disposable, MALDI and SEAC probes for peptide and protein analysis (as stated in the introduction of this thesis).

Traditional probes for MALDI-MS are made of steel (which is a conductive material); non-traditional probes are made of polymeric (plastic materials). These are usually coated with a thin layer of gold after the sample deposition step and prior to the MALDI-MS measurement.

Nonmetallic sample supports for MALDI MS can increase measurement sensitivity when compared to metallic MALDI probes (a more in-depth explanation can be found in section 1.3.1, p.11).

Empirically, their higher sensitivity when compared to metallic probes is based on

- surface-sample interactions in which the competitive ions are removed from the target, thus improving the signal-to-noise ratio and thereby the sensitivity of the measurement;[61]
- the "wettability" of the surface. On hydrophobic surfaces, water droplets simply rest on the surface, without actually wetting it to any significant extent (i.e. large contact angle values), which makes the surface appropriate for the formation of matrix:complex micropatterned structures (sweet spots), hence increasing the relative concentration of the analyte in the laser-irradiated spot.[57]

Unfortunately, some protein measurements using MALDI-TOF/MS require to acquire more measurement points (i.e. longer laser irraditation) at low laser fluencies because TOF mass analyzers are biased toward low molecular weights, and because not all proteins are able to be equally ionized (i.e. same efficiency) using MALDI. As a result of the longer laser irradiations, mass resolution decreases for the combined spectra of all individual measurement points, reducing the increased sensitivity obtained when nonmetallic targets rather than metallic targets are used (see Figure 1 in this manuscript for illustration).

The loss in resolution for the combined spectra has been experimentally quantified in this manuscript. It is based on the production of photo-electrons by UV-laser irradiation during the desorption/ionization step of the MALDI process. The formation of photo-electrons was proposed by Karas *et al.*;[63] later experimental confirmation was provided by other researchers.[51,64-68] This proposal states that a substantial number of photo-electrons is

produced during the irradiation of the matrix with a UV-laser. Moreover, it is the presence of these free photo-electrons that accounts for the lack of the higher charge ion-phase molecules that give MALDI-MS its characteristic mass spectra, in particular when MALDI-MS is compared, for example, to electrospray ionization, another ionization technique for proteins/peptides analysis in mass spectrometry.

Isolating materials such as nonconductive plastics are unable to dissipate the photo-electrons created by the MALDI process. The hypothesis, which we proved in this publication, was that the accumulation of photo-electrons generates an electrical charge, which creates local perturbations in the electric field between the sample and the acceleration plate. This charge defocuses gas-phase ion molecules by increasing their kinetic energy. The increase of kinetic energy can be experimentally observed as an increase in the *m/z* ratio in a time-of-flight analyzer (see section 1.2.1, p.7). Therefore, in accumulated spectra measured on a nonconductive material, the full width at half maximum of a peak is proportional to the laser energy (i.e. number of laser shots) used during acquisition, hence the number of laser irradiations (see Figure 1 of this manuscript for illustration of this effect).

The charging effect can be suppressed by three different approaches:

- the probe itself is made of or contains a conductive material, which will keep electrons from accumulating on the surface (e.g. conductive polymers, gold layers, etc.);
- since the nonconductive material behaves as a dielectric in a capacitor when used in a positive mode, the reduction of the nonconductive MALDI target thickness increases the flow of electrons through the polymer to the base plate;
- the addition of an electron trap which can remove the produced electrons in the first instances of laser irradiation.

Although we studied all three options for suppressing the charging effect on non-conductive materials, in this manuscript we only focus on the addition of an electron trap. In this manuscript the charging effect on disposable nonconductive plastic MALDI chips (pMALDI chips) was reduced by co-crystallizing an effective electron scavenger such as 1,1'-dimethyl-4,4'-bipyridinium (methyl viologen, MV^{2+}) dichloride hydrate (MV) with the matrix.

Under the supervision of Dr. Aleš Svatoš and with the help of Dr. Alexander Muck, I started to elucidate the mechanism of the charging effect. Dr. Alexander Muck and I prepared the microfabrication facilities necessary to run this project. I performed all experiments, measured the charging effect for four different proteins, and performed the statistics studies quantifying their deviation. Dr. Alexander Muck helped to visualize the charging effect during the protein profiling measurement of a human plasma sample.

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Dissipation of charge on MALDI-TOF polymeric chips using an electron-acceptor: analysis of proteins

Alfredo J. Ibáñez, Alexander Muck and Aleš Svatoš*

Mass Spectrometry Research Group, Max Planck Institute for Chemical Ecology, 07745 Jena, Germany

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Disposable polymeric devices used for direct sample pretreatment/signal enhancement and MALDI-MS analyses of biomolecules suffer from the accumulation of electric charge and related positive mass shifts due to photoelectric effects on non-conductive surfaces after irradiation with lasers. The effects of surface charging on the mass shift of protein spectra during MALDI-TOF/MS measurements on copolymeric sample array chips were studied. Methyl viologen is used to monitor the development of charge and is demonstrated to be an effective electron scavenger. The use of such reducible species leads to increased accuracy, signal homogeneity, and resolution for mass spectral measurements of proteins in mixtures with sinapinic and 2,5-dihydroxybenzoic (DHB) acids. This approach can be used on a wide range of nonconductive support materials. Copyright © 2007 John Wiley & Sons, Ltd.

Supplementary electronic material for this paper is available in Wiley InterScience at http://www.interscience.wiley.com/jpages/1076-5174/suppmat/

KEYWORDS: array; methyl viologen; matrix; mass shift; surface charging

INTRODUCTION

The increasing numbers of protein analyses done in laboratories[1] and in clinical[2] practice raise questions about the effectiveness of the pretreatment of samples and the associated costs. Mass fingerprinting (peptide mass fingerprint, PMF) after controlled proteolysis, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and database searching are the methods of choice for high-throughput protein analysis in complex mixtures.

Recently, innovative nonmetallic disposable MALDI sample platforms have been introduced as a cost-effective alternative to conventional metallic target plates. The initial simple designs, such as covering metallic target plates with a self-adhesive tape,[3] blotting proteins on poly(vinylidenefluoride) (PVDF) membrane followed by *in situ* digestion using trypsin,[4,5] or direct protein desorption from isoelectric-focusing (IEF) strips,[6] have recently developed into specialized MALDI arrays.[7] For example, selectively adsorbed phosphorylated and glycosylated proteins were digested *in situ* with trypsin and the resulting peptides used for PMF and post-translation modification analyses on surface enhanced laser desorption/ionisation (SELDI) targets.[8] In addition, novel platforms for automatic chip-based sample and matrix deposition on MALDI targets[9] have been developed, and these show potential for high-throughput measurements. Combinations of electrospray deposition of peptides into array wells and simultaneous matrix deposition by shearing matrix solutions into such wells[10] testify to the progress the field has made.

In the future, the design of low-cost formats of high-density polymeric disposable sample arrays will be particularly important. Polymer high-density nanovial arrays have been introduced for high-throughput PMF in MALDI-MS.[11] Recently, we have established a flexible atmospheric molding fabrication protocol for the rapid replication of planar microstructures.[12,13] This protocol enables the fabrication of low- to high-density polymeric MALDI arrays (pMALDI) with highly tunable hydrophobicity for adsorbing specific proteins from complex matrices.[14]

However, the use of nonconductive polymers such as MALDI arrays (sample supports) for highly precise protein and peptide MALDI-TOF measurements is limited. These limitations result when the surface layers of polymers are charged by the photoelectrons that form after irradiation with fluent UV-laser beams. Such photoelectrons are not effectively dissipated on polymer target surfaces, and the local distortions within the electric field that is formed in the area of the sampling zone result in ion masses shifted to higher values.[3,15]

The formation of such photoelectrons has been widely believed to be caused by UV (photon) irradiation.[3] An additional source of electrons may also be the surface of the MALDI matrix, as has been shown for the 2,5-dihydroxybenzoic acid (DHB) matrix.[15] The charging problem of the nonconductive polymers was solved by using composite carbon/polymeric conductive surfaces[16] or nonconductive target PVDF membrane), on which the samples

*Correspondence to: Aleš Svatoš, Mass Spectrometry Research Group, Max Planck Institute for Chemical Ecology, 07745 Jena, Germany. E-mail: svatos@ice.mpg.de

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were deposited before being covered with gold films that were then ground to a null potential.[17]

However, such solutions are time-consuming and expensive. Instead, we have concentrated on acquiring a broad analytical understanding of the charging effect on fully polymeric chips. In the following sections, we suggest a new approach using an electron acceptor additive in order to avoid charging effects on plastic surfaces. We discuss this approach here for the first time in relation to protein samples and with respect to mass accuracy and resolution.

EXPERIMENTAL

Chemicals

3,5-Dimethoxy-4-hydroxycinnamic acid (sinapinic acid, SA), 2,5-DHB, trifluoroacetic acid (TFA), bovine insulin (INS, ave. MW 5734 Da), chicken egg white lysozyme (LYS, ave. MW 14 307 Da), human hemoglobin (HH, ave. MW of units A and B 15 126 and 15 867 Da, respectively), horse heart myoglobin (HHM, ave. MW 16 951 Da) and carbonic anhydrase I (CAI, ave. MW 28 739 Da) from human erythrocytes, lyophilized human plasma, 1,1′-dimethyl-4,4′-bipyridinium (methyl viologen, MV^{2+}) dichloride hydrate (MV), and acetonitrile were purchased from Sigma-Aldrich. Methyl methacrylate (MMA), butyl methacrylate (BMA) (containing 5 ppm hydroquinone inhibitor), benzoin methyl ether (α-methoxyphenylacetophenone, BME) and activated alumina (grade CG20) were obtained from Polysciences (Warrington, PA, USA).

Device fabrication

The rapid prototyping of polymeric array sample support chips (pMALDI chips) was performed using a modified atmospheric molding procedure.[14] The poly(MMA-co-BMA) polymer was prepared in a 1 : 6.4 molar ratio (1/9 v/v). The solution aliquot (5 ml) was pre-exposed to UV light (365 nm, 2 × 8 W, Carl Roth, Karlsruhe Germany) for approx. 40 min. to obtain a thick 'pre-polymer' molding solution with the addition of 4 mg/ml BME initiator. The resulting oligomer was degassed by sonication and infused into the microfabricated sandwich mold, where it was polymerized for 1 h.

Sample preparation

The MALDI matrix was prepared by dissolving 10 mg/ml SA in a mixture of 0.1% TFA : acetonitrile (6 : 4 v/v). The MV stock solution (10 mM) was prepared in water. The protein stock solutions were prepared in 100 μM concentration in 0.1% TFA, pH 3.4. All working concentrations were obtained by diluting the samples with 0.1% TFA each day. The plasma was reconstituted to its original volume (5 ml) by deionized water and acidified by adding 0.1% TFA (1/1 v/v). A 100 μl aliquot was precipitated with 50% acetonitrile (1/1 v/v). The solution was spun at 10 621 g using a microcentrifuge and aliquots of the supernatant were used for further measurements. The 2,5-DHB was prepared in a mixture of water : acetonitrile (7 : 3 v/v). All protein solutions and the matrix were made in deionized water (Milli-Q, Millipore, Bedford, MA, USA). The proteins were mixed in a 1 : 1 ratio with the matrix and deposited on the polymeric pMALDI chips using the dried droplet technique.

Mass spectrometer

The spectra were acquired using a MALDI-TOF mass spectrometer (TofSpec 2E; Micromass, Manchester, UK) operated in a linear mode. Desorption/ionization was accomplished using a nitrogen UV laser (337 nm, 4 ns pulses of max. energy 180 μJ). If not mentioned otherwise, the grade of laser energy set at coarse was 50, at fine was 15% of max. energy (laser E_{eff} = 13.5 μJ/pulse); laser frequency was 2 Hz. The positive ions were subjected to 22.5 kV accelerating potential and detected with a microchannel plate detector (MCP, detection voltage 3600 V). Matrix ions were suppressed using a low mass (m/z 1500) cutoff. In order to study the influence of surface charging on spectra acquisition, 20 mass spectra (each collected from 20 laser shots) were recorded consecutively on the same matrix spot. The averaged spectra were baseline subtracted and centroided at 80% height of the peaks. The degree of surface charging was characterized by the mass shift of the studied analyte in ppm, and normalized using the spectra obtained from the first 20 laser shots as a reference. The data were recorded using a PC workstation running MassLynx 3.5 software (Micromass, Manchester, UK). The microphotographs of the plastic chips were acquired with a digital camera EOS D30 (Canon, Inc.).

Generation of surface charge

The charge was introduced to the sample well surface by irradiating identical locations with a laser for long exposure periods. To ensure a good analytical signal from such locations, the amounts of INS, HH, HHM, and CAI deposited on each target well were increased three times to 5, 90, 45, and 75 pmol, respectively, in comparison with standard MALDI measurements. MV was added to the protein mixture only if stated.

Safety considerations

Alkyl methacrylates and benzoin methyl ether are toxic substances. Other chemicals used are irritants. Accidental inhalation, ingestion, or skin contact with these chemicals should be avoided. Because UV light may cause damage to skin and eyes, protective goggles and gloves should be used. The polymerization should be carried out in a ventilated fume hood.

RESULTS AND DISCUSSION

Charge accumulation on a polymeric surface

Irradiation of protein samples on MMA polymeric and MMA/BMA copolymeric targets with low laser fluencies, carried out on higher concentration and purity samples,[14] did not result in any significant charging effects. However, when a study of complex protein mixtures was started, longer irradiations were used and many scans were needed to increase the sensitivity of the measurements (e.g. for human plasma samples); pronounced mass shifts to higher values were observed (2000–5000 ppm). Additionally, the mass resolution decreased due to the accumulation of scans with gradually increasing m/z values.

To study these effects, 3 pmol of INS solution were mixed with the SA matrix and deposited on a sample well

J. Mass Spectrom. 2007; **42**: 634–640
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of the polymeric pMALDI chip (MMA/BMA).[14] The energy of the laser was adjusted to provide optimum S/N ratios (*ca* 13.5 μJ/laser shot). The charge accumulation in a given sample well and its effect on mass measurements during consecutive spectrum acquisition at a single irradiated spot are displayed in Fig. 1. The reference m/z values of INS on a metallic and plastic sample well (Fig. 1, a1 and b1, respectively) were measured from the first average of 3 spectra (~total 0.8 mJ energy). The following set of 3 consecutive spectra obtained after the reference measurement resulted in a 2700 ppm shift of the INS molecular peak on the plastic target from its reference value; meanwhile on the metallic target there was no visible mass shift (Fig. 1, b2 and a2, respectively). After the acquisition of a further 15 consecutive spectra (total ~4.0 mJ), the m/z shifts of INS molecular peak on the plastic and the metallic substrates were approximately 3500 and 300 ppm, respectively (Fig. 1, b5, and a5). The consequence of the mass shift of the m/z values can be clearly understood when measured INS $[M + H]^+$ spectra are summed (Fig. 1(A) and (B)). The combined INS $[M + H]^+$ peak measured on plastic shows a lower resolution (239) in comparison with the one measured on metal (819). Moreover, the total intensity for the combined INS spectrum is less than that expected from the combination of its individual spectra. These effects might seriously affect the analysis of biomolecules on plastic MALDI supports in general. It should be noted that the INS signal intensity on the plastic was *ca* 7 times higher than that of the metallic target.

Further experiments focused on spatial probing of the accumulated charge. The m/z values of INS $[M + H]^+$ were consecutively measured at a different radial distance (between 100 and 400 μm) after applying ~1.4 mJ to the well center to monitor the spatial diffusion of charge (data not shown). All mass shifts measured were between 2500 and 3000 ppm and represented a plateau of the exponential increase of INS mass shift. Various spatial recording orders were tested with little influence on these shifts. This fact suggests that the electrons generated on the surface diffuse to a certain extent through the mixed sample/matrix layer and that each additional spectra collection is influenced by the charge propagation in the well. Such a charge buildup affects the mass spectral data for samples mixed with matrix and deposited over the whole area of the well. The high mobility of charges was also observed in another matrix (DHB) by Setz and Knochenmuss.[18] DHB was doped with DCM (4-dicyanomethylene-2-methyl-6-p-dimethylaminostyryl-4H-pyran) dye and the energy transfer was studied in a single DHB crystal.

In contrast, no charge movement was found when an INS mass spectrum was recorded from a nearby well center (at a distance of 2.5 mm from a previous collection point, the inter-well effect) after irradiation with 1000 laser shots. The measured mass shift was 558 ± 288 ppm. This confirms the hypothesis that charge is being transferred through the matrix. However, it does not move on polymeric surfaces even at short distances, and it accumulates on nonconductive surfaces. Therefore the mass shift cannot be eliminated by irradiating distant

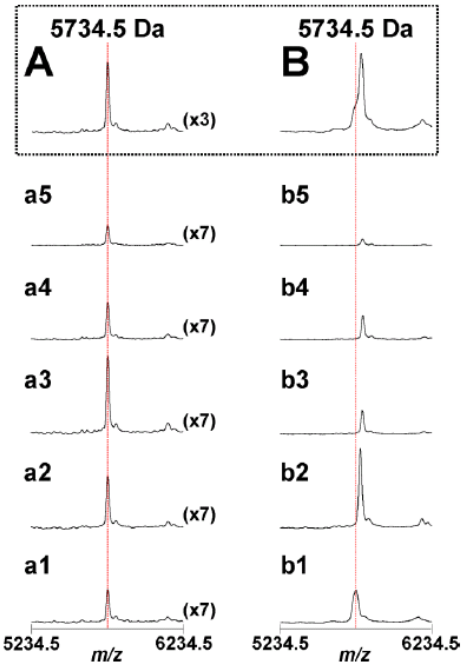

Figure 1. Combined spectrum (18 scans) of bovine INS (molecular peak *m/z* 5734.5 Da) measured from the same spot on metallic and polymeric MALDI-TOF sample targets (A and B respectively). Below: monitoring of a mass shift of INS $[M + H]^+$ peak due to the accumulation of charge during laser irradiation by 0.8, 1.6, 2.4, 3.2 and 4.0 mJ (1, 2, 3, 4, and 5 respectively, three scans per spectrum).

places on the well, but if the sample/matrix mixture is deposited as small dots on the plastic surface, every measurement from any dot should be equally accurate. The subsequent summed individual measurements will provide values practically unaltered by the charging of polymeric surfaces.

Monitoring charge accumulation

The accumulation of charge on the surface of the poly(MMA-co-BMA) chip during irradiation by a nitrogen laser is demonstrated in Fig. 2. A compound capable of a reversible oxidation/reduction process (with a related color change) was used to indicate the generation of free electrons on the pMALDI chips. A 3 μmol/μl solution of MV in 0.1% TFA was mixed in 1/1 v/v ratio with SA, and 1 μl aliquot was deposited on the polymeric sample well and crystallized. No color change was observed for the mixed sample without laser irradiation (Fig. 2(A)). Thirty minutes. after continuous laser irradiation (27 μJ/shot) focused on one spot on the target, a pronounced color change to dark blue, representing the reduction of MV^{2+} to the corresponding radical cation $MV^{\bullet+}$, was observed (Fig. 2(B)). After the plastic chip was

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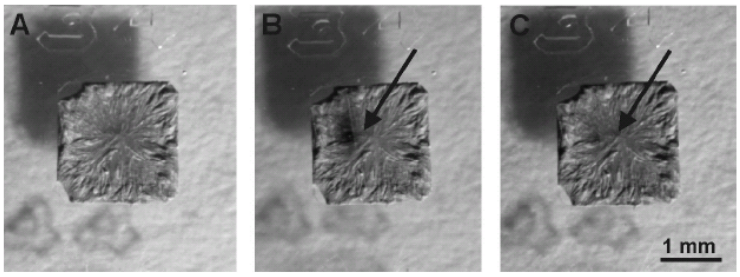

Figure 2. Monitoring of charge accumulation on a plastic sample array chip in the MALDI-TOF source: microphotographs of a copolymeric sample well with the addition of 1.5 µmol methyl viologen as electron acceptor. MV co-crystallized with SA before irradiation with 337 nm N_2 laser (A), the same spot after 10 800 pulses of the laser (~97.2 mJ, 274 nmol) at 15% max. energy, and the identical spot after irradiation and removal from the vacuum (C).

removed from the evacuated ion source, oxygen from atmosphere reoxidized the methyl viologen radical cation to its original MV^{2+} state. This process is characterized as well by a color change from dark blue to colorless (Fig. 2(C)). The observed color changes are in accordance with previously observed color changes of MV^{2+} upon irradiation with UV or visible light.[19,20] MV photochemical reactions have been described in zeolite X[19] and in silica-gel matrix[20]; however, no such photoreduction of MV^{2+} has been described in crystalline organic acids (a matrix in this study) with a UV abs. maximum close to the UV light wavelength.

Mass shift suppression

Such an electron acceptor (MV^{2+}) is an effective additive to the matrix during MALDI-TOF protein analyses and results in mass spectra with minimalized mass shifts. The effect of adding MV^{2+} to the sample mixed with SA matrix for mass spectral measurements of HHM is shown in Fig. 3. In comparison to nearly 5300 ppm maximal mass shifts caused by charging the polymer chip surface without MV^{2+} (recorded without changing the laser position; Fig. 3(A)), increasing the molar ratio of MV *versus* protein in the range of 1–3 (Fig. 3(B), (C) and (D) respectively) suppresses more than 50% of the surface charge and of the related positive mass shift of the molecular peak to *ca* max. 2700 ppm. The kinetics of the charge generation, displayed as mass shifts of the studied protein, are considerably influenced by the addition of an electron acceptor. The hyperbolical profile of charge buildup with no electron acceptor (Fig. 3(A)) is changed to a sigmoidal profile after applying an increasing amount of MV. Ideally, if MV is used in combination with changes in the laser position after each of 20 shots, and total laser energies are lower than a *ca* 0.3 mJ/irradiated spot, the mass shifts of the HHM peak will be below 400 ppm (*ca* 6.8 Da). This value is lower than that usually assigned to represent the calibration accuracy of mass spectrometers operating in the linear mode (typically 0.2%, corresponding to ~33.9 Da or 2000 ppm for the studied myoglobin).

The intra-well charge accumulation experiments (see above) were repeated for INS using SA doped with MV (135 pmol; SA:MV = 595 : 1 molar ratio) and an identical measurement protocol. As expected, no movement of charge

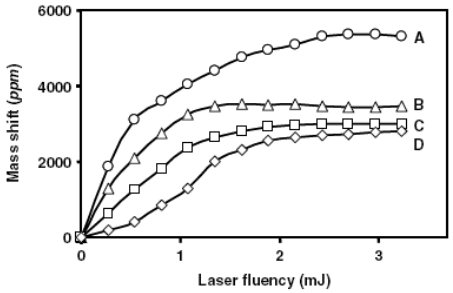

Figure 3. Increase of myoglobin mass shift on a plastic surface as a function of laser energy (A) and its suppression after adding methyl viologen (MV); 45 pmol MV (B), 90 pmol MV (C), and 135 pmol MV (D), corresponding to molar ratios of SA : MV 1784 : 1, 892 : 1, and 595 : 1, respectively.

from the center, irradiated by 1000 laser shots, to different parts of the well was observed, and the average mass shift was *ca* 950 ± 400 ppm.

The MV-dependent decrease of signal intensity in SA matrix preparations was measured for INS at 3.5 mJ laser energy. If the signal on metallic targets is normalized to 100% the favorable increase of signal intensity (I) on plastic target (0 ppm MV, I = 303%) gradually diminishes with addition of MV (50 pmol, I = 127%; 150 pmol. I = 88%), showing that charge dissipation is not 'cost free'.

As shown by the Zenobi[3,21,22] and Knochenmuss[15,23] groups, photoelectrons can be emitted from the matrix that has been deposited on nonconductive surfaces. Here we suggest a mechanism for MV action in electron scavenging. For the DHB matrix ionization potential, (IP = 8.05 eV)[15,21] the electron emission is notable at laser (355 nm) energy higher than 50 µJ. Most of our experiments were performed with SA, which has a lower IP (7.3 eV).[21] For N_2 laser total energy in two 337 nm quanta (2 × 3.68 eV) is 7.36eV and comparable with SA matrix IP. On nonconductive materials the formed electrons can escape from the matrix, creating a charge imbalance on the matrix surface as a result.[15,23] Redox potential $MV^{2+}/MV^{\bullet+}$ (−0.46 V),[24] can be recalculated

to IP using correlation[25] to be *ca* 6.3 eV. This IP is low enough to enable acceptance of electrons liberated from SA by laser irradiation. The absorption maxima for MV^{2+}, for its corresponding radical cation $MV^{\bullet+}$, and for SA are 256, 603, and 340 nm, respectively. Because the laser wavelength used (337 nm) is nearest to the absorption maximum of SA, the matrix should absorb the most energy. If photoelectrons are formed during this process, MV^{2+} will trap them and $MV^{\bullet+}$ will be formed. This prevents electron escape and conserves the charge balance (Scheme 1). Additionally, the rapid propagation of charge in SA was observed here (see above) and in the DHB matrix.[18] Then $MV^{2+}/MV^{\bullet+}$ reduction can take place not only on the irradiated surface, but in the whole sample volume. The exponential increase in mass shift (Fig. 3) can be attributed to such a process.

When 1 mJ of monochromatic light (337 nm) is used (Fig. 3), it corresponds to 2.8 nmol of photons. Supposing a theoretical 100% efficiency of electron generation and estimating the area of target irradiated by laser (based on Fig. 2) to be in the range of 0.0025–0.01 mm^2 (which represents *ca* 0.0625–0.25% of the 2 × 2 mm chip well area) and the amount of MV (3 µmol/well) in the irradiated area to range between 16.8 and 30 nmols, then the estimated efficacy of MV electron trapping would be between 9 and 17%.

Application scope

The electron acceptor properties of MV as an additive to the SA matrix for measurements of proteins on polymeric arrays were studied for a mixture of model proteins (Table 1). INS, HH, HHM and CA I were co-crystallized in the wells of the copolymer MALDI chip with and without the addition of 135 pmol MV. The m/z mass shifts of the molecular protein peaks for each of 20 consecutive laser shots (vs the m/z values obtained by centroiding the initial 20 laser shots used as reference) are shown in Table 1. The mass shifts increased with the increasing molecular weight of proteins and with increasing laser irradiation yet without the addition of an electron scavenger, ranging from ~4000 to 7000 ppm for maximum 100 laser irradiations. Such maximal numbers of laser irradiations could be used in MALDI-TOF protein analysis in case of weak signals. Thus, large mass shifts could seriously hinder the quality of data obtained on nonconductive polymer arrays. In contrast, only minimal mass shifts were observed for those measurements to which MV was added (80 laser shots, m/z shifts <800 ppm). The positive effect of an electron acceptor was further observed by the increased spectral resolution. The resolution of molecular peaks of averaged spectra corresponding to max. 100 laser irradiations increased by approx. 9–57% when using MV (for INS and CA I, respectively).

$$\text{No MV}: SA + h\nu = SA^{\bullet+} + e^- \text{ charge buildup possible}$$

$$\text{With MV}: SA + h\nu = SA^{\bullet+} + e^-$$

$$MV^{2+} + e^- = MV^{\bullet+}$$

Scheme 1. Photoelectric processes during irradiation of samples co-crystalized with SA on fully-polymeric supports.

The analytical versatility of the electron scavenger approach is further demonstrated in Fig. 4 for MALDI-TOF/MS analysis of human plasma lipoproteins. The suppression of mass shifts after the application of MV was evaluated for protoapolipoprotein proApoC-II, and apolipoproteins ApoC-I, C-III, H-III, and A-I in the range of 6.6–28.1 kDa. As with the model proteins, a suppression of mass shifts and an increase of resolution were observed. For the centered spectrum averaged over the entire acquisition (160 laser shots), the mass shift of Apolipoprotein C-I was suppressed from 1720 to 330 ppm (by ~80%) when an MV electron acceptor was added. The resolution of the molecular peak increased from 150 to 200 FWHM (~23%) in the linear mode when MV was used. The comparison of spectral data obtained from the last portion of the acquisition (e.g. the last 40 laser irradiations, where the highest concentration of charge was induced), exhibited an even higher degree of mass shift suppression after methyl viologen was applied (ApoC-I molecular peak shift decreased from 5280 to 755 ppm, ~86%). The use of methyl viologen and the resulting increased mass resolution also positively influenced the spectral intensity (16% peak intensity increase for ApoC-I, improving the sensitivity of such measurements). However, the 20 × increase in sensitivity reported previously for the DHB matrix doped with $CuCl_2$ on PEEK surface was not observed.[22]

Additionally, the DHB matrix was used to test the charge suppression of MV in different matrix systems. The DHB matrix formed large crystals on the hydrophobic surfaces of our pMALDI chip when the conventional solvent system[22] was used, and the sample intensity and reproducibility were

Table 1. Spectral characteristics of a model protein mixture on co-polymeric pMALDI chip with and without the addition of MV^{2+} electron acceptor (135 pmol)

	Average mass error (ppm)[a] without/with MV^{2+}				
Energy (mJ)	INS	HHα	HHβ	HHM	CA I
0.54	1300/160	1891/180	1771/150	1861/180	2730/160
0.81	2246/440	2923/370	2976/370	3112/410	4398/410
1.08	3001/860	3594/800	3634/840	3614/840	5750/780
1.35	3480/1250	4131/1273	4162/1300	4050/1280	6773/1350
Res.[b]	264/288	176/194	97/119	238/278	76/120

[a] The data result from 9 independent experiments and are referenced to the first 20 laser shots (0.27 mJ).

[b] Resolution (FWHM) of spectra collected from 100 shots.

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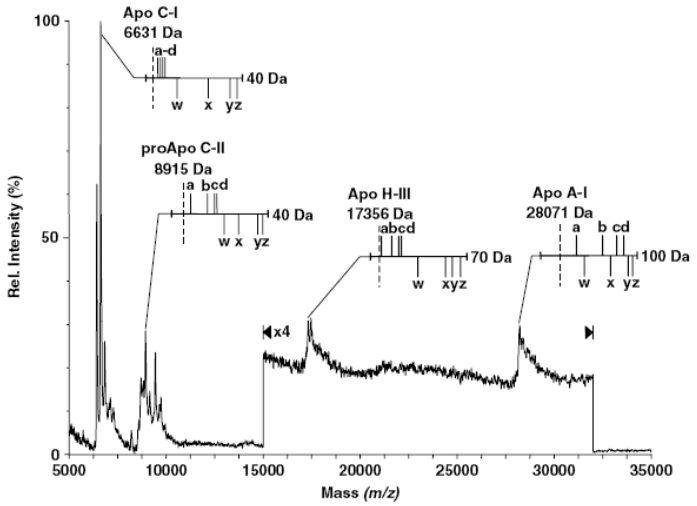

Figure 4. Mass spectrum of human plasma precipitated with 50% MeCN recorded using an SA matrix with the addition of MV (SA : MV 357 : 1) averaged from 160 laser shots. The inset compares the shifts of peaks centroided from consecutive scans with (upper trace, a–d) and without (lower trace, w-z) MV addition; each scan corresponds to 40 laser shots. The spectrum in the range 15 000–32 500 *m/z* was magnified (4x).

rather poor, especially for proteins with high molecular weights. For the DHB doped with MV, accuracy improved as it reportedly did (see above) for smaller proteins (e.g. INS). For α-cyano-4-hydroxycinnamic acid (alpha matrix), adding of MV did not effectively suppress the mass shift of analyzed peptides. The IP of the alpha matrix is even higher (8.3 eV)[21] than that of the DHB matrix, and electrons are not trapped effectively (see Suppl. Fig. S1 for details).

CONCLUSIONS

Laser irradiation of protein MALDI-TOF/MS samples on fully polymeric sample support arrays results in the accumulation of charge on the polymer surface and in considerable mass shifts as well as decreasing mass resolution. The characteristics of this photoelectric process have been described using an MV matrix additive. It effectively scavenges such charges and is compatible with a broad range of polymer materials currently used to prepare planar sample support devices. Such electron acceptor substances could be widely applied in laboratories where advanced polymeric supports are used for MALDI-TOF/MS measurements of biomolecules. Further aspects of protein MALDI-TOF mass spectrometry on polymeric arrays (new effective electron scavengers, the use of high frequency lasers during TOF/TOF experiments) are the focus of our ongoing research.

Supplementary material

Supplementary electronic material for this paper is available in Wiley InterScience at: http://www.interscience.wiley.com/jpages/1076-5174/suppmat/.

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REFERENCES

1. Yates JR. Mass spectral analysis in proteomics. *Annu. Rev. Biophys. Biomol. Struct.* 2004; **33**: 297.
2. Kolch W, Mischak H, Chalmers MJ, Pitt A, Marshall AG. Clinical proteomics: a question of technology. *Rapid Commun. Mass Spectrom.* 2004; **18**: 2365.
3. Frankevich VF, Zhang J, Friess SD, Dashtiev M, Zenobi R. Role of electrons in laser desorption/ionization mass spectrometry. *Anal. Chem.* 2003; **75**: 6063.
4. Vestling MM, Fenselau C. Poly(vinylidene difluoride) membranes as the interface between laser desorption mass spectrometry, gel electrophoresis, and in situ proteolysis. *Anal. Chem.* 1994; **66**: 471.
5. Nakanishi T, Ohtsu I, Furuta M, Ando E, Nishimura O. Direct MS/MS analysis of proteins blotted on membranes by a matrix-assisted laser desorption/ionization-quadrupole ion trap-time-of-flight tandem mass spectrometer. *J. Proteome Res.* 2005; **4**: 743.
6. Loo RRO, Mitchell C, Stevenson TI, Martin SA, Hines WM, Juhasz P, Patterson DH, Peltier JM, Loo JA, Andrews PC. Sensitivity and mass accuracy for proteins analyzed directly from polyacrylamide gels: implications for proteome mapping. *Electrophoresis* 1997; **18**: 382.
7. Alfonso C, Budimir N, Fournier F, Tabet JC. Activated surfaces for laser desorption mass spectrometry: application for peptide and protein analysis. *Curr. Pharm. Des.* 2005; **11**: 2559.
8. Ge Y, Gibbs BF, Masse R. Complete chemical and enzymatic treatment of phosphorylated and glycosylated proteins on proteinchip arrays. *Anal. Chem.* 2005; **77**: 3644.

9. Wheeler AR, Moon H, Kim CJ, Loo JA, Garrell RL. Electrowetting-based microfluidics for analysis of peptides and proteins by matrix-assisted laser desorption/ionization mass spectrometry. *Anal. Chem.* 2004; **76**: 4833.
10. Moerman R, Knoll J, Apetrei C, van den Doel RL, van Dedem GWK. Quantitative analysis in nanoliter wells by prefilling of wells using electrospray deposition followed by sample introduction with a coverslip method. *Anal. Chem.* 2005; **77**: 225.
11. Markó-Varga G, Ekström S, Helldin G, Nillson J, Laurell T. Disposable polymeric high-density nanovial arrays for matrix assisted laser desorption/ionizationtime of flight-mass spectrometry: I. Microstructure development and manufacturing. *Electrophoresis* 2001; **22**: 3978.
12. Muck A, Wang J, Jacobs M, Chen G, Chatrathi MP, Jurka V, Výborný Z, Schöning MJ. Fabrication of poly(methyl methacrylate) microfluidic chips by atmospheric molding. *Anal. Chem.* 2004; **76**: 2290.
13. Muck A, Svatoš A. Atmospheric molded poly(methylmethacrylate) microchip emitters for sheathless electrospray. *Rapid Commun. Mass Spectrom.* 2004; **18**: 1459.
14. Muck A, Nešněrová P, Pichová I, Svatoš A. Fast prototyping of hydrophobic disposable polymer support arrays for matrix-assisted laser desorption/ionization-time of flight-mass spectrometry of proteins by atmospheric molding. *Electrophoresis* 2005; **26**: 2835.
15. Knochenmuss R. Photoionization pathways and free electrons in UV-MALDI. *Anal. Chem.* 2004; **76**: 3179.
16. Li XP, Wilm M, Franz T. Silicone/graphite coating for on-target desalting and improved peptide mapping performance of matrix-assisted laser desorption/ionization-mass spectrometry targets in proteomic experiments. *Proteomics* 2005; **5**: 1460.
17. Scherl A, Zimmermann-Ivol CG, Di Dio J, Vaezzadeh AR, Binz PA, Amez-Droz P, Cochard R, Sanchez JC, Glückmann M, Hochstrasser DF. Gold coating of non-conductive membranes before matrix-assisted laser desorption/ionization tandem mass spectrometric analysis prevents charging effect. *Rapid Commun. Mass Spectrom.* 2005; **19**: 605.
18. Setz PD, Knochenmuss R. Exciton mobility and trapping in a MALDI matrix. *J. Phys. Chem. B* 2005; **109**: 22 947.
19. Ranjit KT, Kevan L. Photoreduction of methyl viologen in zeolite X. *J. Phys. Chem. B* 2002; **106**: 1104.
20. Maruszewski K, Jasiorski M, Hreniak D, Strek W. Electroreduction of methyl viologen in methanol and silicate thin films prepared by the sol–gel method. *J. Mol. Struct.* 2001; **597**: 273.
21. Frankevich V, Zhang J, Dashtiev M, Zenobi R. Production and fragmentation of multiply charged ions in 'electron-free' matrix-assisted laser desorption/ionization. *Rapid Commun. Mass Spectrom.* 2003; **17**: 2343.
22. Dashtiev M, Frankevich V, Zenobi R. Signal enhancement in matrix-assisted laser desorption/ionization by doping with Cu(II) chloride. *Rapid Commun. Mass Spectrom.* 2005; **19**: 289.
23. Knochenmuss R, Zhigilei LV. Molecular dynamics model of ultraviolet matrix-assisted laser desorption/ionization including ionization processes. *J. Phys. Chem. B* 2005; **109**: 22 947.
24. Stombaugh NA, Sundquist JE, Burris RH, Orme-Johnson WH. Oxidation-reduction properties of several low potential iron-sulfur proteins and of methylviologen. *Biochemistry* 1976; **15**: 2633.
25. Miller LL, Nordblow GD, Mayeda E. A simple, comprehensive correlation of organic oxidation and ionization potentials. *J. Org. Chem.* 1972; **37**: 916.

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2.2 Manuscript II

Atmospheric molding of ionic copolymer MALDI-TOF/MS arrays: A new tool for protein identification/profiling

Alexander Muck, Alfredo J. Ibáñez, Einar J. Stauber, Madina Mansourova, Aleš Svatoš

Electrophoresis **2006**, *27*, 4952–4959

Two-dimensional (2D) gel electrophoresis is traditionally used for the separation and display of complex mixtures of proteins on a single gel according their isoelectric points and molecular mass (see section 1.1, p.3). Comparative proteomics is based on contrasting the differences between two proteomes from different states of the same or similar cell/organisms after applying a specific stimulus, or after one of them suffering from a natural change (e.g. disease-related). By comparing two gels, a control vs. a sample, scientists can determine which spots have been up- or down-regulated due to the internal/external (e.g. time/disease respecitvely) stimuli. Likewise, SEAC-MS (see section 1.2.2, p.10), can provide similar qualitative data. In SEAC-MS the sample is preconcentrated and fractionated (i.e. adsorbed) on the surface of the SEAC probe from complex sample mixtures under different washing conditions.[69-72] The cellular expression pattern obtained by SEAC-MS analysis can reveal which proteins have been up- or down-regulated.[73,74]

Common surfaces used for SEAC probe fabrication include chromatography-like stationary phases, such as weak-positive ion exchange, hydrophobic surface, strong anion exchanger, etc.[69-72] Unfortunately, this technology, which was first commercialized by Ciphergen Biosystems (under the name of SELDI) and now is produced/marketed by Bio-Rad Laboratories, cannot always provide low-cost or custom-shaped SEAC-MS probes.

Low-cost, custom-shaped surfaces for SEAC-typed analysis can be produced by polymerizing tailor-made chips (i.e. pMALDI chips).[60] In manuscript II, the fabrication of an ionic pMALDI chip is presented. Ionic pMALDI chips have the potential to selective fractionate analytes according to their pI and the ionic strength and/or pH of the media in which they are applied onto the pMALDI surface, after removing non-specific binding events. Such a system can prove useful for protein mixtures, which are extracted in a surfactant- or salt-rich media, and for the subproteome profiling of a protein mixture, by identifying each individual components based on their pI and total mass, and meanwhile simultaneously removing MALDI-MS interferences. (manuscript II)

Although we studied all these capabilities for the ionic pMALDI chip, in manuscript II, we focus mainly on the use of ionic monomers for the subproteome profiling of complex protein mixtures by either (a) standard protein mixture containing lysozyme, β-lactoglobulin A, trypsinogen and carbonic anhydrase I; or (b) a native occurring protein complex such as the transmembrane photosystem I from the green algae *Chlamydomonas reinhardtii*.

Under the supervision of Dr. Aleš Svatoš, our team expanded the initial concept published by Muck et al. in 2005, in which the authors proposed the feasibility of using ion

exchanger groups as active surfaces.[61] Dr. Muck started this project by developing the negatively charged polymers (carboxy- and sulfoxy- modified chips). Meanwhile, I synthesized the positive polymeric substrates based on different ion exchanger chromatography systems, i.e. strong anion exchangers (SAX), and weak anion exchangers (WAX). Due to the lack of miscibility between the quaternary amine monomer and our backbone polymer, during the UV-initiated polymerization, such chips required an alternative method of fabrication. This new approach was made in collaboration with Dr. Madina Mansourova. This publication was prepared by Dr. Muck; my contribution was to provide the MALDI-TOF/MS measurements, meanwhile Einer Stauber provided the transmembrane photosystem I protein complex from green algae *Chlamydomonas reinhardtii*.

Alexander Muck
Alfredo J. Ibáñez
Einar J. Stauber
Madina Mansourova
Aleš Svatoš

Mass Spectrometry Group,
Max Planck Institute for
Chemical Ecology,
Jena, Germany

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Research Article

Atmospheric molding of ionic copolymer MALDI-TOF/MS arrays: A new tool for protein identification/profiling

An atmospheric molding protocol has been used to prepare an ionic methacrylate-based copolymer sample support chips for MALDI (pMALDI)-MS by targeting selected groups of various monomers copolymerized during molding, namely, carboxy, sulfo, dimethylalkyamino, and trimethylalkylammonium groups. The new disposable array chips provide analyte-oriented enhancement of protein adsorption to the modified substrates without requiring complicated surface coating or derivatization. The MALDI-MS performance of the new ionic copolymer chips was evaluated for lysozyme, β-lactoglobulin A, trypsinogen and carbonic anhydrase I using washing with solutions prepared in pH or ionic strength steps. On cationic chips, the proteins are washed out at pH lower than their p*I* values, and on anionic chips at pH higher than their p*I* values. The ability of the microfabricated pMALDI chip set to selectively adsorb different proteins from real samples and to significantly increase their MS-signal was documented for the transmembrane photosystem I protein complex from the green alga *Chlamydomonas reinhardtii*. The proteins were almost exclusively adsorbed according to calculated p*I* values and grand average of hydropathy (GRAVY) indexes. The new disposable chips reduce manipulation times and increase measurement sensitivity for real-world proteomic samples. The simple atmospheric molding procedure enables additional proteomic operations to be incorporated on disposable MALDI-MS integrated platforms.

Keywords: Adsorption / Ionic interactions / Plastic chip / Polymer modification / Protein mixture analysis
DOI 10.1002/elps.200600391

1 Introduction

One of the preferred analytical methods in proteomics is MALDI-TOF/MS [1], which allows large numbers of samples to be measured in a short time, without compromising mass accuracy and sensitivity [2]. Such measurements have been made even more productive by microfabrication and the preparation of microarrays [3–14] for integrated sample pretreatment and analysis. When used for analysis of complex mixtures, MALDI ionization suffers from various drawbacks, especially the loss of analyte signal intensity due to sample impurities or individual component competition for protons.

In the past 10 years, the development of innovative MALDI probes such as patterned hydrophobic and ionic layers on gold surface [4–6] or polymeric supports [7–9] has proven to be a viable alternative to previous traditional methods (*e.g.* solid-phase extraction systems, microdialysis, or AnchorChips™) of sample preparation and measurement. When proteins or peptides from such surfaces are analyzed, the sample is adsorbed on the surface and then matrix is added. This method is widely used in studies of biofilms, *e.g.* adsorption of proteins/peptides from biofluids (tears) [9]. One study investigating the efficacy of the peptide adsorption and desorption during the MALDI ionization process provided evidence for the difficult desorption of peptides from surfaces with high binding affinities to the molecules studied [8]. The polymers used were commercially

Correspondence: Dr. Aleš Svatoš, Mass Spectrometry Research Group, Max Planck Institute for Chemical Ecology, Hans-Knöll-Str. 8, D-07745 Jena, Germany
E-mail: svatos@ice.mpg.de
Fax: +49-3641-571-701

Abbreviations: **βLG**, β-lactoglobulin A; **CA I**, carbonic anhydrase I; **DMAPMA**, 3-(*N*,*N*-dimethylamino)propyl methacrylate; **LYS**, lysozyme; **MAA**, methacrylic acid; **MMA**, methyl methacrylate; **SEMA**, 2-sulfoethyl methacrylate, **pMALDI chip**, copolymer sample support chip for MALDI; **TMAPMA**, 3-(*N*,*N*,*N*-trimethylammonium)propyl methacrylate; **PSI**, photosystem I; **LHCI**, light harvesting complex I; **SA**, sinapinic acid; **TRP**, trypsinogen

supplied with limited possibilities to choose the proper functionality on the surface. Parallel studies in this area resulted in the surface-enhanced laser desorption/ionization method (SELDI), which combines chemical interactions with affinity binding and the specially constructed target surface that enhances the ionization of molecules of interest, providing a diverse and far-ranging repertoire [10].

The potential advantages of using microfabricated polymeric disposable sample supports to selectively preseparate (adsorb) proteins of interest directly on MALDI-TOF/MS platforms followed by spectral measurements has been recognized by several groups [11–14]. However, most commercial polymer materials used to fabricate polymeric MALDI-TOF support chips are only slightly hydrophobic and carry minimally ionizable functional groups. This reduces the versatility of such devices, especially when considering the high number of ionizable functional groups in a protein chain (and the applicability of p*I*-based affinity interactions). Therefore, fabrication methods enabling incorporation of specific interaction sites on hyphenated plastic MALDI-TOF sample chips are urgently needed.

In a recent communication [12], we described a new atmospheric molding protocol for the fabrication of hydrophobic polymer chips. Using this procedure, which offers rapid fabrication time, almost any "tailor-made" copolymer material corresponding to the analyte character can be obtained without tedious multistep surface derivatization reactions (*e.g.* SELDI). Monomers with the desired functionalities are copolymerized and incorporated throughout the polymeric material. Thus, active surfaces are prepared practically without surface modification, avoiding the risk of high variability in the chemical yield of derivatization reaction (Fig. 1).

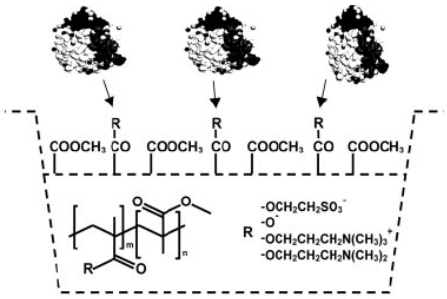

Figure 1. Atmospheric molded ionizable copolymeric MALDI-TOF chip surface for integrated protein preconcentration/spectral acquisition.

As a contribution to the ongoing efforts to develop new "gel-free" proteomic methods, this report describes the first application of the rapid fabrication procedure to the design of ionic copolymer sample support chips for MALDI (pMALDI chips). The optimal conditions for and the favorable performance of the new MALDI-TOF ionic copolymer chips for integrated protein preconcentration/spectral identification are described in the following sections.

2 Materials and methods

2.1 Chemicals, solutions, and proteins

Sinapinic acid (SA), TFA, lysozyme (LYS), β-lactoglobulin A (βLG), trypsinogen (TRP), and carbonic anhydrase I (CA I) were purchased from Sigma. Methyl methacrylate (MMA), methacrylic acid (MAA), 2-sulfoethyl methacrylate (SEMA), 3-(*N,N*-dimethylamino)propyl methacrylate (DMAPMA), butyl methacrylate (BMA), [3-(methacryloyl-oxo)propyl]-trimethylammonium iodide (all containing hydroquinone inhibitor), benzoin methyl ether, and activated alumina (Grade CG20) were obtained from Polysciences (Warrington, PA).

The photosystem I-light harvesting complex I (PSI-LHCI) complex was obtained from the thylakoid membranes of the arginine auxotrophic *Chlamydomonas reinhardtii* strain CC425/424, which were grown in liquid TAP media (a cell growth Tris-acetate-phosphate buffer containing salt nutrients and trace elements) supplemented with 50 μg/μL L-arginine under constant light (55 μmol photons m^{-2} s^{-1}) and agitation on a rotary shaker at 120 rpm. Thylakoid membranes were prepared as described in [15]. PSI-LHCI particles were enriched from thylakoids that have been solubilized in 0.9% w/v *n*-dodecyl-β-D-maltoside and then centrifuged through a sucrose gradient containing 0.05% w/v *n*-dodecyl-β-D-maltoside as described previously [16]. Chlorophyll concentrations were determined as described in [17].

2.2 Device fabrication

The rapid prototyping of pMALDI chips was performed using a modified atmospheric molding procedure in which 3-D sample zone arrays were fabricated on a silicon substrate and transferred by *in situ* polymerization to the polymeric material in a thin atmospheric pressure sandwich mold. The masters were prepared from 100 mm diameter p-type <100> orientation silicon wafers by means of soft-photolithography and wet chemical etching. The complete fabrication procedure has been described elsewhere [12, 18]. The cation-exchanger polymers, MAA/MMA and SEMA/MMA, were copolymer-

ized using molar ratios of 1:13.3 and 1:32.4, respectively (corresponding to 10 and 6% v/v of MAA and SEMA in the total volume of the polymerized mixtures, respectively). These ratios were based on previous atmospheric molding experiments [13]. For fabricating the anion-exchanger polymers, DMAPMA/MMA were copolymerized in a 1:13.3 molar ratio. The surface was further activated for 1 min with a freshly distilled methyl iodide (1 μL/well) to yield a quaternary amine 3-(*N,N,N*-trimethylammonium)-propyl methacrylate (TMAPMA) [19].

2.3 Sample preparation

The MALDI matrix was prepared by dissolving 10 mg/mL SA in a mixture of 0.1% TFA:ACN (6:4 v/v). All aqueous solutions were made in high-purity water filtered through a Milli-Q water system (Millipore, Bedford, MA). Different pH solutions (4, 6, 8 and 10) were prepared from dilutions of 0.1% TFA (pH 4, 6) and 0.1% NH_4OH (pH 8, 10) to minimize the effects of ionic strength during the adsorption of proteins from solutions at different pH values. The protein (LYS, βLG, and CA I; 100 μM each) stock solutions were prepared in the above-mentioned pH series. The TRP working solutions (200 μM) had to be freshly prepared every day. Samples for the pH selectivity study were freshly prepared from the stocks. To ensure reproducible adsorption of proteins with different molecular weights, the sample solutions (1.5 μL) were repeatedly (20 times) introduced to the surface of the ionic copolymer chips from a 10 μL pipette tip.

2.4 Apparatus

The spectra were acquired using a MALDI-TOF instrument (TofSpec 2E; Micromass, Manchester, UK) operated in a linear mode. Desorption/ionization was accomplished using a nitrogen UV laser (337 nm, 4-ns pulses of max. energy 180 μJ). The positive ions were subjected to a 20 kV accelerating potential and detected by a microchannel plate (MCP) detector (3600 V). Matrix ions were suppressed using low-mass (*m/z* 1200) cut-offs. The spectra were accumulated from seven points using 15 laser shots per point. The data were recorded using a PC workstation running MassLynx 3.5 software (Micromass, Manchester, UK).

A nano-LC-MS/MS system was used to analyze *C. reinhardtii* proteins. The HPLC system consisted of an Agilent 1100 series autosampler and a micro-LC pump (Agilent, Palo Alto, CA). The injected peptides were first loaded onto an RP Nanoease C18 trap column (0.180 × 24 mm, Waters, Milford, MA), then eluted and separated on an RP capillary column (PicoFrit™, 5 μm, BioBasic® C18, 75 μm × 10 cm, tip 15 μm, New Objective, Woburn, MA) by a 60-min linear multistep gradient of 2.5–95% ACN in 0.1% formic acid/0.1% formic acid at a flow rate of approximately 0.4 μL/min after the split. The HPLC was directly coupled to an LTQ linear IT mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with a nanospray ionization source. The proteins were identified by the BioWorks™ program version 3.1 (Thermo Electron) The protein database for *C. reinhardtii* was obtained from the National Center for Biotechnology Information (NCBI). Peptide identification was evaluated using the 'Xcorr *vs.* Charge State' filter set to a minimum of 1.5, 2.0, and 2.5 for singly, doubly, or triply charged precursor ions, respectively.

The ^{1}H NMR spectra were recorded with Bruker AVANCE DRX 500 nuclear magnetic resonance spectrometer (Bruker, Billerica, MA) at 500.13 MHz. All spectra were measured in $CDCl_3$. Chemical shifts are given in δ values relative to tetramethylsilane $\delta = 0$. Typically, 256 scans were accumulated per spectrum.

3 Results and discussion

The fast prototyping of medium- to high-density copolymer arrays for MALDI ionization introduced recently by our group [12] was tested for the group of monomers containing ionic functions. Several anionic and cationic alkyl methacrylate monomers, such as MAA, SEMA, and DMAPMA have been copolymerized with the methyl methacrylate MMA backbone to yield a specific strong/weak ionic surface charge of the pMALDI substrates. The ionogenic functionalities could thus attract oppositely charged groups of protein chains in solutions with the appropriate pH (Fig. 1). The molding and demolding process was straightforward, as with hydrophobic copolymers [12]. The demolding procedure for highly anionic SEMA/MMA chips was observed to be more difficult and longer sonication times were needed for highly hydrophilic chips to be removed from the silicon master. Our attempts to prepare the TMAPMA/MMA chip directly using [3-(methacryloyloxo)propyl]-trimethylammonium iodide monomer were unsuccessful due to the poor solubility of the monomer in methyl methacrylate (higher hydrophobicity) and slow polymerization. The TMAPMA/MMA chip was prepared by simple chemical activation of DMAPMA/MMA chip with methyl iodide. The synthesis of the TMAPMA/MMA copolymer in a similar ratio was confirmed by ^{1}H NMR [13.80:1, according to signals of OCH_3:$N^+(CH_3)_3$]. ^{1}H NMR (400 MHz, $CDCl_3$), δ = 3.65 (s, OCH_3), 3.45 [m, $N^+(CH_3)_3$], 2.00–1.80 (m, -CH_2- side chain polymer), 1.80, 1.30, 1.10 (m, -CH_2- side chain polymer), 0.95, 0.75 (2s, -CH_3 backbone polymer). Overall, the fast

atmospheric molding procedure yielded ion-exchange chips that covered a range from strong cationic to strong anionic exchangers in short fabrication times (less than 2 h) and enabled novel chip chemistries to be evaluated quickly.

Although the processes of protein adsorption to polymer surfaces are rather elusive and not yet fully described, it is widely accepted that the mechanism for hydrophobic surfaces is driven by entropy, and for ionic surfaces, by entropy and enthalpy [20]. The interaction steps include diffusion of the protein to the surface, removal of its solvation layer, possible removal of water or ion molecules from the surface, and reversible adsorption of the protein to the surface. The nonspecific binding and signals of unbound proteins on the polymer surface were counterbalanced by a post-adsorption washing step with a protein-free solution, making the MALDI-TOF MS signals mainly attributable to proteins which were strongly bound to the chip surface.

To evaluate different interactions of proteins with the surface of prepared chips, a mixture of four proteins with different molecular weight and p*I* values from 5 to 11 was prepared. Figure 2 depicts the selective adsorption of LYS (p*I* 11), βLG (p*I* 5), TRP (p*I* 9) and CA I (p*I* 7) protein mixture (5, 35, 50, and 5 pmol, respectively) on a strongly anionic poly(2-sulfoethyl methacrylate-*co*-methyl metha-

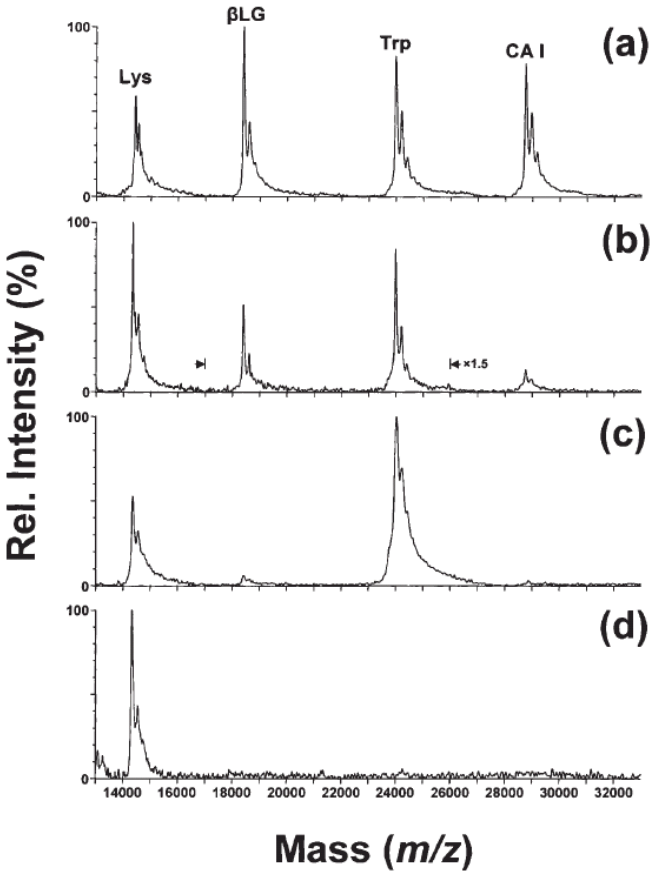

Figure 2. Selective adsorption of LYS, βLG, TRP, and CA I protein mixture (5, 35, 50, and 5 pmol, respectively) on the anionic SEMA/MMA chip: mass spectral protein profile after deposition of the complete sample solution (a), compared to mass spectra of the adsorbed proteins at pH of 6, 8, and 10 (b, c, and d, respectively).

crylate) copolymer (SEMA/MMA) pMALDI chip. To enable comparable ionization conditions after the adsorption/washing step under different pH conditions, the sample wells were acidified by adding of 0.1% TFA and SA solution in a 1:1 ratio (1 μL) and co-crystallized; last, MALDI-TOF/MS spectra were recorded from the chip. When the protein mixture at pH 4 was simply deposited on the copolymer chip, well-resolved molecular peaks of all model proteins were detected (Fig. 2a). After increasing the pH of the protein solution stepwise from 4 to 6 (Fig. 2b) and washing it with the identical buffer, a distinct adsorption profile and selective preconcentration of LYS, TRP, and βLG was observed (p*I* values 11, 9 and 5, respectively) on the negatively charged chip. A slightly irregular behavior was observed for βLG, which possesses localized positive charge groups (lysyl or arginyl residues) [21, 22] in its tertiary structure that could interact with the negatively charged surface. Those groups enabled βLG to be adsorbed even from solutions with pH values slightly higher than its p*I* value. By further increasing the solution pH to 8, the adsorption of βLG was strongly suppressed (Fig. 2c); at pH value 10 (Fig. 2d) only LYS (p*I* 11) showed adsorption to the surface. The complete adsorption profiles of the studied proteins on all weak and strong cationic and anionic pMALDI chips are compared in Fig. 3, using the previously mentioned protein concentrations. The protein mixture was adsorbed in the same manner on a DMAPMA/MAA weak cationic (A), TMAPMA/MAA strongly cationic (B), MAA/MMA weak anionic (C), and SEMA/MMA strongly anionic (D) pMALDI chips at pH values ranging from 4 to 10. The proteins preferentially adsorbed at pH values that were clearly higher than their p*I* (carrying a negative charge) when adsorbed to a positive surface. Meanwhile, the opposite effect took place when the proteins were adsorbed at pH lower than their p*I* (for negative charge). Such profiles could be used to optimize the conditions for detecting proteins of interest in complex protein mixtures.

As the ion-exchange chromatographic systems suggest, the adsorption of proteins could be also influenced by varying the salt concentration in the sample buffer (Table 1). Based on the strength of the protein/functional group surface interaction, the peak protein intensities were suppressed when stronger ion concentrations were used (ranging between 0 and 120 mM ammonium acetate). An exponential decrease in peak protein intensities has been observed for MALDI-TOF measurements on both positive (cationic TMAPMA/MMA chip in pH 10) and negative (anionic MAA/MMA in pH 4) as well as hydrophobic (BMA/MMA, pH 4) chips after washing them with elution buffers of increasing ionic strength. The increase of ammonium acetate salt concentration in the protein solution and the related raise of ionic strength amplify

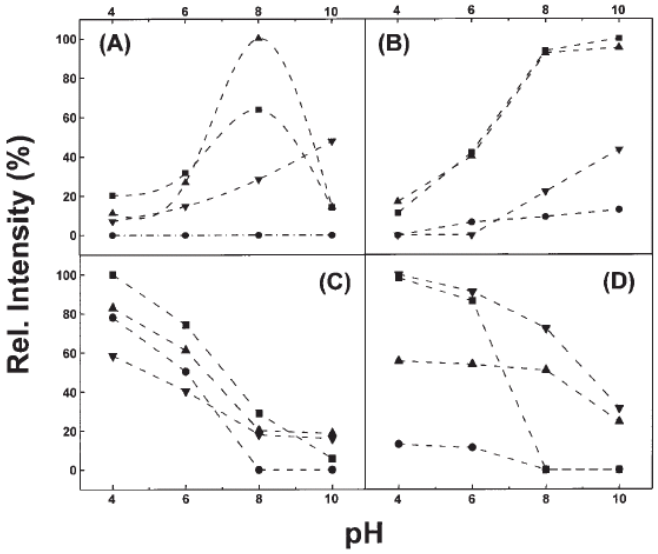

Figure 3. Adsorption profiles of LYS (▼), βLG (■), TRP (▲), and CA I (●) protein mixture (5, 35, 50, and 5 pmol, respectively) on DMAPMA/MAA weakly cationic (A), TMAPMA/MAA strongly cationic (B), MAA/MMA weakly anionic (C) and SEMA/MMA strongly anionic (D) pMALDI chips at different pH values. Adsorption conditions: 2 μL, ammonium acetate buffer. The intensities are scaled (100%) to the intensity of the strongest signal for every chip.

Table 1. Comparison of the adsorption profiles of model proteins from the cationic TMAPMA/MMA, anionic MAA/MMA and the hydrophobic BMA/MMA arrays

Protein	Surface	TOF response (mM salt)[a]					
		(0)	(8)	(15)	(40)	(80)	(120)
β-LG	+[b]	412.0	89.0	60.0	66.0	62.0	59.0
	−[c]	425.0	123.8	113.5	155.3	162.0	180.0
	Hydrophobic[d]	407.0	455.5	366.0	172.8	153.5	114.2
CA I	+	53.0	35.0	40.0	41.0	41.0	45.0
	−	500.0	21.0	17.5	20.3	20.4	19.0
	Hydrophobic	500.0	14.0	14.8	12.0	10.0	10.6
TRP	+	393.0	91.0	110.0	79.0	72.0	59.0
	−	492.0	91.3	85.0	87.0	91.2	102.0
	Hydrophobic	438.0	117.0	69.5	77.0	44.0	44.0
LYS	+	400.0	49.2	72.0	65.5	58.3	59.5
	−	278.0	40.2	33.8	61.3	76.0	58.8
	Hydrophobic	333.0	110.0	73.5	66.0	71.5	49.2

a) Elution using the given amount of ammonium bicarbonate.
b) Poly[3-(*N,N,N*-trimethylammonum)propyl methacrylate-*co*-methyl methacrylate].
c) Poly(methacrylic acid -*co*-methyl methacrylate).
d) Poly(butyl methacrylate-*co*-methyl methacrylate). The absolute intensities were averaged from the initial seven scans.

desorption of the proteins from the surface, which is demonstrated as decreased MALDI-TOF signals from the arrays. The initial rapid desorption of the protein peaks around the 20 mM salt concentration is followed by a further slower MALDI signal decrease in increasing concentrations of the buffer. A combination of pH-dependent absorption and ionic strength increase desorption could be used to selectively enrich similar proteins from complex mixtures directly on the MS measurement platform.

To further demonstrate the applicability of the ionic pMALDI chip platform for real-world proteomic analysis, we have tested the identification of PSI and the associated LHCI proteins from the green alga *C. reinhardtii* [23]. We selected the PSI-LHCI as a model system because it is a trans-membrane complex that binds pigments and comprises over 16 proteins [24] that vary greatly in p*I* and hydrophobicity. Together, these factors provided several challenges for our analytical system. To identify proteins, proteomic investigations of PSI-LHCI have traditionally used 2-DE and LC-MS/MS of tryptic peptides after protein solubilization [25].

However, this analytical approach is sometimes cumbersome and time-consuming [25]. Therefore, a new, straightforward pMALDI method offers an additional tool for the research on photosynthetic protein complexes. The ability of the ionic chips to analyze these proteins was tested in complex solubilizing buffers directly after protein extraction and/or prefractionation using sucrose gradients (Fig. 4). Two microliters of samples containing approximately 16 μg of protein (~3 μg chlorophyll), 0.05% w/v *n*-dodecyl-β-D-maltoside and ~0.6 M sucrose was adsorbed on a weak/strong anion exchanger and a weak cation exchanger pMALDI chip. The sample zones were after washing with 1.2 μL of 0.1% TFA overlayed with SA solution. The resulting TOF-MS spectrum, recorded on the TMAPMA/MMA strong anion exchanger chip (Fig. 4a), showed more defined PSI protein peaks than did its weak anion counterpart (figure not shown). Compared to this result, the weak cation chips presented two additional proteins, PSI-E and PSI-D (Fig. 4b), probably due to their more basic character or nonspecific interaction with the surface. In contrast, only the strongest peak of PSI-K protein could be identified in the spectrum obtained from a steel target (Fig. 4c). For comparison of the analytical efficiency and to confirm the hypothesis that the nonspecific adsorption of proteins is due to protein-protein interactions with already immobilized proteins on the cation exchanger chip (MMA/MAA), the adsorbed proteins from 16 MMA/MAA wells were collected in a 200-μL Eppendorf tube and were vacuum-dried. The collected samples were then reconstituted in 13 μL ammonium acetate (25 mM, pH 7.8) and 50 ng trypsin were added in 1 μL of 50 mM acetic acid (pH 4). The in-solution digestion with trypsin was carried out overnight at 37°C and

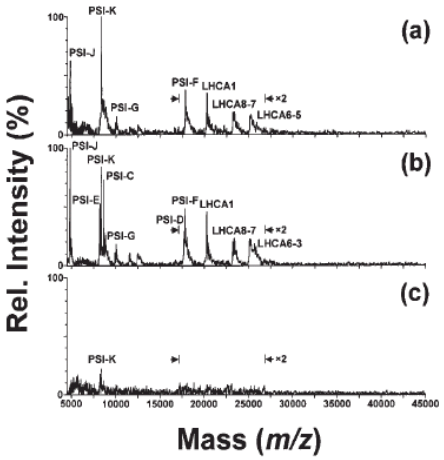

Figure 4. Mass spectra of the PSI and LHCI proteins (16 μg of protein and 3 μg of chlorophyll) from *Chlamydomonas rheinhardtii* using on-chip sample adsorption and salts and impurities removal from a solubilizing buffer containing 0.05% w/v *n*-dodecyl-β-D-maltoside and ~0.6 M sucrose on TMAPMA/MMA strong anion exchanger (a) and a weak cation exchanger (MAA/MAA) 1:9 v/v (b) pMALDI chips compared to spectrum obtained from a steel target (c).

analyzed using an LC-LTQ system, which allowed parallel protein identification. The results of the comparison are summarized in Table 2. The parallel identifications showed a high degree of similarity with only two larger proteins PSI-A, and PSI-B, being detected only with the LC-MS system. These two proteins form the core of PSI-LHCI with which smaller proteins may bind. This example shows that the adsorptions of specific proteins from complex mixtures can be inhibited by a formation of multiprotein complexes, where only the core protein is preferentially adsorbed due to specific chip surface-protein interactions, thereby effectively blocking the surface of the chip.

Additionally, the profile of obtained peptides differs from that of in-solution digestion. We speculate here that structural rearrangements of the protein occurred due to the partly denaturing character of protein adsorptions to functionalized copolymeric surfaces. This could be readily utilized to enhance protein sequence coverage by producing additional peptides not formed during in-solution digestion.

Table 2. Identification of PSI and LHCI proteins from *Chlamydomonas rheinhardtii* using on-chip protein adsorption to a weak cation exchanger pMALDI chip (MAA) with direct linear MALDI-TOF and a parallel identification using LC-MS/MS after elution of these proteins from chips and in-solution trypsin digestion

Molecular mass (Da)	pMALDI-TOF/MS	LC-MS/MS
4 748.5	PSI-J	
8 124.2	PSI-E	PSI-E
8 424.8	PSI-K	PSI-K
8 860.3	PSI-C	
10 023.2	PSI-G	
17 708.5	PSI-D	PSI-D
17 933.8	PSI-F	
20 351.1	LHCA-1	LHCA-1
23 328.7	LHCA-8	LHCA-8
23 424.6	LHCA-7	LHCA-7
25 158.2	LHCA-6	LHCA-6
25 242.0	LHCA-5	LHCA-5
25 699.5	LHCA-3	LHCA-3
25 883.6	LHCA-4	LHCA-4
82 108.7		PSI-B
83 153.5		PSI-A

4 Concluding remarks

The versatility of the atmospheric bulk-polymerization method was demonstrated by replicating a scale of different MALDI probes with similar functional groups to those present in ion-exchange columns. Using diverse chemistries on the pMALDI chip, we have further demonstrated the utility of the platform for specific adsorption/desorption of proteins from complex mixtures and their further identification after off-chip proteolysis. Currently, we are studying the chip well/analyte interactions at the molecular level in more depth using atomic force microscopy to predict the most appropriate surface functional groups for each application.

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5 References

[1] Karas, M., Hillenkamp, F., *Anal. Chem.* 1998, *60*, 2299–2301.
[2] Righetti, P. G., *Electrophoresis* 2004, *25*, 2111–2127.

[3] Sauer, S., Lange, B. M. H., Gobom, J., Nyarsik, L. *et al.*, *Nat. Rev. Genetics* 2005, *6*, 465–476.

[4] Warren, M. E., Brockman, A. H., Orlando, R., *Anal. Chem.* 1998, *70*, 3757–3761.

[5] Xu, Y., Watson, J. T., Bruening, M. L., *Anal. Chem.* 2003, *75*, 185–190.

[6] Xu, Y., Bruening, M. L., Watson, J. T., *Anal. Chem.* 2004, *76*, 3106–3111.

[7] Walker, A. K., Wu, Y., Timmons, R. B., Kinsel, G. R., Nelson, K. D., *Anal. Chem.* 1999, *71*, 268–272.

[8] Walker, A. K., Qiu, H., Wu, Y., Timmons, R. B., Kinsel, G. R., *Anal. Biochem.* 1999, *271*, 123–130.

[9] Griesser, H. J., Kingshott, P., McArthur, S. L., McLean, K. M. *et al.*, *Biomaterials* 2004, *25*, 4861–4875.

[10] Tang, N., Tornatore, P., Weinberger, S. R., *Mass Spectrom. Rev.* 2004, *23*, 34–44.

[11] Markó–Varga, Gy., Ekström, S., Helldin, G., Nillson, J., Laurell, T., *Electrophoresis* 2001, *22*, 3978–3983 and 3984–3992.

[12] Muck, A., Nešněrová, P., Pichová, I., Svatoš, A., *Electrophoresis* 2005, *26*, 2835–2842.

[13] Wang, J., Muck, A., Chatrathi, M. P., Chen, G. *et al.*, *Lab Chip*, 2005, *5*, 226–230.

[14] Guo, X., Chan–Park, M. B., Yoon, S. F., Chun, J. H. *et al.*, *Anal. Chem.* 2006, *78*, 3249–3256.

[15] Chua, N. H., Bennoun, P., *Proc. Natl. Acad. Sci. USA.* 1975 *72*, 2175–2779.

[16] Hippler, M., Drepper, F., Farah, J., Rochaix, J. D., *Biochemistry* 1997, *36*, 6343–6349.

[17] Porra, R. J., Thompson, W. A., Kriedemann, P. E., *Biochim. Biophys. Acta* 1989, *975*, 384–394.

[18] Muck, A., Svatoš, A., *Rapid Commun. Mass Spectrom.* 2004, *18*, 1459–1464.

[19] Sommer, H. Z., Lipp, H. I., Jackson, L. L., *J. Org. Chem.* 1971, *36*, 824–828.

[20] Doherty, E. A. S., Meagher, R. J., Albarghouthi, M. N., Barron, A., *Electrophoresis* 2003, *24*, 34–54.

[21] Kuwata, K., Hoshino, M., Forge, V., Era, S. *et al.*, *Protein Sci.* 1999, *8*, 2541–2545.

[22] Uhrínová, S., Smith, M. H., Jameson, G. B., Uhrín, D., Sawyer, L., Barlow, P. N., *Biochemistry* 2000, *39*, 3565–3574.

[23] Nield, J., Redding, K., Hippler, M., *Eukaryotic Cell* 2004, *3*, 1370–1380.

[24] Nelson, N., Ben-Shem, A., *Bio Essays* 2005, *27*, 914–922.

[25] Stauber, E. J., Fink, A., Markert, C., Kruse, O. *et al.*, *Eukaryotic Cell* 2003, *2*, 978–994.

2.3 Manuscript III

Metal-chelating plastic MALDI (pMALDI) chips for the enhancement of phosphorylated-peptide/protein signals

Alfredo J. Ibáñez, Alexander Muck, and Aleš Svatoš

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Beyond the vast number of proteins coded by the genome, several factors may increase protein heterogeneity, such as splicing variants, genetic variation between individuals, and posttranslational modifications.

The latter type of variation refers either to the proteolytic cleavage of the protein or to the modification of one or more amino acid residues in its structure by covalently adding a modifying group (such as acetate, phosphate, various lipids and carbohydrates), hence changing the protein activity/function inside the cell.[75]

As mentioned in section 1.2, mass spectrometry analyzers and in particular MALDI-MS have become powerful tools for the analysis of proteins due to their reliability, speed and sensitivity. The elucidation of the phosphoproteome of an organism can improve our understanding of various cell functions, such as signal transduction, metabolic maintenance, cell devision, etc.[75] However, the use of MALDI-TOF/MS for this kind of analysis remains troublesome because[36] (see manuscript III) (a) various amounts of protein are expressed (wide dynamic range of analysis), (b) the stoichiometry of phosphorylation at each site can be variable; and (c) the ionization efficiencies of phosphorylated peptides/proteins can differ significantly compared to those of nonphosphorylated peptides/proteins (i.e., ion suppression effects).

In this publication, we showed that plastic MALDI (pMALDI) chips can overcome these challenges by using a surface modified with metal ion affinity probes. These probes have been previously tested in traditional affinity chromatography for the sensitivity enhancement of phosphopeptides and phosphoproteins via the selective retention (i.e. strong adsorption) of the phosphate groups toward the surface of the stationary phase, and the ease elution once the interference is removed by adding a chelating agent.

Under the supervision of Dr. Aleš Svatoš I was given the freedom to plan all experiments. I synthesized all the new polymeric substrates based on ion metal affinity chromatography systems, and ran the comparative studies using a commercially available phosphopeptide enrichment kit. The supportive information for the paper using a LC-MS/MS instrument for the identification of the phosphopeptides using the neutral loss technique was provided by Dr. Alexander Muck.

technical notes Journal of proteome research

Metal-Chelating Plastic MALDI (pMALDI) Chips for the Enhancement of Phosphorylated-Peptide/Protein Signals

Alfredo J. Ibáñez, Alexander Muck, and Aleš Svatoš*

Mass Spectrometry Research Group, Max Planck Institute for Chemical Ecology, Hans-Knöll-Strasse 8, 07745 Jena, Germany

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Abstract: A disposable polymeric pMALDI array with a universal metal cation-chelatable surface for pretreatment/signal enhancement of phosphoproteins and/or phosphopeptides in complex samples was developed. Acrylic acid *N*-hydroxysuccinimide ester and methyl methacrylate monomers were copolymerized in thin layer molds in a 1:13.3 molar ratio and subsequently treated with *N*α,*N*α-bis(carboxymethyl)-L-lysine to obtain a structured planar MALDI array. The prepared NTA pMALDI chip array was activated with metal cations (e.g., Ga(III), Ni(II)), and the selectivities for phosphopeptides (e.g., trypsin-digested α-casein (α-Cas), and phospho-angiotensin II (p-Ang)) were evaluated using MALDI-TOF/MS. The highest selectivity for proteins was observed for the Ni(II)−NTA chip. The p-Ang was enriched in the presence of BSA tryptic peptides ca. 5 times and represented the major peak after sample adsorption/washing on Ga(III)−NTA chip. The performance of the Ga(III)-chip, tested on α-Cas tryptic digest, is fully comparable to commercial systems. Additionally, higher MW peptides and limited methionine oxidation were observed with the chip. A combination of selective absorption of phosphoproteins on Ni(II)-chips and the further enrichment of digested phosphopeptides on the Ga(III)-chip can prove to be very useful for fast identification of unknown proteins using MALDI-TOF/MS.

Keywords: phosphoproteomics • nickel(II) • gallium(III) • selective adsorption • phosphopeptide • phosphoprotein

Introduction

Originally scientists believed that proteomics was a technique that would examine a whole proteome with a single, integrated set of analytical operations; today, researchers understand that this may not be possible. Beyond the vast number of proteins coded by the genome, there are several factors which may increase their heterogeneity, such as splicing variants, genetic variation between individuals, and post-translational modifications (PTM).[1,2] Phosphorylation modification is one of the many PTM that are critical for protein function and activity.[3,4] It has been calculated that one-third of mammalian proteins is covalently bound to a phosphate group after their expression.[5,6] Thus, determining the phosphorylation state/sites of a protein is now essential in the study of many types of regulations.

Mass spectrometry has become a more effective tool for analysis of proteins phosphorylated during physiological changes than the traditional isotopic labeling (^{32}P)/gel separation approach.[7,8] However, performing a comprehensive and high-throughput phosphoproteomic analysis is still challenging because (a) various amounts of protein are expressed, (b) the stoichiometry of phosphorylation at each site can be variable and dynamically changing, and (c) the ionization efficiencies of phosphorylated peptides/proteins can differ significantly when compared to those of nonphosphorylated peptides/proteins (i.e., ion suppression effects).[9]

To improve the quality of the phosphoproteome analysis, ion metal affinity systems can be used. The principle of the IMAC methods can be explained using the Pearson classification, which postulates that metal ions can be divided into three categories (hard, intermediate, and soft) based on their preferential reactivity to nucleophile groups present in amino acids (such as the negative charge oxygen present in the phosphate group).[10] By the proper selection of metal ions, systems like SwellGel gallium discs,[11,12] solid-phase extraction (IMAC SPE),[13] and Millipore ZipTip$_{MC}$[14] have proved useful for alleviating ion suppression effects. However, despite their success, they cannot be coupled with mass spectrometer instruments directly, thus, decreasing the speed of analysis. For the on-line phosphopeptide enrichment/separation, numerous methods were developed, for example, LC/MS using ion metal affinity chromatography (IMAC columns).[10,15−17] In addition to the previously mentioned methods, MALDI-TOF/MS analyses using metal oxide-covered silicon surfaces,[18] immobilized IMAC beads applied to the MALDI probe,[19−22] or gold-coated MALDI targets with chemically grafted Fe(III)−NTA complexes[23] were also introduced.

In recent communications,[24−26] we demonstrated that plastic MALDI chips (pMALDI) can present selective chemical-reactive groups to the surface of the polymer without having to perform tedious multistep surface derivatization reactions. To contribute to ongoing efforts in the field of phosphoproteomics, we developed a universal chelating pMALDI system, which can be used to identify phosphopeptides and phosphoproteins, when combined to Ga^{3+} or Ni^{2+}, respectively.

* To whom correspondence should be addressed. Dr. Aleš Svatoš, Mass Spectrometry Research Group, Max Planck Institute for Chemical Ecology, Hans-Knöll-Str. 8, D-07745 Jena, Germany. E-mail, svatos@ice.mpg.de; fax, +49-3641-571701.

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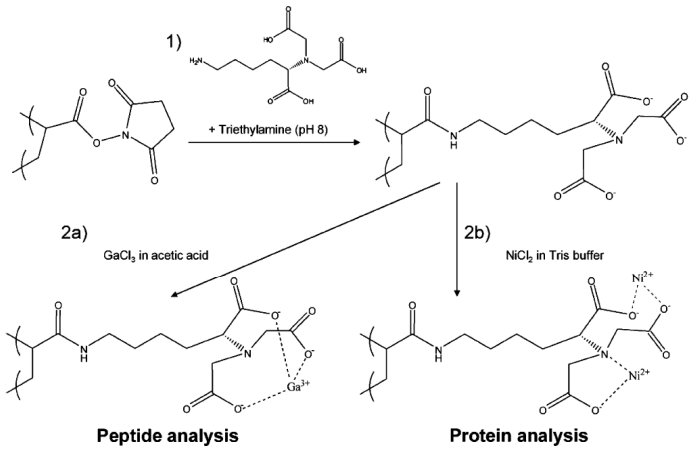

Figure 1. Modification steps of the atmospherically molded pMALDI-TOF chip surface for the capture of metal ions. (1) A copolymerized (AA-NHS:MMA) polymer is reacted with *N*α,*N*α-bis(carboxymethyl)-L-lysine hydrate in triethylamine (pH 8.0). (2a) For the adsorption of peptides, the surface was modified with $GaCl_3$ in 25 mM acetic acid (pH 2.5). (2b) For the adsorption of proteins, the surface was modified with $NiCl_2$ in 20 mM Tris buffer (pH 7.7).

Materials and Methods

Chemicals and Proteins. 3,5-Dimethoxy-4-hydroxycinnamic acid (sinapinic acid, SA), 2,5 dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (α-matrix), trifluoroacetic acid (TFA), β-lactoglobulin A (β-Lac A), alpha-casein (α-Cas), desphosphorylated alpha-casein (dp-α-Cas), horse heart myoglobin (Myo), carbonic anhydrase I (CA I), TPCK treated trypsin (Trp-TPCK), acrylic acid *N*-hydroxy-succinimide ester (AA-NHS), *N*α,*N*α-bis(carboxymethyl)-L-lysine hydrate (NH_2-C_4-NTA), gallium(III) chloride, nickel(II) chloride, copper(II) chloride, and iron(III) chloride were purchased from Sigma. Methyl methacrylate (MMA), containing hydroquinone inhibitor, benzoin methyl ether (BME), and activated alumina (Grade CG20), were obtained from Polysciences (Warrington, PA). Human angiotensin II (Ang) and phosphorylated human angiotensin II (p-Ang) were purchased from CALBIOCHEM (San Diego, CA).

Chip Fabrication. The rapid prototyping of pMALDI chips was performed using a modified atmospheric molding procedure in which three-dimensional sample zone arrays were fabricated on a silicon substrate and were used to template polymeric chips. The masters were prepared from 100 mm diameter p-type <100> orientation silicon wafers using soft-photolithography and wet chemical etching. The complete fabrication procedure has been described elsewhere.[24,27] The atmospheric molding *in situ* polymerization technique yields a negative copy of the silicon substrate in a thin sandwich mold. The acrylic acid *N*-hydroxysuccinimide ester (AA-NHS) and methyl methacrylate (MMA) monomers were copolymerized in a 1:13.3 molar ratio (1 g of AA-NHS with 5.576 mL of MMA and 0.4% (w/v) BME) under UV exposure in a sandwich mold (glass plate + spacer + silicon master). The molding and demolding process of AA-NHS:MMA was straightforward, as was the previously reported process with the hydrophobic[24] and ionic copolymers.[25]

Fabrication of the NTA-Metal System. Prior to immobilizing the metal ion to the pMALDI chip, the copolymer surface was cleaned (that means flushed by aspirating and dispensing) 50 times with 2.0 μL of 2 M HCl and rinsed with DI water. The copolymeric surface was then neutralized by flushing it with 2.0 μL of triethylamine, and by repeating the rinsing step. The modification involved spotting a 2.0 μL of 0.5 M NH_2-C_4-NTA in triethylamine (pH 8.0) on the surface and incubating it for 1 h at room temperature or overnight at 4 °C (Figure 1.1). Subsequently, the remaining active pMALDI surface was blocked with 2.0 μL of 5 mM Tris buffer (pH 9.0) and then flushed with 2.0 μL of double deionized MilliQ water (ddH_2O; Millipore, Bedford, MA). Finally, the pMALDI chip was incubated with 2.5 μL of a metal ion chloride solution containing either 100 mM Ga^{3+} in 25 mM acetic acid (pH 2.5) or 100 mM Ni^{2+} in 20 mM Tris (pH 7.8) for 1 h at 4 °C and shaken at 200 rpm (Figure 1.2). The remaining supernatant solution was removed, and the surface of the pMALDI chip was flushed either with 1.5 μL of 25 mM acetic acid containing acetonitrile (3%) or with 1.5 μL of ddH_2O for the Ga^{3+}− or Ni^{2+}−pMALDI chip, respectively.

Caution: The process of dissolving $GaCl_3$ in water is extremely exothermic, and it produces HCl gas. Protective clothing must be worn and a fume hood must be used. $GaCl_3$ solution is toxic and may produce spasms and burns.

Sample Preparation. The MALDI matrix was prepared by dissolving 10 mg/mL of α-matrix, DHB, or sinapinic acid in a mixture of methanol/acetonitrile (1:1 v/v), ddH_2O/acetonitrile (7:3), or ddH_2O/acetonitrile (6:4 v/v), respectively. All aqueous solutions were made in high-purity water (ddH_2O). The protein stock solutions were prepared in 300 μM concentration in 0.1% TFA (pH 3.4) to ensure their stability. Unless otherwise stated, the working solutions were prepared daily in 25 mM acetic acid containing acetonitrile (3%) at pH 2.5 for Ga^{3+} phosphopeptide

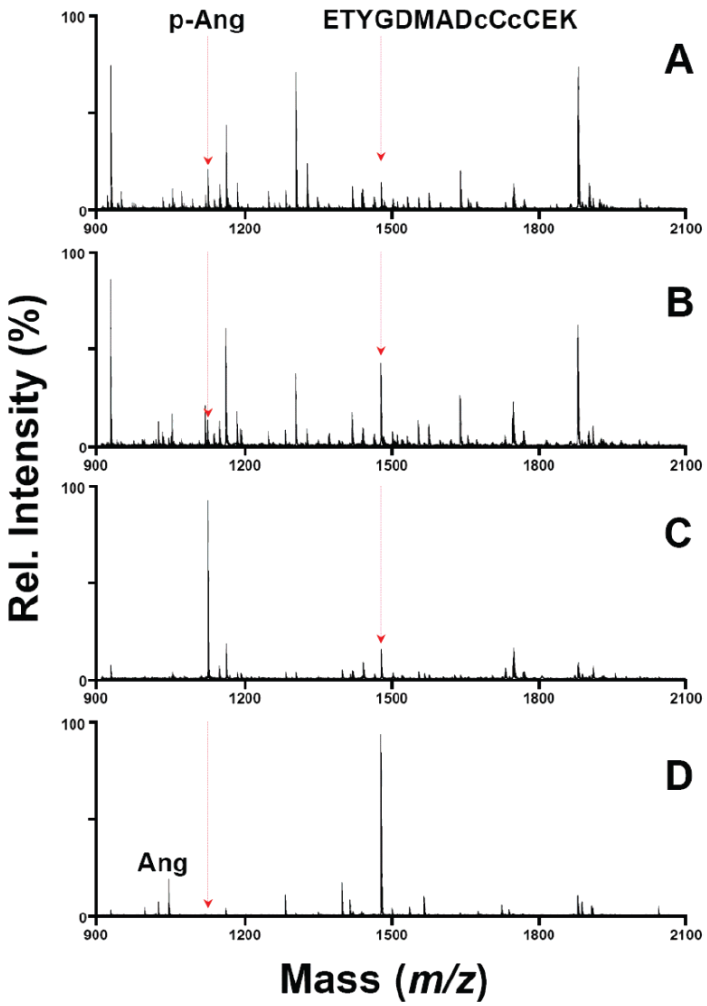

Figure 2. Phosphate-selective peptide enrichment of 1.0 pmol of phosphorylated human angeotensin II (p-Ang) in the presence of 3.5 pmol of standard BSAdig (all mass spectra normalized to 1.25 × 10^4 counts). (A) Peptide mixture deposited and measured on a metallic MALDI target. (B) Peptide mixture deposited and measured on a Ga^{3+}–pMALDI target. (C) Peptide mixture adsorbed, washed, and measured on a Ga^{3+}–pMALDI chip. (D) Conditions as in C; 1.0 pmol of Ang in 3.5 pmol BSAdig peptide mixture.

analysis; or 20 mM Tris buffer at pH 7.7 for Ni^{2+} phosphoprotein analysis. The digestion of 25 μg of α-casein (α-Cas) was carried out overnight at 37 °C, using Trp-TPCK in a (1:100 ratio) in 1 mL of 25 mM ammonium carbonate buffer (pH 8.0). Afterward, the digestion buffer was evaporated under vacuum, and the sample was reconstituted in 1 mL of 25 mM acetic acid containing acetonitrile (3%) at pH 2.5.

Apparatus. The protein mass spectra were acquired using a MALDI-TOF/MS instrument (TofSpec 2E; Micromass, Manchester, U.K.) operated in a linear mode. Desorption/ionization was accomplished using a nitrogen UV laser (337 nm, 0.5 Hz, 18 μJ per pulse). The positive ions were subjected to a 20.0 kV accelerating potential with 0 V set to the sample plate, and detected by a microchannel plate (MCP) detector (3.6 kV).

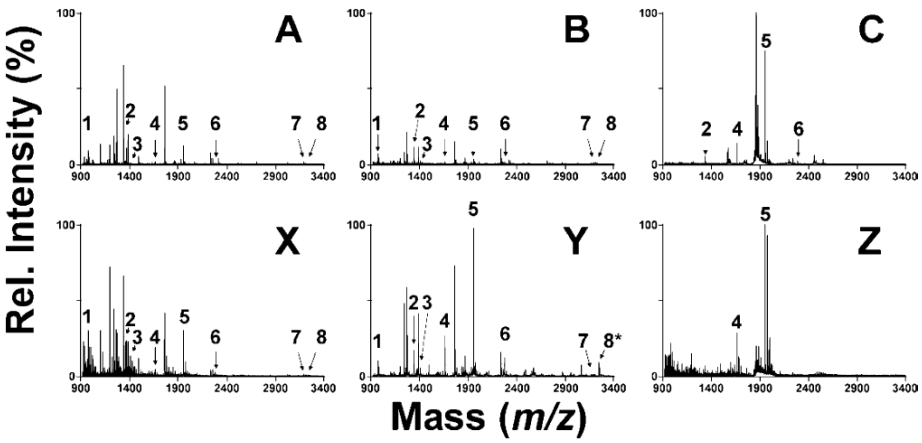

Figure 3. Mass spectra of 2 μL of α-Cas tryptic peptide mixture (50 μg) cocrystalized with α-matrix on steel plate (A), after adsorption/washing steps on a Ga^{3+}−pMALDI chip (B), and on a steel plate after enrichment using the PHOS-Select Iron kit (C), in addition to the mass spectra corresponding to the same measurements using DHB (X, Y, and Z; respectively) as a matrix. All spectra were normalized to 2.9×10^4 counts.

Matrix ions were suppressed using low mass (m/z 2000) cutoffs. The spectra were accumulated from 9 points using 15 laser shots per point. The data were recorded using a PC workstation running MassLynx 3.5 software (Micromass, Manchester, U.K.). A MALDI Micro MX mass spectrometer (Waters, Milford, MA) was used in reflectron mode for phosphopeptide analysis. The instrument operated with 5 kV set on the sample plate, −12 kV on the extraction grid, pulse voltage of 1.95 kV, and 2.35 kV detector voltages. A nitrogen laser (337 nm, 5 Hz, 50 μJ per pulse) was used for ionization. MassLynx v4.0 software (Waters) served for data acquisition, and each spectrum was composed of 10 laser pulses. For both instruments, the pMALDI chip was fixed to a standard metallic plate with an adhesive tape and introduced at the source. Carbamidomethylated BSA tryptic digest (BSAdig) was used to calibrate the mass spectrometer (MPrep, Waters). The signal from singly protonated human glu-fibrinopeptide B (1570.6774 Th) served as an external lock-mass reference.

^{1}H NMR spectra were recorded with Bruker AVANCE DRX 500 nuclear magnetic resonance spectrometer (Bruker, Billerica, MA) at 500.13 MHz. All spectra were measured in $CDCl_3$. Chemical shifts are given in δ values relative to tetramethylsilane $\delta = 0.0$. Typically, 256 scans were accumulated per spectrum.

Results and Discussion

In this paper, we fabricated an amine reactive functionalized polymeric chip by quickly and simply copolymerizing AA-NHS with MMA. The activation of the monomer surface with NH_2-C_4-NTA (Figure 1.1) was monitored first by TLC in trials using the AA-NHS monomer with MeOH/DCM, 1:20, as the mobile phase. The reagents and the product of the reaction were spotted and allowed to run for 10 min. The product spot (modified nitrilotriacetic acrylic acid) eluted in a similar way to the one of NH_2-C_4-NTA (observed under UV). The result was verified using a permanganate solution to oxidize the double bond of the acrylic acid, in which only the product and the AA-NHS spot gave positive signals.

Later, the same reaction was examined in the polymer mixture using NMR; the decrease of the $\delta = 2.89$ (-CH_2-) signals from the *N*-hydroxysuccinimide residue, and the increase of -CH_2- signals of the side chain, in particular $\delta = 2.96$ (-NH-CH_2-) of the product were monitored; using $\delta = 3.65$ (-OCH_3-) as an internal standard.

The pMALDI chip flexibly chelates different metal ions (Figure 1.2). Hard metal ions, such as Fe^{3+}, Ga^{3+}, and Al^{3+}, have been proved to bind selectively phosphopeptides at low pH values.[1,12] Ga^{3+} was used because it has been reported to possess a higher selectivity toward phosphopeptides, in comparison with the other hard metal ions.[12] To demonstrate the selectivity of our Ga^{3+}−pMALDI system, 1 pmol p-Ang (DRVpY-IHPF, 1126.5721 Th) was mixed with 3.5 pmol of digested BSA solution. BSA is a nonphosphorylated protein (MW ca. 66 KDa); when completely digested, it produces >60 distinct peptides, of which two-thirds are acidic and may constitute a possible interference for the phosphate-Ga^{3+} recognition.[28] The complexity of the mass spectra as well as the low intensity for the p-Ang could be observed when the mixture was applied using the "dry-droplet" technique on a metallic target and Ga^{3+}−pMALDI chip (Figure 2, panels A and B, respectively). The most intense signal of p-Ang was obtained, when the sample was adsorbed/washed on the Ga^{3+}−pMALDI chip (Figure 2C). To verify these results, a similar mixture using Ang (DRVYIHPF, 1046.1687 Th) was also adsorbed/washed on the Ga^{3+}−pMALDI target (Figure 2D). In this case, the most intense signal was from a BSAdig peptide (ETYGDMADcCcCEK, 1479.3021 Th), which presumably can chelate Ga^{3+} via two adjacent carboxymethylated cysteines.

The effects of different matrices on the Ga^{3+}−pMALDI chip were analyzed, by comparing the signal of a tryptic alpha-casein (α-Cas) digest with a commercial method, PHOS-Select Iron Affinity Gel provided by Sigma (Figure 3, Table 1). When

Table 1. List of Phosphorylated Peptides from α-Cas Tryptic Peptide Mixture in Figure 3

peptide no.	α-Cas subunit	MC	mass	sequence	no. P
1	S2	0	970.4074	NMAINPSK	1
2	S2	1	1339.6076	QEKNMAINPSK	1
3	S2	0	1411.6076	EQLSTSEENSK	2
4	S1	0	1660.7941	VPQLEIVPNSAEER	1
5	S1	1	1951.9524	YKVPQLEIVPNSAEER	1
6	S1	1	2257.1046	VPQLEIVPNSAEERLHSMK	1
7	S1	2	3164.5568	VPQLEIVPNSAEERLHSMKE GIHAQQK	1
8	S1	1	3227.2094	QMEAESISSSEEIVPNSVEQ KHIQK	5
8*	S1	1	3249.2094	QMEAESISSSEEIVPNSVEQ KHIQK + Na^+	5

α-matrix was used for the phosphopeptide analysis, the intensity of the phosphorylated peptides was quite low (Figure 3B), comparable to that obtained by simple deposition on a steel target (Figure 3A), and lower when compared to that obtained by the commercial kit (Figure 3C). This results can be explained by the strong interaction between phosphorylated peptides and the Ga^{3+}−pMALDI surface, which restrains them from properly cocrystallizing with the matrix.[20] When DHB was used, which is a metal-chelating agent as well as a MALDI matrix,[20] higher signals were observed after pMALDI preconcentration (Figure 3Y) than that for simple deposition on a steel target using DHB matrix (Figure 3X). Moreover, the signal intensities obtained on the Ga^{3+}−pMALDI were fully comparable to those obtained using the PHOS-Select Iron kit (Figure 3Z), proving that the Ga^{3+}−pMALDI system is comparable to one of the commercial available systems for phosphopeptide analysis. Additionally, the pMALDI targets provide more phosphorylated peptides, in particular for the high-mass range, which may contribute to better identification of the digested protein, and less pronounced methionine oxidation than that for PHOS-Select Iron kit.

Although other researchers have observed the interaction between hard metal ions and phosphorylated proteins,[29−31] our attempts to enhance the selective absorption of a whole protein based exclusively on the interactions between Ga^{3+} or Fe^{3+}, and the phosphorylated moieties at the surface of a protein were not successful (data not shown). We had published that a protein cannot bind to a negative pMALDI surface, if the pH value of the media is higher than its p*I*.[25] In addition, according to the Pearson metal ion definition, at higher pH values, the affinities of the intermediate metal ions may be similar to the hard ones.[10] Therefore, by working at higher pH with intermediate metal ion, the phosphorylated proteins can be selectively adsorbed on a metal-chelated-pMALDI chip. From the two intermediate metal ions that were evaluated (Ni^{2+} and Cu^{2+}), Ni^{2+} presented the best results for adsorbing phosphorylated proteins. In Figure 4, the adsorption of α-Cas at pH 4.5, 7.0, and 8.0 (Figure 4, panels A, B, and C, respectively), and between a bare NTA- and a Ni^{2+}−pMALDI chips (three repeats) was compared. As expected, the NTA−pMALDI chips were able to strongly adsorb the protein; meanwhile, the pH value was lower than 4.98 (p*I* of α-Cas).[22] In comparison, the presence of chelated Ni^{2+} contributed to the protein adsorption also in the pH range between 7.0 and 8.5.

To further prove the selectivity of the Ni^{2+}−pMALDI chips, an equimolar (10 pmol/μL) mixture of Myo, α-Cas, and CA I (p*I*: 6.80, 4.98, 6.60, respectively) was prepared in 20 mM Tris buffer (pH 7.7). To evaluate the selective adsorption of α-Cas, two control measurements were run: measuring the mixture on a Ni^{2+}−pMALDI chip, without adsorption/washing step; and on a NTA−pMALDI chip after being adsorbed, and consecutively flushed with ddH_2O (Figure 5, panels A and B, respectively). The selectivity toward α-Cas could be clearly seen when comparing the protein mixture which was adsorbed/washed, with the one which was only spotted on the Ni^{2+}−pMALDI (Figure 5, panels C and A, respectively). An additional control measurement, using dephosphorylated α-Cas (80%) instead of α-Cas in the protein mixture, was used to verify the selectivity of the surface toward the phosphate groups present on the surface of α-Cas (Figure 5D). Similar selectivity was observed when the PHOS-Select Iron kit from Sigma was modified using Ni^{2+} as the active affinity metal ion component. Unfortunately, it was noticed that histidine-rich protein, such as β-Lac A, could compete for the binding sites on the surface, which is in accordance to selectivity of Ni^{2+} (data not shown). Nevertheless, the ability to reduce possible phosphorylated candidates in a protein mixture during a databases search could significantly increase the identification score when combined with the phosphopeptide analysis of the same mixture.

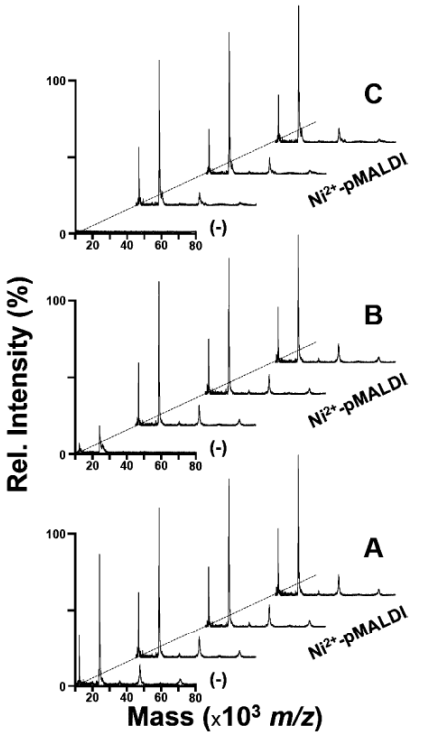

Figure 4. Comparison of α-Cas adsorption on a Ni^{2+}−pMALDI chip and a cation exchanger pMALDI chip based on bare NTA−pMALDI (-) at different pH values. (A) Acidic (pH 4.5), (B) neutral (pH∼7.0); and (C) basic (pH 8.0).

In conclusion, the versatility of the pMALDI chips to chelate different metal ions was demonstrated by replicating a range of different IMAC pMALDI probes. Using the Ga(III)−pMALDI

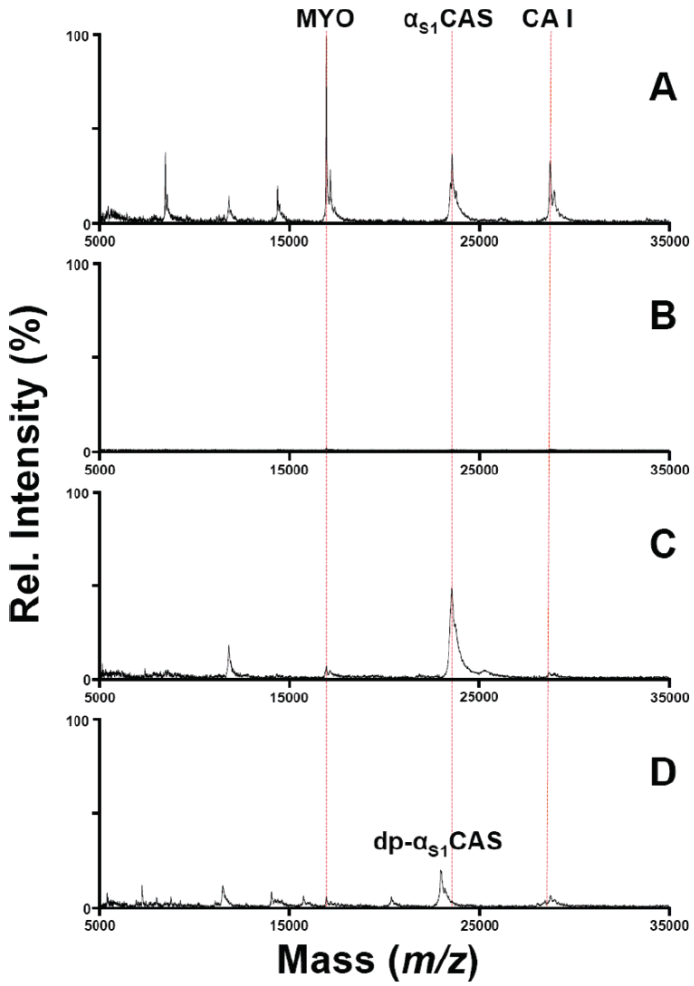

Figure 5. Phosphate-based protein enrichment of 5.0 pmol α-Cas from a protein mixture containing equimolar amounts of Myo, and CA I dissolved in 20 mM Tris buffer (pH 7.8). (A) Protein mixture deposited and measured on a Ni^{2+}−pMALDI chip. (B) Two microliters of protein mixture adsorbed, flushed with ddH_2O, and measured on a bare NTA−pMALDI chip. (C) Two microliters of protein mixture adsorbed, flushed with ddH_2O, and measured on a Ni^{2+}−pMALDI chip. (D) Protein mixture containing 5.0 pmol of dephosphorylated α-Cas (dp- α-Cas, 80%), Myo, and CA I dissolved in 20 mM Tris buffer (pH 7.8), adsorbed, flushed with ddH_2O, and measured on a Ni^{2+}−pMALDI chip.

chip, we obtained a comparable performance to commercial systems for the measurement of phosphopeptides. Additionally, peptides with higher MW and limited methionine oxidation were observed with the chip. Although the Ni^{2+}−pMALDI chip presented a selectivity toward His- as well as phospho-rich proteins, it is the combination of *m*/*z* values for phosphorylated protein candidates (obtained using the Ni^{2+}−pMALDI), with the values of digested phosphorylated peptides of the protein mixture (using the Ga^{3+}−pMALDI) that could prove to be very useful for the fast identification of unknown phosphoproteins using MALDI-TOF/MS.

Currently, we are studying new approaches to improve the selectivity toward whole phosphorylated proteins by using different recognition elements such as other hard metal ions

in combination with different chelating agents, antibodies, and enzymes.

Abbreviations: MALDI, matrix adsorbed laser desorption/ionization; TOF, time-of-flight; MS, mass spectrometry; pMALDI, plastic MALDI supports; LC, liquid chromatography; HCl, hydrochloric acid; TPCK, L-(tosylamido-2-phenyl) ethyl chloromethyl ketone; NTA, nitrilotriacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; DI water, deionized water; ddH_2O, double-deionized MiliQ water; MeOH, methanol (99.9%); DCM, dichloromethane; $CDCl_3$, deuterochloroform.

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References

(1) Mirzaei, H.; Regnier, F. Reduction of non-specific binding in Ga-(III) immobilized metal affinity chromatography for phosphopeptides by using endoproteinase glu-C as the digestive enzyme. *J. Chromatogr.. B* **2005**, *817*, 23−34.
(2) Garavelli, J. S. The RESID Database of Protein Modifications as a resource and annotation tool. *Proteomics* **2004**, *4*, 1527−1533.
(3) Hirsh, A. K. H.; Fischer, F. R.; Diederich, F. Phosphate recognition in structural biology. *Angew. Chem., Int. Ed. Engl.* **2007**, *46*, 338−352.
(4) Bodenmiller, B.; Mueller, L. N.; Mueller, M.; Domon, B.; Aebersold, R. Reproducible isolation of distinct, overlapping segments of the phosphoproteome. *Nat. Methods.* **2007**, *4*, 231−237.
(5) Xu, C.-F.; Wang, H.; Li, D.; Kong, X.-P.; Neubert, T. A. Selective enrichment and fractionation of phosphopeptides from peptide mixtures by isoelectric focusing after methyl esterification. *Anal. Chem.* **2007**, *79* (5), 2007−2014.
(6) Zhang, H.; Hunter, G. K.; Goldberg, H. A.; Lajoie, G. A.; Yeung, K. K.-C. An integrated procedure of selective injection, sample stacking and fractionation of phosphopeptides for MALDI MS analysis. *Anal. Chim. Acta* **2007**, *581*, 268−280.
(7) Riggs, L.; Seeley, E. H.; Regnier, F. E. Quantification of phosphoproteins with global internal standard technology. *J. Chromatogr., B* **2005**, *817*, 89−96.
(8) Tsai, L.-C.; Hsieh, Y.-J.; Lyu, P.-C.; Yu, J.-S. Anti-phosphopeptide antibody, P-STM as a novel tool for detecting mitotic phosphoproteins: identification of lamins A and C as two major targets. *J. Cell. Biochem.* **2005**, *94*, 967−981.
(9) Goshe, M. B. Characterizing phosphoproteins and phosphoproteomes using mass spectrometry. *Brief. Funct. Genomics Proteomics*, **2006**, *4*, 363−376.
(10) Chaga, G. S. Twenty-five years of immobilized metal ion affinity chromatography: past, present and future. *J. Biochem. Biophys. Methods* **2001**, *49*, 313−334.
(11) Haney, P. J.; Draveling, C.; Durski, W.; Romanowich, K.; Qoronfleth, M. W. SwellGel: a sample preparation affinity chromatography technology for high throughput proteomic applications. *Protein Expression Purif.* **2003**, *28*, 270−279.
(12) Kange, R.; Ulrike, S.; Granberg, M.; Lindberg, U.; Ekstrand, G.; Ek, B.; Gustafsson, M. Comparison of different IMAC techniques used for enrichment of phosphorylated peptides. *J. Biomol. Technol.* **2005**, *16*, 91−103.
(13) Aprilita, N. H.; Huck, C. W.; Bakry, R.; Feuerstein, I.; Stetcher, G.; Morandell, S.; Huang, H.-L.; Stasyk, T.; Huber, L. A.; Bonn, G. K. Poly(Glycidyl Methacrylate/ Divinylbenzene)-IDA-Fe^{III} in phosphoproteomics. *J. Proteome Res.* **2005**, *4*, 2312−2319.
(14) Mann, K.; Olsen, J. V.; Maček, B.; Gnad, F.; Mann, M. Phosphoproteins of the chicken eggshell calcified layer. *Proteomics* **2007**, *7*, 106−115.
(15) Shu, H.; Chen, S.; Bi, Q.; Mumby, M.; Brekken, D. Identification of phosphoproteins and their phosphorylation sites in the WEHI-231 B Lymphoma cell line. *Mol. Cell. Proteomics* **2004**, *3*, 279−286.
(16) Posewitz, M.; Tempst P. Immobilized Gallium(III) affinity chromatography of phosphopetides. *Anal. Chem.* **1999**, *71*, 2883−2892.
(17) Feuerstein, I.; Morandell, S.; Stecher, G.; Huck, C. W.; Stasyk, T.; Huang, H.-L.; Teis, D.; Huber, L.; Bonn, G. Phosphoproteomic analysis using immobilized metal ion affinity chromatography on the basis of cellulose powder. *Proteomics* **2005**, *5*, 46−54.
(18) Zhou, H.; Xu, S.; Ye, M.; Feng, S.; Pan, C.; Jiang, X.; Li, X.; Han, G.; Fu, Y.; Zou, H. Zirconium Phosphonate-modified porous silicon for highly specific capture of phosphoproteins and MALDI-TOF MS analysis. *J. Proteome Res.* **2006**, *5*, 2431−2437.
(19) Zhou, W.; Merrick, B. A.; Khaledi, M. G.; Tomer, K. B. Detection and sequencing of phosphopetides affinity bound to immobilized metal ion beads by matrix assisted laser desorption/ionization mass spectrometry. *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 273−282.
(20) Hart, S. R.; Waterfield, M. D.; Burlingame, A. L.; Cramer, R. Factors governing the solubilisation of phosphopeptides retained on ferric NTA IMAC beads and their analysis by MALDI-TOF/MS. *J. Am. Soc. Mass Spectrom.* **2002**, *13*, 1042−1061.
(21) Lo, C.-Y.; Chen, W.-Y.; Chen, C.-T.; Chen, Y.-C. Rapid enrichment of phosphopeptides from tryptic digests of proteins using iron oxide nanocomposites of magnetic particles coated with zirconia as the concentrating probes. *J. Proteome Res.* **2007**, *6*, 887−893.
(22) Feuerstein, I.; Najam-ul-Haq, M.; Rainer, M.; Trojer, L.; Bakry, R.; Aprilita, N. H.; Stetcher, G.; Huck, C. W.; Bonn, G. K. Material-enhanced laser desorption/ionization (MELDI) − A new protein profiling tool utilizing specific carrier materials for time of flight mass spectrometry analysis. *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 1203−1208.
(23) Dunn, J.; Watson, J. T.; Bruening, M. L. Detection of phosphopeptides using Fe(III)-nitrilotriacetate complexes immobilized on a MALDI plate. *Anal. Chem.* **2006**, *78*, 1574−1580.
(24) Muck, A.; Nešněrová, P.; Pichová, I.; Svatoš, A. Fast prototyping of hydrophobic disposable polymer support arrays for matrix-assisted laser desorption/ionization-time of flight-mass spectrometry of proteins by atmospheric molding. *Electrophoresis* **2005**, *26*, 2835−2842.
(25) Muck, A.; Ibáñez, A. J.; Stauber, E. J.; Mansourova, M.; Svatoš, A. Atmospheric molding of ionic copolymer MALDI-TOF/MS arrays: a new tool for protein identification/ profiling. *Electrophoresis* **2006**, *27*, 4952−4959.
(26) Ibáñez, A. J.; Muck, A.; Halim, V. A.; Svatoš, A. Trypsin-linked copolymer MALDI chips for fast protein identification. *J. Proteome Res.* **2007**, *6*, 1183−1189.
(27) Svatoš, A.; Muck, A. Patent application, DE102004053458A1.
(28) Stensballe, A.; Andersen, S.; Jensen, O. N. Characterization of phosphoproteins from electrophoretic gels by nanoscale Fe(III) affinity chromatography with off-line mass spectrometry analysis. *Proteomics* **2001**, *1*, 207−222.
(29) Andersson, L.; Porath, J. Isolation of phosphoproteins by immobilized metal (Fe^{3+}) affinity chromatography. *Anal. Biochem.* **1986**, *154*, 250−254.
(30) Myszyńska, G.; Dobrowolska, G.; Medin, A.; Ekman, P.; Porath, J. O. Model studies on iron (III) ion affinity chromatography II. Interaction of immobilized iron(III) ions with phosphorylated amino acids, peptides and proteins. *J. Chromatogr.* **1992**, *604*, 19−28.
(31) Wolschin, F.; Wienkoop, S.; Weckwerth, W. Enrichment of phosphorylated proteins and peptides from complex mixtures using metal oxide/hydroxide affinity chromatography (MOAC). *Proteomics* **2005**, *5*, 4389−4397.

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2.4 Manuscript IV

Trypsin-linked copolymer MALDI chips for fast protein identification

Alfredo J. Ibáñez, Alexander Muck, Vincentius A. Halim, and Aleš Svatoš

Journal of Proteome Research **2007**, *6*, 1183-1189

One of the most traditional ways to visualize the changes in protein expression is the combination of 2D gel electrophoresis separation coupled to peptide mass fingerprint identification (PMF). PMF identification consists of the digestion of an isolated protein by sequence-specific protease, and the further comparison of the yielded peptide mass spectra by MALDI-MS against a database.[31-34]

The advantages of using specific proteases such as trypsin (EC. 3.4.21.4) for PMF analysis is that under standard digestion conditions, trypsin exclusively cleaves peptide bonds, which are located at C-terminal to arginine and lysine residues, producing fragments with an average size of 10-12 amino acids.[75] These peptide sizes are optimal for MS ionization techniques such as MALDI and ESI, and also for *de novo* sequencing methods using MS/MS techniques.

Major two drawbacks that impede the application of trypsin for proteomic studies are (a) the enzyme undergoes rapid autolysis at basic pH and 37ºC (i.e. conditions in which the enzyme is also used for PMF identification), hence it must be kept at a lower concentration than the protein (i.e. enzyme/protein ratio 1/100), increasing the time for a tryptic digestion to an average of 16 hours; and, (b) general digestion protocols yield high amounts of digestion volume (i.e. 10 μL); such amounts are not compatible with the volume used for PMF identification by MALDI-MS (i.e. 0.5-1.2 μL).[31-34]

In this manuscript we overcame these drawbacks by using an immobilized enzyme (e.g. trypsin) bioreactor on a confined volume (i.e. pMALDI chip microwell) to carry out the protein endoproteolysis in a biologically compatible environment. Because of the preconcentration effect associated with the immobilization of enzymes in confine small volumes, highly purified enzymes or great amounts of protein are not required. Furthermore, the immobilization of the enzyme increases enzymatic stabilization, protecting enzymes from heat, organic solvents, and pH without loss of catalytic activity.

Based on the work published by Dr. Muck *et al.*[60] and under the supervision of Dr. Aleš Svatoš, I was given the freedom to plan all experiments. I synthesized the new polymeric substrates and developed the protocol for the effective immobilization of enzymes on the surface. Digestion conditions were optimized in collaboration with Dr. Vincentius Halim and Dr. Muck. Furthermore, Dr. Muck provided complementary information for the peptide identification and proteolytic enzyme mechanisms by running in-solution digestion samples on LC-MS/MS instrument as references.

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Trypsin-Linked Copolymer MALDI Chips for Fast Protein Identification

Alfredo J. Ibáñez, Alexander Muck, Vincentius Halim, and Aleš Svatoš*

Mass Spectrometry Research Group, Max Planck Institute for Chemical Ecology, Hans-Knöll-Strasse 8, 07745 Jena, Germany

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For the first time, trypsin-linked copolymer poly(methyl methacrylate-*co*-2-amino-ethyl methacrylamide) MALDI-TOF 100-sample array chips for integrated proteomic sample preparation/measurements have been fabricated using a simple atmospheric molding protocol. The enzyme link on the polymeric chip surface has been created by covalently binding ethylene glycol disuccinate bis(sulfo-*N*-succinimidyl) ester with the amine functionalities of the chip well surface and subsequent reaction of the linker with 1.3 nmol trypsin. The superior performance of the new chips is demonstrated for the enzymatic digestion of individual proteins (500 fmol) of 5−60 kDa size. A mixture of 500 fmol cytochrome *C*, bovine serum albumin, human hemoglobin, and horse myoglobin was deposited in the trypsin-linked sample well, followed by 15−60 min of on-chip digestion. Subsequent peptide mass fingerprinting using protein-database-searching software identified all four proteins. The combination of hydrophobic pMALDI arrays and novel enzyme-linked chips minimizes sample-handling times and enhances the analytical information collected by offering intact protein mass measurements combined with enzymatic cleavage and the peptide mass fingerprint. Our concept can be readily extended to further high-throughput enzyme activity screening and protein processing.

Keywords: biochip • digestion • immobilized enzyme • polymer modification • off-line MALDI-TOF/MS • protein mixtures

Introduction

One of the preferred methods for identifying proteins in the ever-growing field of proteomic research is peptide mass fingerprinting using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS).[1−4] This technique involves the sequence-specific enzymatic cleavage of an intact protein by a protease (for example, trypsin) obtained after 2D-PAGE, liquid chromatography, or capillary zone electrophoresis separation step, and the subsequent identification of the protein using MALDI-TOF mass spectra to compare the peptide fragments to a theoretical digestion pattern in a database.[2,3]

Although this approach is widespread in proteomic studies, the current in-solution and in-gel digest protocols have two main drawbacks:[1−4] (i) the typical time for a tryptic in-solution/in-gel digestion is 16 h, because the protein/trypsin ratio should be kept very high (100:1) to reduce the number of protease autodigestion peptides in the mass spectra; and (ii) a general in-gel/in-solution digestion protocol yields ca. 3−10 μL of a digest solution; however, it is difficult to apply the whole volume to a typical stainless-steel MALDI plate with a maximum capacity of ca. 2 μL. The self-digestion of proteases can be minimized by the acetylation of N-termini, lysine, and arginine residues.[5−7] However, it is difficult to avoid diluting peptide samples. One solution is to integrate and miniaturize sample preparation prior to the MALDI-TOF/MS measurement, since sample preparation usually involves multiple transfers which may significantly reduce sample amounts, in particular when the quantity of available protein is low.[1,2,8]

In recent years, innovative approaches, such as the use of trypsin-modified polymethacrylate,[9] the adsorption of trypsin directly on a metallic MALDI plate,[10,11] the use of nitrocellulose membranes for protein characterization,[12] an elastomeric device (for example, a sample concentrator) coupled with a metallic MALDI plate,[13] or chemically modified MALDI probes such as SELDI,[14] have obviated the need to handle samples prior to carrying out the MALDI-TOF/MS measurements.

In a recent communication, we described a new atmospheric molding protocol for fabricating hydrophobic- and ionic-modified polymer chips.[15,16] Using this rapid fabrication procedure, we have selectively added chemically reactive functionalities to the surface of the polymer without having to perform tedious multistep surface derivatization reactions.[17,18] These functionalities can be used to link a wide range of enzymes. When the enzyme is immobilized, only minimal autolysis products are observed. In addition to this, miniaturizing the complete digestion procedure further enhances the speed of the reaction, allowing digestion to take place in minutes.[19,20]

* Address correspondence to Dr. Aleš Svatoš, Mass Spectrometry Research Group, Max Planck Institute for Chemical Ecology, Hans-Knöll-Str. 8, D-07745 Jena, Germany. E-mail, svatos@ice.mpg.de; fax, +49-3641-571701.

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To contribute to ongoing efforts to develop integrated and fast digestion protocols, the following sections describe the first fabrication and analytical performance of a trypsin-linked copolymeric pMALDI-TOF/MS chip.

Materials and Methods

Chemicals and Proteins. Sinapinic acid (SA), α-cyano-4-hydroxycinnamic acid (α-matrix), trifluoroacetic acid (TFA), trichloroacetic acid (TCA), DL-dithiothreitol (DTT), iodoacetamide, cytochrome *C* (Cyt C), bovine serum albumin (BSA, fraction V), lysozyme (Lys), β-lactoglobulin A (β-Lac A), casein (Cas), human hemoglobin (H.Hem), horse myoglobin (Myo), carbonic anhydrase I (CA I), trypsin (TRP-TPCK treated), alkaline phosphatase (AP), 4-nitrophenyl phosphate (pNPP), and ethylene glycol disuccinate bis(sulfo-*N*-succinimidyl) ester (sulfo-EGS) were purchased from Sigma. Methyl methacrylate (MMA) and butyl methacrylate (BMA), both containing hydroquinone inhibitor, *tert*-butoxycarbonyl-2-aminoethyl methylacrylamide (*t*-Boc-AEMA), benzoin methyl ether (BME), and activated alumina (Grade CG20), were obtained from Polysciences (Warrington, PA). Acetic acid *N*-hydroxysuccinimide ester (AA-NHS) was from MP Biomedical (Solon, OH). Carbamidomethylated-BSA (CAM-BSA) was prepared by first reducing the BSA in a solution of 10 mM DTT in 25 mM ammonium bicarbonate for 1 h at 56 °C, and then by alkylating the reduced BSA with 55 mM iodoacetamide in 25 mM ammonium bicarbonate in the dark for 45 min at room temperature. The reduced CAM-BSA was precipitated by adding 3 M TCA and then incubated for 2 h on ice. Finally, the protein solution was centrifuged, and the pellet was washed three times with 1 mL of 25 mM ammonium bicarbonate.

Chip Fabrication. The rapid prototyping of pMALDI chips was performed using a modified atmospheric molding procedure in which three-dimensional sample zone arrays were fabricated on a silicon substrate and were used to template polymeric chips. The masters were prepared from 100 mm diameter p-type <100> orientation silicon wafers by means of soft-photolithography and wet chemical etching. The complete fabrication procedure has been described elsewhere.[15,16] The atmospheric molding *in situ* polymerization technique yields a negative copy of the silicon substrate in a thin sandwich mold. The *t*-Boc-*N*-aminoethyl methacrylate and methyl methacrylate monomers were copolymerized in a 1:27 molar ratio (0.5 g of *t*-Boc-AEMA mixed with 4 mL of MMA and 0.4% (w/v) BME) under UV exposure in a sandwich mold (glass plate + spacer + silicon master). The molding and demolding process of *t*-Boc-AEMA/MMA was straightforward, as with the hydrophobic and ionic copolymers previously reported.[15,16]

Enzyme Immobilization. Prior to linking trypsin to the pMALDI chip, the copolymer surface was activated, that is, flushed 50 times with 1.2 μL of a $HCl_{(co)}$/MeOH mixture (1:1) in 0.5% TFA. After the surface activation step, the pMALDI surface was cleaned: it was rinsed with DI water (2 L) and then sonicated for 5 min. The copolymeric surface was then neutralized by flushing with 0.1 M ammonium bicarbonate (pH 9.0) (20×), and by repeating the washing step. The modification involved adding 1.5 μL of 5 mM sulfo-EGS and incubating it for 30 min; subsequently, the pMALDI surface was flushed with 1.2 μL of PBS (0.1 M sodium phosphate buffer and 0.3 M sodium chloride, pH 7.4), and 1.5 μL of a solution containing 20 mg/mL trypsin was added. The enzyme was incubated for 1 h at 4 °C and shaken at 200 rpm. The remaining supernatant solution was removed from the pMALDI chip before it was used. The active surface was flushed with 1.5 μL of Tris buffer (0.1 M, pH 7.6; 15×) and finally cleaned by reapplying the washing step. The trypsin-linked chips could be immediately used or stored. In the latter case, 1.5 μL of 50 mM acetic acid (pH 4.5) was applied on the trypsin-linked wells. Remaining reagents and solvents were evaporated in a vacuum to ensure their long-term stability.[21]

Sample Preparation. The MALDI matrix was prepared by dissolving 10 mg/mL of α-matrix in a mixture of methanol/acetonitrile (1:1, v/v). All aqueous solutions were made in high purity water filtered through a Milli-Q water system (Millipore, Bedford, MA). The protein stock solutions were prepared in 150 μM concentration in 0.1% TFA (pH 3.4) to ensure their stability. Unless otherwise stated, the working solutions were prepared daily in a 0.5 μM concentration in 25 mM ammonium bicarbonate buffer, pH 7.8. To avoid the evaporation of the buffer solution during the digestion process, the trypsin-linked pMALDI chip was placed inside a humid chamber. This chamber consisted of a sealed plastic box containing water-soaked foam where the pMALDI chip rested. After the digestion of the proteins and evaporation of digestion buffer in a vacuum, the peptides could be directly measured on the chip by mixing 0.1% TFA in a 1:1 ratio with α-matrix and depositing the mixture on the enzymatic polymeric pMALDI chips using the dried droplet technique. Alternatively, the peptides could be extracted from the chip using a mixture of 0.1% TFA/acetonitrile in a 1:1 ratio.

Apparatus. The spectra were acquired using a MALDI-TOF/MS instrument (TofSpec 2E; Micromass, Manchester, U.K.) operated in a linear and reflectron mode. Desorption/ionization was accomplished using a nitrogen UV laser (337 nm, 4 ns pulses of max energy 180 μJ). The positive ions were subjected to a 20.0 kV accelerating potential with 0 V set to the sample plate, reflected by a reflectron electrode (25.0 kV), and detected by a microchannel plate (MCP) detector (1.9 kV). Matrix ions were suppressed using low mass (m/z 500, reflectron, and m/z 2000, linear mode) cutoffs. The spectra were accumulated from 5 points using 15 laser shots per point. The data were recorded using a PC workstation running MassLynx 3.5 software (Micromass, Manchester, U.K.). A MALDI micro MX mass spectrometer (Waters, Milford, MA) was used in reflectron mode for monitoring protein digestion. The instrument operated with 5 kV set on the sample plate, −12 kV on the extraction grid, pulse voltage of 1.95 kV, and 2.35 kV detector voltages. A nitrogen laser (337 nm, 5 Hz, 50 μJ per pulse) was used for ionization. MassLynx v4.0 software served for data acquisition (Waters), and each spectrum was composed of 10 laser pulses. For both instruments, the pMALDI chip was fixed to a standard metallic plate and introduced at the source. BSA tryptic digest was used to calibrate the mass spectrometer (MPrep, Waters). The signal from protonated human glu-fibrinopeptide B (1570.6774 Da) served as an external lock-mass reference. ProteinLynx Global Server Browser v.2.2 program, from Waters, was used for baseline subtraction and smoothing, de-isotoping, and peptide mass fingerprinting using a Swiss-Prot database downloaded on March 20, 2006. The search parameters were peptide tolerance of 80 ppm and minimum four peptides found, estimated calibration error of 0.05 Da, 1 possible missed cleavage, carbamidomethylation of cysteins, and possible oxidations of methionines when appropriate.

^{1}H NMR spectra were recorded with Bruker AVANCE DRX 500 nuclear magnetic resonance spectrometer (Bruker, Billerica, MA) at 500.13 MHz. All spectra were measured in $CDCl_3$.

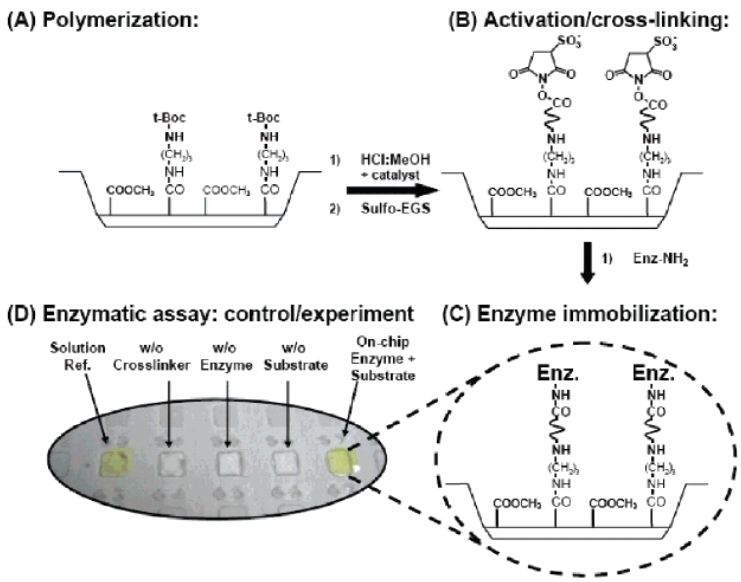

Figure 1. Modifying steps of the atmospherically molded pMALDI-TOF chip surface to capture enzymes. (A) Copolymerization of *t*-Boc-AEMA/MMA; (B) polymer activation with $HCl_{(cc)}$/MeOH/0.5% TFA; and addition of a cross-linker (sulfo-EGS; the linker chain is depicted by wavy lines); (C) incubation of the enzyme (Enz., alkaline phosphatase or trypsin); (D) comparison of the reaction using immobilized and nonimmobilized alkaline phosphatase.

Chemical shifts are given in δ values relative to tetramethylsilane $\delta = 0.0$. Typically, 256 scans were accumulated per spectrum.

Results and Discussion

It has been shown previously that amine-functionalized polymeric chips can be fabricated by rather lengthy chemical modifications of the methylmethacrylate surfaces.[17,18] For this paper, we fabricated the amine-functionalized polymeric chips by quickly and simply copolymerizing *t*-Boc-AEMA and MMA. The *t*-Boc-AEMA monomer was selected instead of AEMA hydrochloride to avoid the chemical pretreatments associated with the hydrochloride monomer.[22] Prior to activation, the copolymerization ratio (27:1) was verified using ^{1}H NMR (400 MHz, $CDCl_3$), according to a ratio of $OCH_3/N(COC(CH_3)_3)$ signals.

The deprotection of *tert*-butyl groups was confirmed by the decrease of the $\delta = 1.42$ (s, $NCOC(CH_3)_3$) signal, and the increase of $\delta = 2.05$ (br, $-CH_2-NH_2$), 3.30 (br, $-CH_2-NH_3^+$), using $\delta = 3.65$ (s, $-OCH_3$) as an internal standard.

Figure 1 demonstrates the flexibility of the pMALDI chip for straightforward modifications with different enzymes. The copolymeric surface (Figure 1A) can be easily activated by acid hydrolysis, and the resulting amino-functionalities can be reacted with the ethylene glycol linker molecule (Figure 1B), allowing the enzyme that is subsequently attached to function normally (Figure 1C). The efficiency of the washing and blocking procedures prevents nonspecific enzymatic activity by removing the enzymes that are not covalently bound from the polymeric surface. Alkaline phosphatase was selected to demonstrate the successful functionalization of the pMALDI chip. Alkaline phosphatase is specifically bound to the chip surface where it catalyzes the hydrolysis of the *p*-nitrophenylphosphate to a yellow *p*-nitrophenol (Figure 1D), whereas no such color change, or enzyme activity, occurs in the wells with a nonspecifically adsorbed enzyme.

In Figure 2, the MALDI-TOF/MS spectra of tryptic digests of 500 fmol Cyt C are shown. An optimally sized cross-linker[23] enables the enzymatic activity of the immobilized enzyme (Figure 2B,C) to mimic the enzymatic behavior of the in-solution enzyme (Figure 2A). Linking the enzyme enhances enzymatic activity, as demonstrated in the reduction of the peak at m/z 1633.82 with one miscleavage and the improvement in the signal of the digested fragments between m/z 1000 and 1600. Moreover, the similar S/N ratios in Figure 2B,C attest to how thoroughly the buffers and salts used during the modification were washed away; washing is necessary because salts may lower the signal during *in situ* measurements.

An S/N ratio is not the only parameter used to determine the identification of a protein. The efficiency of the peptide mass fingerprinting approach relates mainly to two analytical parameters obtained when comparing a MALDI-TOF/MS spectrum to a database: (i) the sum of peptides identified in a protein digest and (ii) the peptide coverage of the protein sequence. The amount of time is known to influence the number of missed cleavages in a protein,[12] thus, affecting protein coverage and peptide number. Figure 3 shows the influence of digestion time on the protein identification using

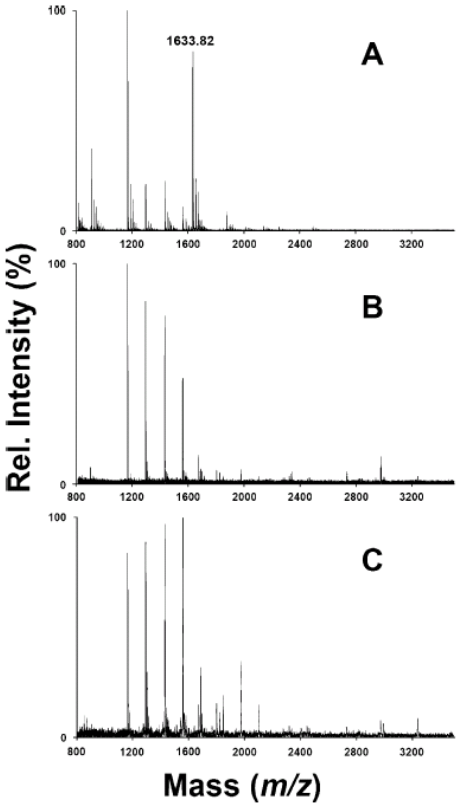

Figure 2. Comparison of in-solution and on-pMALDI chip digestion of Cyt C (500 fmol). (A) In-solution digestion (100:1). (B) On-pMALDI chip digestion, subsequently measured on a metallic target. (C) On-pMALDI chip digestion, subsequently measured *in situ*.

the database-searching software. The trypsin-linked chip offers measurable signals of the CAM-BSA after only 15 min of digestion, thus, allowing the user to manually identify the protein. The automatic software identification can be performed in the next 15 min when the S/N ratio is much higher. An improved ratio of digestion efficiency-to-digestion time was observed when the heating chamber was preheated 1 h prior to digestion. Since the goal is to achieve an accurate and efficient high-throughput digestion platform for proteomic studies, the digestion time was set to 1 h at 37 °C, an amount of time which is still much lower than the 16 h used for in-gel/in-solution digestion. Additionally, the high-throughput of MALDI plates (96 or 100 wells/plate) makes the time required for the overall plate analysis comparable to on-line digestion methods (35 s per digestion/sample × 100 samples ~1 h).

The digestion parameters, that is, the number of peptides produced and protein coverage, for proteins produced by in-gel/in-solution digestions also depend highly on the structural

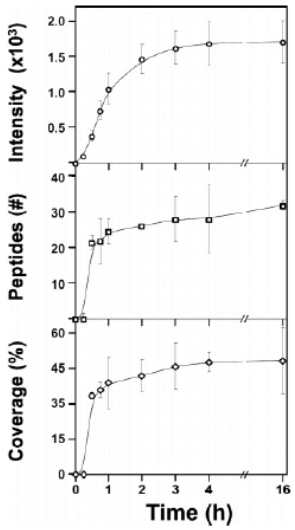

Figure 3. Influence of digestion time on the ability of the database identification software to correctly identify carbamidomethyl-BSA. All digestions were carried out using 1 pmol/μL CAM-BSA in 25 mmol/L ammonium bicarbonate buffer (pH 7.8), at 37 °C.

properties of the protein to be analyzed because proteins with rigid structures, such as heme proteins, tend to be resistant to digestion.[20] To study the efficiency of our system, eight proteins with molecular weights between 5 and 60 kDa were used. Figure 4 shows the MALDI-TOF/MS spectra of the individual digests performed directly on the chip. The results of different well measurements for the same protein yielded a similar sequence coverage and a similar number of peptides; these peptides were later used to identify each individual protein, proving the high reproducibility of the digestion process (data not shown).

The ability of the trypsin-linked pMALDI chip to identify a protein after being stored at different temperature conditions, −20, 37 °C, and room temperature, was also studied. The 30-day-old pMALDI chip still offered reasonable S/N ratios for the digestion of CAM-BSA (Figure 5), after being reconstituted with water and 25 mM ammonium bicarbonate. The results of the CAM-BSA digestion in these chips were compared to the results obtained on a freshly prepared trypsin-linked pMALDI chip. As shown in the inset in Figure 5, the number of identified peptides and the protein coverage obtained were sufficient to allow a positive database identification of the CAM-BSA. In addition, stability and reproducibility were improved when the enzyme was blocked with 1 μL of 50 mM AA-NHS in PBS. However, because this reagent decreased the intensity of peptide signals by 30%, the blocking agent was not used during further experiments.

The application of the trypsin-linked pMALDI chip as a reactor after measuring the protein mixture containing 500 fmol of Cyt C, Myo, H.Hem, and BSA each, on a hydrophobic BMA/MMA pMALDI chip[15] is demonstrated in Figure 6A. After

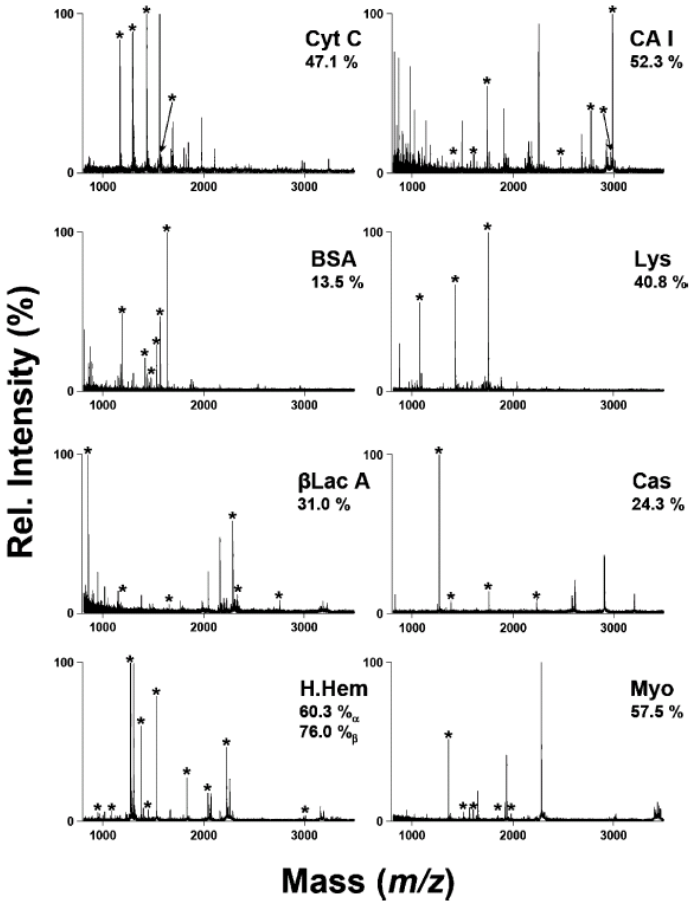

Figure 4. MALDI-TOF MS spectra of peptides obtained after digesting diverse proteins using the trypsin-linked pMALDI-chip (*in situ* measurements). The corresponding sequence coverage percentage is based on the peptide fragments recognized by PGLS (*). Conditions: 500 fmol/μL of protein in 25 mmol/L ammonium bicarbonate buffer (pH 7.8), 1 h incubation at 37 °C. Identified sequences and the modifications observed are given in Supporting Information as Table ST1.

MALDI-TOF/MS protein measurement, the protein mixture was extracted from an adjacent well of the hydrophobic chip using 1 μL of 30% acetonitrile/25 mM ammonium bicarbonate. The sample was then transferred onto the trypsin-linked chip, and an immediate proteolysis was performed after adding 0.5 μL of 25 mM ammonium bicarbonate. The analysis of the resulting peptide fragments (Figure 6B) enabled all four proteins deposited on the BMA/MMA hydrophobic chip to be positively identified. Signals for the mixture of both proteins and the related peptides are clearly visible; however, the effects of ion competition can be observed in both the lower signals for H.Hem in the linear mode and the strong signals for peptides of H.Hem in the reflectron mode. In the case of even more complex protein mixtures, the ion signals for some proteins could be strongly suppressed, preventing them from being identified. In such cases, a prefractionation of proteins and/or peptides would be necessary to limit the sample complexity.

Combining m/z values of intact proteins with those of digested peptides is very useful for identifying unknown proteins using MALDI-TOF/MS. Knowing the correct protein mass would limit the number of possible candidates from databases to consider and, thus, significantly increase the identification score.

Trypsin-linked chips may be coupled with SPE- and nanoLC-protein separation, thus, combining the high separation power with the fast parallel digestion and subsequent MALDI-TOF/

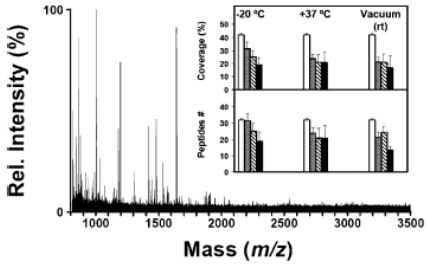

Figure 5. MALDI-TOF MS spectrum obtained from the digestion of 1 pmol/μL CAM-BSA by a 30-day-old trypsin-modified pMALDI chip stored at −20 °C. The inset shows the number of peptides and protein coverage given by the PGLS program under different times and storage conditions; digestion parameters as in Figure 4.

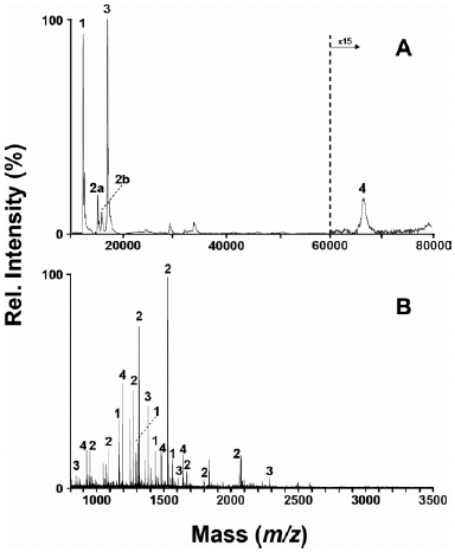

Figure 6. (A) MALDI-TOF MS spectra (linear mode) of Cyt C (1), H.Hem (2), Myo (3), and BSA (4) (500 fmol/μL each) on a BMA/MMA. (B) MALDI-TOF MS spectra (reflectron mode) of a mixture of peptides after digestion (conditions as in Figure 4). The proteins were desorbed from the BMA/MMA chip (panel A) using 30% acetonitrile/25 mM ammonium bicarbonate mixture (pH 7.8). Peptides are marked with the numbers used for proteins in panel A.

MS measurements. Currently, we are studying new approaches to combine both the "gel-free" protein analysis/separation and the high-throughput off-line digestion/peptide identification in search of faster and more robust methods.

Abbreviations: *t*-Boc-AEMA, *tert*-butoxycarbonyl-2-aminoethyl methacrylamide; AEMA, 2-aminoethyl methacrylamide; MMA, methyl methacrylate; PMF, peptide mass fingerprint; pMALDI chip, copolymer sample support array for matrix-assisted laser desorption/ionization; PMMA, poly(methyl methacrylate); SELDI-MS, surface-enhanced laser desorption/ionization mass spectrometry.

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Supporting Information Available: Table listing the determined sequences and observed modifications using the PLGS server. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Roe, M. R.; Griffin, T. J. Gel-free mass spectrometry-based high throughput proteomics: Tools for studying biological response of proteins and proteomes. *Proteomics* **2006**, *6*, 4678−4687.
(2) Peterson, D. S.; Rohr, T.; Svec, F.; Fréchet, J. M. J. High-throughput peptide mass mapping using a microdevice containing trypsin immobilized on a porous polymer monolith coupled to MALDI TOF and ESI TOF mass spectrometers. *J. Proteome Res.* **2002**, *1*, 563−568.
(3) Gobom, J.; Nordhoff, E.; Ekman, R.; Roepstorff, P. Rapid microscale proteolysis of proteins for MALDI-MS peptide mapping using immobilized trypsin. *Int. J. Mass Spectrom. Ion Processes* **1997**, *169/170*, 153−163.
(4) Park, Z-Y.; Russell, D. H. Identification of individual proteins in complex protein mixtures by high-resolution, high-mass-accuracy MALDI TOF-mass spectrometry analysis of in-solution thermal denaturation/enzymatic digestion. *Anal. Chem.* **2001**, *73*, 2558−2564.
(5) Rice, R. H.; Means, G. E.; Brown, W. D. Stabilization of bovine trypsin by reductive methylation. *Biochem. Biophys. Acta* **1977**, *492*, 316−321.
(6) Finehout, E. J.; Cantor, J. R.; Lee, K. H. Kinetic characterization of sequencing grade modified trypsin. *Proteomics* **2005**, *5*, 2319−2321.
(7) Murphy, A.; Fágáin, C. Ó. Stability characteristics of chemically-modified soluble trypsin. *J. Biotechnol.* **1996**, *49*, 163−171.
(8) Quadroni, M.; James, P. Proteomics and automation. *Electrophoresis* **1999**, *20*, 664−667.
(9) Turkova J.; Blaha, K.; Malanikova, M.; Vancurova, D.; Svec, F.; Kalal, J. Methacrylate gels with epoxide groups as supports for immobilization of enzymes in pH range 3−12. *Biochem. Biophys. Acta.* **1978**, *524*, 162−169.
(10) Colangelo, J.; Orlando, R. On-target exoglycosidase digestions/MALDI-MS for determining the primary structures of carbohydrate chains. *Anal. Chem.* **1999**, *71*, 1479−1482.
(11) Harris, W. A.; Reilly, J. P. On-probe digestion of bacterial proteins for MALDI-MS. *Anal. Chem.* **2002**, *74*, 4410−4416.
(12) Luque-Garcia, J. L.; Zhou, G.; Sun, T.-T.; Neubert, T. A. Use of nitrocellulose membranes for protein characterization by matrix-assisted laser desorption/ionization mass spectrometry. *Anal. Chem.* **2006**, *78*, 5102−5108.
(13) Chen, X.; Murawski, A.; Kuang, G.; Sexton, D. J.; Galbraith, W. Sample preparation for MALDI mass spectrometry using an elastomeric device reversibly sealed on the MALDI target. *Anal. Chem.* **2006**, *78*, 6160−6168.
(14) Su, J.; Rajapaksha, T. W.; Peter, M. E.; Mrksich, M. Assays of endogenous caspase activities: A comparison of mass spectrometry and fluorescence formats. *Anal. Chem.* **2006**, *78*, 4945−4951.
(15) Muck, A.; Nešněrová, P.; Pichová, I.; Svatoš, A. Fast prototyping of hydrophobic disposable polymer support arrays for matrix-assisted laser desorption/ionization-time of flight-mass spectrometry of proteins by atmospheric molding. *Electrophoresis* **2005**, *26*, 2835−2842.
(16) Muck, A.; Ibáñez, A. J.; Stauber, E. J.; Mansourova, M.; Svatoš, A. Atmospheric molding of ionic copolymer MALDI-TOF MS arrays: a new tool for protein identification/profiling. *Electrophoresis* **2006**, *27*, 4952−4959.
(17) Soper, S. A.; Henry, A. C.; Vaidya, B.; Galloway, M.; Wabuyele, M.; McCarley, R. L. Surface modification of polymer-based microfluidic devices. *Anal. Chim. Acta* **2002**, *470*, 87−99.

(18) Berhane, B. T.; Limbach, P. A. Functional microfabricated sample targets for matrix-assisted laser desorption/ionization mass spectrometry analysis of ribonucleic acids. *Anal. Chem.* **2003**, *75*, 1997−2003.
(19) Liu. Y.; Lu, H.; Zhong, W.; Song, P.; Kong, J.; Yang, P.; Girault, H. H.; Liu, B. Multilayer-assembled microchip for enzyme immobilization as reactor toward low-level protein identification. *Anal. Chem.* **2006**, *78*, 801−808.
(20) Shui, W.; Fan, J.; Yang, P.; Liu, C.; Zhai, J.; Lei. J.; Yan, Y.; Zhao, D.; Chen, X. Nanopore-based proteolytic reactor for sensitive and comprehensive proteomic analyses. *Anal. Chem.* **2006**, *78*, 4811−4819.
(21) Sipos, T.; Merkel, J. R. An effect of calcium ions on the activity, heat stability, and structure of trypsin. *Biochemistry* **1970**, *9*, 2766−2775.
(22) Wang, J.; Muck, A.; Chatrathi, M. P.; Chen, G.; Mittal, N.; Spillman, S. D.; Obeidat, S. Bulk modification of polymeric microfluidic devices. *Lab Chip* **2005**, *5*, 226−230.
(23) Kuramitz, H.; Natsui, J.; Sugawara, K.; Itoh, S.; Tanaka, S. Electrochemical evaluation of the interaction between endocrine disrupter chemicals and estrogen receptor using 17β-estradiol labeled with daunomycin. *Anal. Chem.* **2002**, *74*, 533−538.

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2.5 Manuscript V

DNA detection using a novel triple read-out optical/AFM/MALDI planar microwell plastic chip.

Alfredo J. Ibáñez, Thomas Schüler, Robert Möller, Wolfang Fritzsche, Hans-Peter Saluz, and Aleš Svatoš

Analytical Chemistry, **submitted**

The use of microarray-based technology is growing rapidly and has had considerable impact on genomic and proteomic research.[8, 76] One crucial component of microarray technology is the surface chemistry of the substrate,[77,78] and the sensitivity/selectivity of the read-out associated with the instrument.[79]

In the past few years, the fabrication process has made huge leaps forward with respect to spot densities, both *in situ*[80] and *ex situ*[81,82]. For example, DNA-chips, the affixed single stranded DNA (ssDNA), known as probes, can be placed in known locations on a single DNA microarray, and their hybridization events with ssDNA targets, carrying fluorescence tags, can be monitored using fluorescence scanners. For that reason, glass, silicon and quartz have been the primary DNA microarray substrates used, due to their low background signal for fluorescence read-out instrumentation.[83]

Although DNA microarray fabrication is a well-established technique, the exploration of new cost-effective substrates and chemical approaches to their production is fast-changing field of research. The speed of change in this field is particularly fuelled by the appearance of new labelling methods, such as metal nanoparticle labels,[84-86] enzymatic labelling,[87,88] quantum-dots,[89,90] etc., and by new available detection methods, such as electrochemical approaches,[91] light-scattering,[92,93] surface plasmon resonance,[94] and label-free methods (e.g. MALDI-TOF/MS).[95,96]

Manuscript V outlines the development of a ready-to-spot, polymeric DNA microarray for the determination of human herpes virus 5 or cytomegalovirus (CMV). The advantage of our polymeric DNA-chip in comparison to traditional DNA microarray materials is that we do not require time-consuming and expensive surface activation procedures for attaching nucleic acid sequences to the surface. In addition, our polymeric DNA-chip is compatible with state-of-the-art DNA detection methods such as laser-induced fluorescence, enzyme-catalyzed optical detection, and MALDI-MS.

The use of these multiple read-out techniques has given us the possibility to compare the sensitivity, selectivity and robustness of all the current state-of-the-art DNA detection methods (optical, AFM, and MALDI) on the same platform, and allowed us to avoid inherent technical differences between dissimilar microarray platforms and experiments. The major advantage of our platform is its versatility for diverse applications, with sensitivity ranging from 10^{-6} to 10^{-15} M. Additionally, the combination of several read-out technologies shows improved sensitivity, reproducibility, and reliability (i.e. reduction of the number of false positives and false negatives results) for DNA detection.

Under the supervision of Prof. Dr. Saluz, and Dr. Aleš Svatoš, my contribution to this project was the material development, and the ssDNA measurements based on fluorescence and MALDI-TOF/MS techniques. The transluminescence and AFM measurements of the binding events on the microarray surface were performed at the Institute of Photonic Technology, IPHT Jena, by Thomas Schüler under the supervision of Dr. Fritzsche and Dr. Möller. Moreover, Drs. Fritzsche and Möller also provided information about (bio)chemical surface modification on silicon.

DNA detection using a novel triple read-out optical/AFM/MALDI planar microwell plastic chip.

Alfredo J. Ibáñez,[1‡] Thomas Schüler,[2‡] Robert Möller,[2] Wolfgang Fritzsche,[3] Hans-Peter Saluz,[4] Aleš Svatoš[1*]

Analytical Chemistry, **submitted**

[1] Mass Spectrometry Research Group, Max Planck Institute for Chemical Ecology, Hans-Knoell-Str. 8, 07745, Jena, Germany

[2] Jenaer Biochip Initiative, Institute for Physical Chemistry, Friedrich Schiller University, Helmholtzweg 4, 07743 Jena Jena, Germany

[3] Institute of Photonic Technology, Albert-Einstein-Str. 9, 07745, Jena, Germany

[4] Leibniz Institute for Natural Product Research and Infection Biology – Hans Knöll Institute, Beutenbergstr. 11a, 07745, Jena, Germany

[‡] Those authors contributed equally to the paper

[*]Correspondence to: Dr. Aleš Svatoš, Mass spectrometry research group, Max Planck Institute for Chemical Ecology, Hans-Knöll-Str. 8, D-07745 Jena, Germany, E-mail: svatos@ice.mpg.de, Fax : +49-3641-571701

Keywords:

DNA chip, enzymatic silver deposition, miss-match detection, CMV, single strand DNA

ABSTRACT

A ready-to-spot disposable DNA chip for specific and sensitive detection of DNA was developed. Plastic co-polymeric substrate chemistry was optimized to selectively couple target DNA with the active surface. The combination of glycidyl and n-butyl methacrylates was found as the best fit for developing a substrate, which limits unspecific adsorption of target DNA molecules or additional polar contaminants in the test samples to the chip surface. The fabricated DNA microarrays have mechanical properties similar to those of glass and silicon substrates and, at the same time, provide chemically reactive surfaces which do not require lengthy chemical modification. An additional advantage of the plastic microchip is its compatibility with different analytical read-out techniques, such as mass spectrometry (MALDI-TOF/MS), optical detection (fluorescence & transluminiscence) and probe microscopy techniques (atomic force microscopy). This multi read-out capability has given us the ability to compare sensitivity, selectivity and robustness of current state-of-the-art bioanalytical methods on the same platform exemplified by successful DNA-based detection of human cytomegalovirus. The obtained sensitivity for enzymatic-enhanced silver deposition (10^{-15} M) surpasses conventional fluorescence read-outs (10^{-11} M). This is achievable due to the plastic chip's low background noise. In addition, the assay's dynamic range (10^{-6} - 10^{-15} M), reproducibility, and reliability (at compromised sensitivity from 10^{-9} M) of the ssDNA speaks for the silver deposition method in combination with MALDI-MS detection.

INTRODUCTION

The use of microarray-based technology is growing rapidly and has had considerable impact on genomic and proteomic research.[1,2] Crucial factors in microarray technology are the surface chemistry of the substrate enabling the defined immobilization of the capture molecules,[3,4] and the sensitivity/selectivity of the read-out associated with the instrument.[5]

Previously, glass, silicon and quartz have been the primary DNA microarray substrates due to their low background signal for fluorescence read-out instrumentation.[6-8] Nevertheless, the chemical groups on the surface of the glass, silicon, and quartz solid structures are not suitable for directly immobilizing biomolecules. Hence, in the past years, chemical modification protocols as well as thin layers coatings have been applied to the surface of glass, silicon, and quartz to bind DNA, RNA, proteins, etc. In addition, new platforms substrates are being constantly developed to improve the selective binding of biomolecules onto surfaces.[9]

Furthermore, the progress in the field of DNA microarray can be described in terms of (a) throughput and (b) sensitivity. The first one refers to huge leaps made in the fabrication process concerning spot densities on silicon substrates, either *in situ*[10] and *ex situ*.[11,12] Nevertheless, there is still a great scientific interest to explore new flexible, and cost-effective substrate materials for fabricating high-throughput and reproducible DNA microarrays;[3, 13] the ideal substrate being one which could provide inherent coupling functionality already in its material.

Recently introduced nanomaterials have led to higher sensitivity and reproducibility of DNA detection. New labeling methods such as enzyme metallography can overcome the deficiencies of fluorescence labels, in particular short lifetime.[9] For genetic diseases diagnosis, and infectious agents detection, labels such as: metal nanoparticle,[14-17] enzymes,[9,18-20] quantum-dots,[21,22] etc. are usually preferred, due to their rapid and simple synthesis, reproducibility, and stability. In addition, sensitivity and

robustness of DNA-chips has also increased due to the use of different read-out detectors; for example: electrochemical approaches,[16,17,20] light-scattering,[15,23] surface plasmon resonance,[24] atomic force microscopy,[25] and label-free methods (e.g. MALDI-TOF/MS).[26-29]

The human herpesvirus 5 or cytomegalovirus (CMV) has the largest genome (~235 kb) of any of the human herpesvirus family, which includes herpes simplex (1 & 2), Epstein-Barr, and varicella-zoster virus.[30,31] Although CMV is not highly contagious, it is the most common congenital infection (80% of the world population is infected),[32] and it is particularly hazardous in immunocompromised individuals[31,32] and pregnant women (e.g. 10% of infected newborns with CMV exhibit permanent mental retardation and auditory damage).[32,33]

Thanks to the developments in the field of miniaturization during the past decades, biosensors, chips, and in particular microarrays have shown great potential for the analysis of viral DNA.[7,8,13] In the case of CMV, microarray technology is starting to replace traditional determination methods such as ELISA and latex agglutination, because it allows multiplexed, parallel analysis. DNA microarrays can monitor the viral load of CMV-infected patients by just using viral cultures from urine, throat swabs, or tissue samples.[32-36]

Matrix-assisted laser desorption/ionization time-of-flight mass analyzers (MALDI-TOF/MS) have become effective tools for DNA studies,[34-36] because in contrast to fluorescence it directly measures a physical property of the DNA (i.e. mass), thus reducing the amounts of false positive signals. Thus, a microarray system that couples both (optical and mass spectrometry) read-out formats promises to be extremely sensitive and selective. Nevertheless, it is evident that the use of silicon-, glass- or quartz-based microarrays do not offer an optimal economy benefit due to the costly material and fabrication techniques involved when compared to polymer microstructures of equal quality in terms of structure resolution and planarity of the surface.[37-39]

In this paper, we describe the development of a low-cost material, which can be readily reacted with biomolecules, in this particular case ssDNA, to generate high-throughput microarray platforms (see Figure 1). As a proof of concept, we compared the performance of a DNA-chip based on a traditional microarray material such as glass with our novel plastic substrate for the analysis of CMV DNA. The following work is an extension of our current results from research into co-polymeric plastic chips, which have so far been used for protein analysis.[40]

EXPERIMENTAL SECTION

Chemicals and DNA sequences. Methyl methacrylate, butyl methacrylate, glycidyl methacrylate, and 2-(methacryloyloxy)ethyldimethyl-(3-sulfopropyl)ammonium hydroxide (all of them containing hydroquinone monomethyl ether as stabilizer), Ultrasensitive Streptavidin−Peroxidase Polymer (S2438), and 3-hydroxypicolinic acid were purchased from Sigma. Benzoin methyl ether (BME), and activated alumina (Grade CG20), were obtained from Polysciences (Warrington, PA, USA). The Horseradish peroxidase silver enhancement kit, EnzMet™ reagent kit, was purchased from Nanoprobes Inc. (Yaphank, NY, USA). Oligonucleotides were obtained from MWG-biotech Inc. (High Point, NC, USA), and had the sequences reported in Table 1. Random size human DNA samples extracted from female placenta cell nuclei and *E. coli* protein cell lysates were from our laboratory stock.

Sample preparation. All stock solutions (100μM) were prepared in double deionized MilliQ water (ddH_2O; Millipore, Bedford, MA, USA). The phosphate buffer solution (PBS) consisted of 0.01 M potassium monobasic phosphate, 0.137 M NaCl, and 0.003 M KCl (adjusted to pH 7.4 with sodium hydroxide). A sodium chloride/sodium citrate (SSC) buffer solution was used for the hybridization and washing steps and consisted of 0.3 M sodium citrate buffer, 3 M sodium chloride, adjusted to pH 7 with HCl.

Apparatus. A MALDI Micro MX mass spectrometer (Waters/Micromass, Manchester, UK) was used in linear (positive and negative ion) mode for the DNA analysis. The instrument operated with 5 kV set on the sample plate, -12 kV on the extraction grid, pulse voltage of 1.95 kV, and 2.35 kV detector voltages. A nitrogen laser (337 nm, 5 Hz, 50 μJ per pulse) was used for ionization. MassLynx v4.0 software (Waters) served for data acquisition, and each spectrum was composed of 10 laser pulses. The pMALDI chip was fixed to a standard metallic plate with adhesive tape and introduced at the source. A mixture containing 20-, 30-, and 40-base long ssDNA fragments was used to calibrate the mass spectrometer (using the *m/z* values given by the quality control department of Eurofins MWG for these ssDNAs). The signal from the 30-base long ssDNA served as an external lock-mass reference.

^{1}H NMR spectra were recorded with Bruker AVANCE DRX 500 nuclear magnetic resonance spectrometer (Bruker, Billerica, MA, USA) at 500.13 MHz. All spectra were measured in $CDCl_3$. The proton chemical shifts in the NMR spectrum are given in δ values relative to tetramethylsilane δ = 0.0. Typically 256 scans were accumulated per spectrum.

Chip fabrication. The rapid prototyping of the co-polymeric DNA microarrays is similar to the one used for plastic MALDI chips, [40-45] dimensions of the pMALDI chip and the well are 42 x 56 x 0.4 mm, and 2.5 (diameter) x 0.03 mm (depth), respectively. It was performed using a modified atmospheric molding procedure in which three-dimensional sample zone arrays were fabricated on a silicon substrate and were used to template polymeric chips. The masters were prepared from 100 mm diameter positive doped, <100> orientation, silicon wafers using soft-photolithography and wet chemical etching. The complete fabrication procedure has been described elsewhere.[41] The atmospheric molding *in situ* polymerization technique yields a negative copy of the silicon substrate in a thin sandwich mold. An aluminium spacer and a polytetrafluoroethylene seal tape was utilized between silicon substrate and the glass cover plate to prepare 0.4 mm thick chips. The decreased thickness of the chip dramatically reduced previously observed charging effect.[44] The molding and demolding process of prepared chips (Figure 2, Table) was straightforward, as was the previously reported.[41-45]

Conjugation of the DNA probes to the epoxide-pMALDI chip. Prior to immobilizing the ssDNA oligonucleotide to the surface, the co-polymer was cleaned and rinsed with ddH_2O and then dried under a stream of nitrogen. A piece of the co-polymeric DNA microarray was cut with scissors and dissolved in $CDCl_3$. The presence of the characteristic proton signals for the epoxide group (2.38, 2.63, and 3.13 PPM), and their relative intensity when compared to the –OCH_2– signal of the backbone side-chain guarantee the quality of the chip even after long-term storage at room temperature in high humidity environment.

The probe immobilization is based on a nucleophilic ring opening reaction (see Figure 2). The ssDNA probes used for our studies were 30 base ssDNA with different complementarities, full match, 1-base mismatch, 3-bases mismatch, and a longer non-

complementary ssDNA probe (0mis, 1mis, 3mis, and NC respectively) to the target CMV DNA sequence (Table 1). Moreover, labeled control probes were used to confirm the degree of labeling of the pMALDI chip surface (Table 1).

The probe immobilization involved the spotting, in each microwell, of 2.0 μL of 10 μM single stranded probe or labeled-probe DNA (ssDNA; see Table 1) in 1 x Arrayit spotting buffer (Telechem, Sunnyvale, CA, US). Then, the DNA-chip was incubated for either 1 hour at 50 ºC or 8 hour at 37 ºC; subsequently, the remaining probe spotting solution was flushed away with ddH_2O, and dried under nitrogen. The remaining active surface was blocked with 50 mM ethanolamine in 0.1M Tris and 0.1% SDS, pH 9, for 15 min. Finally, the chips were flushed with ddH_2O, and dried under nitrogen.

DNA probe array hybridization. For the optical and AFM detection, the biotin-modified target ssDNA of different sizes (i.e. biotin-labeled 20, 30, and 40 bases ssDNA, see Table 1) were used. MALDI-TOF/MS measurements were done with non-labeled target ssDNA of equivalent sizes to those employed for AFM and optical detection (see Table 1). Independent of the analytical detection method used, the target ssDNA (labeled or non-labeled) was dissolved in 1 x SSC + 0.1% SDS and incubated with the DNA-chip at least for 1 hour in a humidity chamber at 37 ºC. This was followed by two washing steps, first in 2 x SSC for 5 min and then in 0.2 x SSC for 5 min, and a drying step under a stream of nitrogen.

Gold nanoparticle deposition for AFM analysis. For the streptavidin gold nanoparticle (5 nm goldparticles; British Biocell, Cardif, UK) labeling, 100 μL of a 1:100 dilution of the original nanoparticle solution was dissolved in PBS with 0.1% BSA. Each chip was incubated with this solution for 1 h at 37 °C in a humidity chamber. Afterward the chips were washed six times for 5 min each in PBS with 0.05% Tween 20 and then briefly rinsed with distilled water to remove any excess of chloride ions.

Silver deposition for optical/AFM analysis. Based on the streptavidin-peroxidase polymer, the substrates were incubated with a 1:1000 dilution of the original solution in PBS with 0.05% Tween 20. A 100 μL portion of this solution was applied and incubated at 20 °C for 1 h on the chip. Then, the pMALDI chip was washed six times for 5 min each in PBS containing 0.05% Tween 20, and then in ddH_2O to remove any excess of unbound enzyme complex and chloride ions, which could interfere with the silver deposition reaction. The enzyme-induced silver deposition was performed using the EnzMet™ reagent kit. The chips were incubated with the EnzMet™ reagent kit for 1, 2, 3, or 5 min. Longer reaction times were used to test whether they can improve the strength of the signal. However, incubation times longer than 5 min led to an increase of non-specific signals (i.e. background) and were not used therefore. To avoid the inactivation of the enzyme, the EnzMet™ reagents were applied immediately after the washing step. The reaction was stopped by rinsing with ddH_2O to wash away the kit solution. After stopping the enzyme-induced silver deposition the enzyme becomes inactive because of the entrapment of the enzyme by the deposited silver.

MALDI-TOF/MS for label-free ssDNA analysis. The pMALDI chip was washed six times for 5 min each in PBS containing 0.05% Tween 20, and then briefly in ddH_2O to remove any excess of buffer and surfactant. Although this treatment was not necessary, it was done to maintain a similar sample handling protocol for the different analytical techniques. Consecutively, the double stranded DNA (dsDNA) was denatured by adding 2

μL ddH_2O to each microwell and increasing the temperature to 98°C for 10 min. To prevent evaporation of water the whole microarray was placed inside a humidity chamber. To avoid re-association of single-stranded DNA, 1.2 μL of 3-hydroxypicolinic acid (25 mg/mL), dissolved in water:acetonitrile (1:1), was immediately added by aspirating and dispensing the solution to each well, and the analyte/matrix mixture was left to crystallize at room temperature.

Safety considerations. *Alkyl methacrylates and benzoin methyl ether are toxic substances. Other used chemicals are irritants. Accidental inhalation, ingestion, or skin contact with these chemicals should be avoided. UV light may cause damage to skin and eyes, protective goggles and gloves should be used. The polymerization should be carried out in a ventilated fume hood.*

RESULTS AND DISCUSSION

The microarray for the detection of human CMV DNA presented in this paper relies on the parallel use of multiple read-out techniques, such as MALDI-TOF/MS, transluminiscence/AFM, fluorescence (see Figure 1). The ability to compare the sensitivity, selectivity and robustness of these different read-outs, using the same platform and identical preparation protocols, provides reliable and sensitive detection of a human CMV DNA sequence. To our knowledge, the present protocol represents the first example of integration of different state-of-the-art DNA read-outs in combination with a low-cost and ready-to-spot polymeric microarray substrate.

Preparation and optimization of the co-polymeric DNA microarray chips. The material chemistry of the plastic DNA microarray chip was extensively studied, because different surfaces properties can be obtained by using different polymer compositions. Therefore, to test the relationship between polymer composition and the MALDI-MS, AFM, and optical transluminescence signals, the chips were prepared by co-polymerizing different mixtures of functionalized monomers. Subsequently, the quality of the spot and the robustness, i.e. tolerance to non-specific adsorption of common interferences present in biological studies such as salts, random DNA sequences, and proteins were studied.

Based on our previous research,[9] the epoxide group was found to be the most suitable linking unity for the immobilization of DNA probes. Hence, the polymeric chips were prepared by co-polymerizing commercially available glycidyl methacrylate (i.e epoxide containing the monomer) with a backbone monomer such as methyl methacrylate (backbone 1 in Figure 2; weakly hydrophobic), butyl methacrylate (backbone 2 in Figure 2; strongly hydrophobic), or methyl methacrylate:[2-(methacryloyloxy)ethyl]dimethyl-(3-sulfopropyl)ammonium hydroxide (backbone 3 in Figure 2; zwitterionic).

To evaluate the quality of the spot (i.e. wettability), the surface coverage was characterized using optical & scanning probe microscopy, and the signal intensity of the DNA ions from recorded MALDI-MS spectra.

In similar way, the robustness of the DNA detection was evaluated using a common sample hybridization protocol to detect CMV DNA target sequences that has dissolved in solutions of increasing complexity, such as spotting buffer (containing only salts as interference), randomly sized human placenta DNA extracts (containing salts and non-complementary DNA as interference), and *E.coli* cell lysates (containing salts, non-complementary DNA, lipids, and proteins as interference).

Based on MALDI-MS measurements and bright field (optical) microscopy (i.e. which measures the light transmitted after the DNA target molecules are silver-labeled on the surface), glycidyl methacrylate:butyl methacrylate (EBMA, i.e. strongly hydrophobic, Figure 2) was selected to further use.

To further optimize EBMA substrates for MALDI-MS, optical and scanning probe microscopy measurements, it was necessary to assure a homogeneous distribution of the epoxide groups on the surface of the plastic substrates. Therefore, monomers (glycidyl methacrylate:butyl methacrylate) were co-polymerized using different molar ratios: 1:13.3, 1:26.6, and 1:39.9. Furthermore, in addition to each monomer ratio, different concentrations of two different 30 base pair capture CMV DNA probes, a ssDNA probe and ready labeled-probe (0mis and B-0mis, respectively), and a 35 base pair non-complementary sequence probe (NC) were dispensed manually into each well (1.5 μL/well).

By using bright field and scanning probe microscopy read-outs (i.e. transluminescence and atomic force microscopy respectively), it was concluded that 10 μM DNA probe immobilized on a 1:13.3 ratio pMALDI chip provided the most efficient and homogeneous distribution of active recognition sites on the polymer surface.

Part of this results are illustrated in Figure 3 were the chip surface was treated with biotin-labeled probe (B-0mis, 10 μM). Then the probe was labeled with gold nanoparticles (Figure 3A) or silver deposition by peroxidase-based enhancement (Figure 3B, 5 min). Surface images obtained at different random positions demonstrated that the sites of reaction were equally distributed across the surface. Moreover, the measurement of different heights of the formed structures in Figure 3 shows that enzymatic labeling (Figure 3B) gives bigger particles (ca 300 nm) than does labeling using gold nanoparticles (Figure 3A). Figure 3C shows the control for the silver deposition labeling method, where a biotin-labeled probe (B-0mis) was applied to a pure butyl methacrylate surface (AFM signal not higher than 10 nm). Therefore, owing to its better signal-to-noise ratio, the Horseradish peroxidase-based labeling system was preferred over the gold nanoparticle labeling system for further experiments.

Sensitivity comparison among state-of-the-art techniques. A comparison between MALDI-TOF/MS and optical measurements (using FITC-labeled and enzymatically-labeled targets) is shown in Figure 4A. The dynamic range of the silver enhancement kit depends on the incubation time of the peroxidase enzyme. The overall dynamic range given by horseradish silver enhancement is much wider (5 fM to 5 μM) than the one observed for FITC-labeled targets (pM to μM),[25] and for MALDI-TOF/MS (high nM to μM).[29]

While FITC-labels tended to photobleach, only negligible signal decay during measurements was observed for enzymatically deposited silver layers. Furthermore, methods involving enzyme-deposited silver layers require simpler detection equipment. These advantages make it more suitable for smart automatic-scan read-out systems than fluorescence tags.

Robustness of the CMV detection based on a plastic DNA-chip. The robustness of the optical microscopy measurements using enzymaticaly-labeled target CMV ssDNA is presented in Figure 4B. Consecutive calibration plots were made after 2 minutes of silver enhancement for the detection of CMV DNA on the plastic DNA-chip in a spotting solution (n=5, RSD=16.7) in the presence of random size human placenta DNA samples (n=3, RSD=7.1), and *E. coli* cell lysates (n=3, RSD=23.9).

Reliability in the DNA identification by using multiple read-out formats. Figure 5 shows a comparison between reliability and validity of the methods. For this experiment the grid (Figure 5A) was divided in two sets: (a) Spots 3-5 contain different 30 base long ssDNA probes, which were hybridized with a 30 base ssDNA target (30mer, Table 1). The nomenclature used to describe the probe in each well depends on the amount of mismatches in their oligonucleotide sequence: 3 mismatches (spot 3), 1 mismatch (spot 4), or completely complementary (spot 5). The oligonucleotide sequence for these probes can be found in Table 1 (3-mis, 1-mis and 0-mis, respectively). (b) Spots 6-7 contains the same completely complementary probe sequence used in spot 5 (0-mis, Table 1), but instead of hybridizing to the 30mer CMV ssDNA target sequence as in the case of spot 5; they were used to study the recognition of two target ssDNA, which posses a complementary sequence but differed in length, i.e. 20 bases long ssDNA target (spot 6) and 40 bases long ssDNA target (spot 7). The oligonucleotide sequence for these targets can be found in Table 1 (20-mer and 40-mer, respectively).

The efficiency of the MALDI-MS, transluminescence and AFM measurements were evaluated using as references the signal from spot 5, and the positive (B-0mis in Table 1 for AFM and 30mer in Table 1 for MALDI), negative (non-complementary probe, NC in Table 1) and blank controls, which were located in spots (1, 2, and 8 respectively).

The transluminescence and AFM read-outs were able to differentiate the hybridization events based on the number of mismatches in the probe sequence (case a), providing qualitative information for the hybridization of the target CMV ssDNA to the complementary DNA (0mis, TTTTTTCAGCATGTGCTCCTTGAT TCTATG), 1-mismatch (1-mis, TTTTTTCAGCATGGGCTCCTTGATTCTATG) and 3-mismatches sequences (3-mis, TTTTTTCAGCATTATCTCCTTGATTCTA TG). However, transluminescence and AFM measurements failed to differentiate the binding events based on the length of similar sequence target ssDNA (case b), since 20-mer (CATAGAATCAAGGAGCACAT) and 40-mer (GGGGGGGGGGC ATAGAATCAAGGAGCACATGCTGAAAAAA) target ssDNA share the same recognition sequence with the 30-mer (CATAGAATCAAGGAGCACATGCTG AAAAAA), hence providing similar enzymatic response and resulting in a high number of false positive identifications.

On the other hand, MALDI-TOF/MS instruments operating in linear mode have an accuracy of 0.2% in relation to the mass measurement. Thus the expected mass errors, ca. 20 Da for typical *m/z* 10,000 values used here, is smaller than the addition or subtraction of the smallest nucleoside monophosphate, deoxycytidine monophosphate (289.2 Da). This makes MALDI-TOF/MS a good read-out instrument for obtaining information regarding the size of the DNA sequence, which cannot be obtained in terms of matching sequence identity only. Moreover, the sensitivity for DNA MALDI-TOF/MS has been reported to be in the order of the femtomoles range, when hydrophobic substrates are used to pre-concentrate the sample DNA in a discrete spot in combination with a decreased amount of matrix.[29]

Therefore by using a read-out method such as MALDI-TOF/MS (see Figure 5C), it is possible to differentiate the length of bound DNA. Therefore, the false positive results observed for case (**b**) when using transluminisence and AFM read-outs can be corrected by comparing the mass spectra of the target DNAs with a standard (such as the one found in spot 1, Figure 5A). Additionally, MALDI-TOF/MS can analysis the non-bound target DNA present after the hybridization step (Figure 5D), as a possible for self-validation. To achieve this, the sample is pre-concentrated in anion exchange pMALDI chip[42] (e.g. one

containing a quaternary amine functionality on its surface) or on a dialysis membrane and later spotted to hydrophobic butyl methacrylate pMALDI chip[41] and subsequently measured using MALDI-TOF/MS, as in the case of Figure 5D.

Unfortunately, although the plastic-based substrates used in this work enhanced the sensitivity of MALDI-MS instruments in comparison to commercial available metallic targets, our MALDI-TOF/MS instrument failed to reach the limit-of-detection achieved by the enzymatic-labeled transluminiscence assay (see Figure 4A). Therefore, *in-situ* DNA amplification (e.g. *in-situ* PCR amplification) prior MALDI-TOF/MS measurement is required.

CONCLUSION

A versatile, ready-to-spot, disposable UV-photopolymerized plastic substrate for gold- and silver- nanoparticle labeled and labeled-free ssDNA detection based on optical, scanning probe microscopy and mass spectrometry was developed. Unlike the conventional silicon, glass or quartz DNA chips substrates previously used, the key advantage of this novel substrate lies in the fact that the plastic material exhibits an inherent activity towards DNA immobilization, so that the additional activation steps required in the case of the above-mentioned standard materials are not needed. Active functional epoxide groups are stable for months, allowing the production chips well before actually spotting with DNA probes. Although a standard DNA sample was used to illustrate the capabilities of this novel DNA microarray substrate, the composition of the co-polymeric DNA-chip was tailored to make it protein non-adhesive. Moreover, the fabrication protocol could be easily modified to prepare medium to high density chips of 10,000 – 50,000 wells with desired shape/size patterns and surface properties, in which the activity of the surface can be probed by infrared spectroscopy or by ^{1}H NMR.

Currently, we are developing an *in-situ* PCR amplification using a robotic-pipetting device, to increase the analysis throughput and to extend the dynamic linear range of the MALDI-TOF/MS measurements toward lower concentrations. Further, we foresee diverse applications for our polymeric substrates such as fast and reliable detection of human pathogens, the study of specific protein-DNA interaction, and genotyping. Additionally, this platform can find applications in areas currently not covered by commercial DNA chips, for example for the study of organisms of toxicological or ecological importance.

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References

(1) Brown, P.O.; Botstein, D. *Nat. Genet.* **1999**, *21*, 33-37.
(2) Pandey, A.; Mann, M. *Nature* **2000**, *405*, 837-846.
(3) Zhou, X.; Wu, L.; Zhou, J. *Langmuir* **2004**, *20*, 8877-8885.
(4) Wong, A.; Krull, U. *Anal. Bioanal. Chem.* **2005**, *383*, 187-200.

(5) Finnskog, D.; Ressine, A.; Laurell, T.; Marko-Varga,G. *J. Proteome Res.* **2004**, *3*, 988-994.
(6) Schena, M. *Microarray Analysis*. Wiley-Liss (November 11, 2002).
(7) Sauer, S.; Lange, B.M.H.; Gobom, J.; Nyarsik, L.; Seitz, H.; Lehrach, H. *Nature Rev. Genet.* **2005**, *6*(6), 465-476.
(8) Wilgenbus, K.K.; Lichter, P. *J. Mol. Med.* **1999**, *77* (11), 761-768
(9) Möller, R.; Powell, R. D.; Hainfeld, J. F.; Fritzsche, W. *Nano Lett.* **2005**, *5*, 1475-1482.
(10) Fodor, S.P.A. *Science* **1999**, *277*, 393-395.
(11) Schena, M.; Shalon, D.; Davis, R.W.; Brown. P.O. *Science* **1995**, *270*, 467-470.
(12) Okamoto, T.; Suzuki, T.; Yamamoto. N. *Nature* **2000**, 18, 438-441.
(13) Wang, J. *Nucleic Acids Res.* **2000**, *28*, 3011-3016.
(14) Mirkin, C. A.; Letsinger, R. L.; Mucic, R. C.; Storhoff; J. J. *Nature* **1996**, 382, 607-609.
(15) Xu, X.; Georganopoulou, D.G.; Hill, H.D.; Mirkin, C.A. *Anal. Chem.* **2007**, *79*(17), 6650-6654.
(16) Wang, J.; Xu, D.; Kawde, A.-N.; Polsky, R. *Anal. Chem.* **2001**, *73*, 5576-5581.
(17) Ambrosi, A.; Castañeda, M.T.; Killard, A.J.; Smyth, M.R.; Alegret, S.; Merkoci, A. *Anal. Chem.* **2007**, *79*, 5232-5240.
(18) Fritzsche, W.; Taton, T. A. *Nanotechnology* **2003**, *14*, R63-R73.
(19) Willner, I.; Baron, R.; Willner, B *Adv. Mater.* **2006**, *18*, 1109-1120.
(20) Wang, J.; Kawde, A.-N.; Jan, M.R. *Biosens Bioelectron.* **2004**, *20*(5), 995-1000.
(21) Zhang, C.Y.; Yeh, H.C.; Kuroki, M.T.; Wang. T.H. *Nat. Mater.* **2005**, *4*, 826-831.
(22) Wang, J. *Small* **2005**, *1*, 1036-1043.
(23) Yguerabide J.; Yguerabide, E.E. *J. Cell. Biochem. Suppl.* **2001**, *37*, 71-81.
(24) Kukanskis, K.; Elkind, J.; Melendez, J.; Murphy, T.; Miller, G.; Garner, H. *Anal. Biochem.* **1999**, *274*, 7-17.
(25) Csaki, A.; Kaplanek, P.; Möller R.; Fritzsche, W. *Nanotechnology* **2003**, *14*, 1262-1268.
(26) Liu, Y.H; Bai, J.; Zhu, Y.; Liang, X.; Siemieniak, D.; Venta, P.J.; Lubman, D.M. *Rapid Commun. Mass Spectrum.* **1995**, *9*, 735-743.
(27) Kirpekar, F.; Nordhoff, E.; Larsen, L.K.; Kristiansen, K.; Roepstorff, P.; Hillenkamp, F. *Nucleic Acids Res.* **1998**, *26*(11), 2554-2559.
(28) Kepper, P.; Reinhardt, R.; Dahl, A.; Lehrach, H.; Sauer, S. *Clin. Chem.* **2006**, *52*, 1303-1310.
(29) Schuerenberg, M.; Laubbert, C.; Eickhoff, H.; Kalkum, M.; Lehrach, H.; Nordhoff, E. *Anal. Chem.* **2000**, *72*, 3436-3442.
(30) Novotny, J.; Rigoutsos, I.; Coleman, D.; Shenk, T. *J. Mol. Biol.* **2001**, *310*, 1151-1166.
(31) Yen-Moore, A.; Straten, M.V.; Carrasco, D.; Evans, T.Y.; Tyring, S.K. *Clin. Dermatol.* **2000**, *18*(4), 423-432.
(32) Yang, S.; Ghanny, S.; Wang, W.; Galante, A.; Dunn, W.; Liu, F.; Soteropoulos, P.; Zhu, H. *J. Virol. Methods* **2006**, *131*, 202-208.
(33) Pagana, K.D.; Pagana T.J. Mosby's manual of diagnostic and laboratory test manual, Mosby St. Louis, MO, 1992, p401-402.
(34) Korimbocus, J.; Scaramozzino, N.; Lacroix, B.; Crance, J.M.; Garin, D.; Vernet, G. *J. Clin. Microbiol.* **2005**, *43*, 3779-3787.

(35) Chambers, J.; Angulo, A.; Amaratunga, D.; Guo, H.; Jiang, Y.; Wan, J.S.; Bittner, A; Frueh, K.; Jackson, M.R.; Peterson, P.A.; Erlander, M.G.; Ghazal, P. *J. Virol.* **1999**, *73*, 5757-5766.
(36) Fujimuro, M.; Nakaso, K.; Nakashima, K.; Sadanari, H.; Hisanori, I.; Teishikata, Y.; Diane Hayward, S.; Yokosawa, H. *Exp. Mol. Pathol.* **2006**, *80*, 124-131.
(37) Marko-Varga, G.; Ekström, S.; Helldin, G.; Nilsson, J.; Laurell, T. *Electrophoresis* **2001**, *22*, 3978-3983.
(38) Ekström, S.; Nilsson, J.; Helldin, G.; Laurell, T.; Marko-Varga, G. *Electrophoresis* **2001**, *22*, 3984-3992.
(39) Berhane, B.T.; Limbach, P.A. *Anal. Chem.* **2003**, *75*, 1997-2003.
(40) Muck, A.; Svatoš, A. *Talanta* **2007**, *74*, 333-341
(41) Muck, A, Nešněrová, P, Pichová, I, Svatoš, A. *Electrophoresis* **2005**, *26*, 2835-2842.
(42) Muck, A.; Ibáñez, A.J.; Stauber, E.J.; Mansuorova, M.; Svatoš, A. *Electrophoresis* **2006**, *27*, 4952-4959.
(43) Ibáñez, A.J.; Muck, A.; Halim, V.A.; Svatoš, A. *J. Proteome Res.* **2007**, *6*, 1183-1189.
(44) Ibáñez, A.J.; Muck, A.; Svatoš, A. *J. Mass Spectrom.* **2007**, *42*, 634-640.
(45) Ibáñez, A.J.; Muck, A.; Svatoš, A. *J. Proteome Res.* **2007**, *6*, 3842-3848.

Table and figure captions

Table 1. DNA sequences of probes and targets used in this work

Function	Abbrev.	Length	Sequence 5'-3'	Modification
Probe	Probe	30	TTT TTT CAG CAT GTG CTC CTT GAT TCT ATG	5´-Aminohexyl
Probe	NC	35	**ACT GAC TGA CTG ACT GAC TGA CTG GGC GGC GAC CT**	5´-Aminohexyl
Probe	3mis	30	TTT TTT CAG CAT **TAT** CTC CTT GAT TCT ATG	5´-Aminohexyl
Probe	1mis	30	TTT TTT CAG CAT G**G**G CTC CTT GAT TCT ATG	5´-Aminohexyl
Probe control	B-probe	30	TTT TTT CAG CAT GTG CTC CTT GAT TCT ATG	5´-Aminohexyl, 3´-Biotin
Probe control	F-probe	30	TTT TTT CAG CAT GTG CTC CTT GAT TCT ATG	5´-Aminohexyl, 3´-FITC
Probe control	B-NC	35	**ACT GAC TGA CTG ACT GAC TGA CTG GGC GGC GAC CT**	5´-Aminohexyl, 3´-Biotin
Probe control	F-NC	35	**ACT GAC TGA CTG ACT GAC TGA CTG GGC GGC GAC CT**	5´-Aminohexyl, 3´-FITC
Probe control	B-3mis	30	TTT TTT CAG CAT **TAT** CTC CTT GAT TCT ATG	5´-Aminohexyl, 3´-Biotin
Probe control	B-1mis	30	TTT TTT CAG CAT G**G**G CTC CTT GAT TCT ATG	5´-Aminohexyl, 3´-Biotin
Target	30mer	30	CAT AGA ATC AAG GAG CAC ATG CTG AAA AAA	
Target	B-30mer	30	CAT AGA ATC AAG GAG CAC ATG CTG AAA AAA	5´-Biotin
Target	F-30mer	30	CAT AGA ATC AAG GAG CAC ATG CTG AAA AAA	5'-FITC
Target	20mer	20	CAT AGA ATC AAG GAG CAC AT	
Target	B-20mer	20	CAT AGA ATC AAG GAG CAC AT	5´-Biotin
Target	40mer	40	GGG GGG GGG GCA TAG AAT CAA GGA GCA CAT GCT GAA	
Target	B-40mer	40	GGG GGG GGG GCA TAG AAT CAA GGA GCA CAT GCT GAA	5´-Biotin

Figure 1. Schematic workflow of the multiple read-outs compatible with the co-polymeric planar DNA microarray chip (DNA-chip). The multiple read-out methods here shown are: (a) MALDI-TOF/MS, (b) fluorescence, and (c) enzymatic silver enhancement.

Figure 2. Scheme of the polymerization reaction, and the flexibility that is possible by combining different backbone monomers with glycydyl methacrylate. The R in the Table represents (1) methyl, (2) butyl and 2-ethyl-2,2-dimethyl-(3-sulfopropyl)ammonium hydroxide substituents.

Figure 3**.** AFM images of the positive control measurements (10μm x 10μm) using gold nanoparticles (A), peroxidase enhance silver deposition after 5 min (B), and peroxidase enhance silver deposition after 5 min on a BMA-based non-reactive DNA-chip (C). DNA probe concentration was 10 mM.

Figure 4. Comparison between the calibration plots using Horseradish peroxidase-based (HRP) DNA detection (i.e. transluminiscence) at different incubation times, FITC-based (i.e. fluorescence), and MALDI-TOF/MS DNA detection. 5 min HRP incubation (♦), 3 min HRP incubation (•), 1 min HRP incubation (■), FITC (▲), MALDI-TOF/MS (▼). (B) Reproducibility of the Horseradish peroxidase-based DNA detection in different environment: non-background (▲), human placenta DNA purified extract (♦), *E. coli* cell lysate (■) using 2 min incubation time.

Figure 5. Reliability and validity of the CMV DNA detection by using two complementary read-out techniques (AFM and MALDI-TOF/MS, **B** & **C**, respectively). The comparison between different probe sequences toward a common 30 base long sequence (30mer) is demonstrated for the wells 2-5: non-complementary (NC), and 30 base ssDNA probes with 3-mismatches (3mis), 1-mismatch (1mis), and the complementary sequence (0mis). The comparison between the analyses of different length target ssDNA samples with similar sequence toward the same probe (0mis) is demonstrated for the wells 5-7 using 30-bases (30mer), 20-bases (20mer) and 40-bases long ssDNA (40mer),

respectively. Positive control (B-0mis for AFM and 30mer for MALDI), and blank signals are observed in wells 1 and 8, respectively. Roman numeration (**D**) represent MALDI-TOF/MS spectra of the not-bound target DNA from the respective immobilization step for the respective Arabic-numbered well; "+" represents $[M+H-5A]^+$ fragment ions of the DNA.

Figures

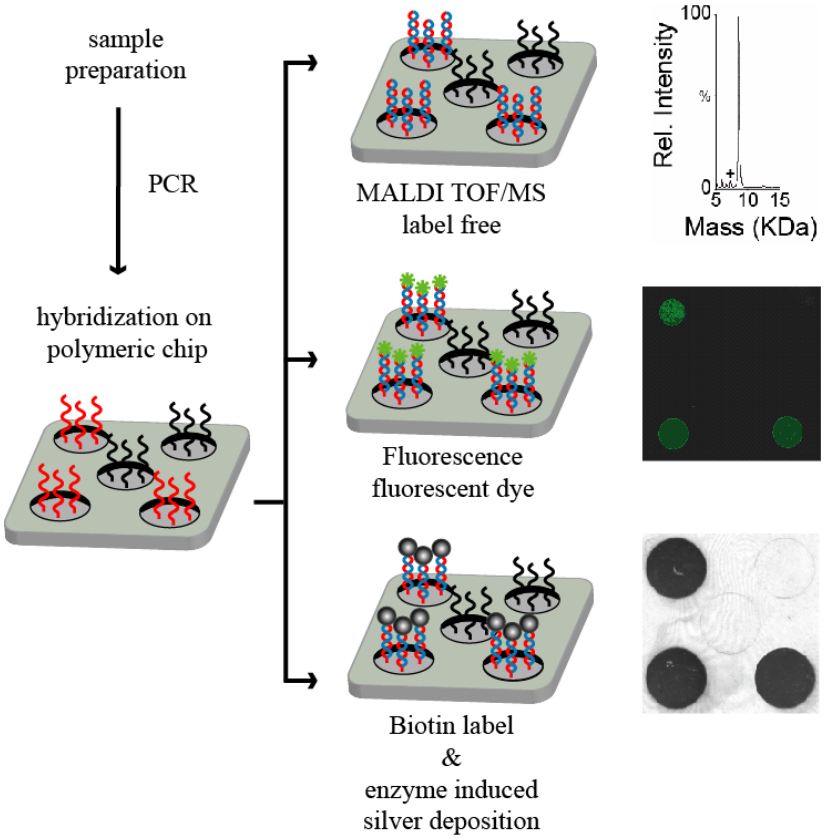
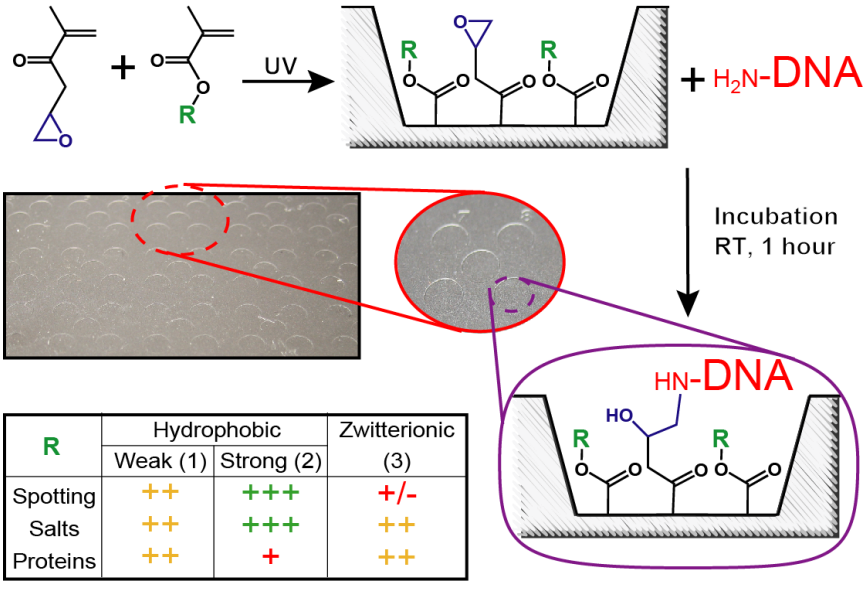


Figure 1.



R	Hydrophobic		Zwitterionic
	Weak (1)	Strong (2)	(3)
Spotting	++	+++	+/-
Salts	++	+++	++
Proteins	++	+	++

Figure 2.

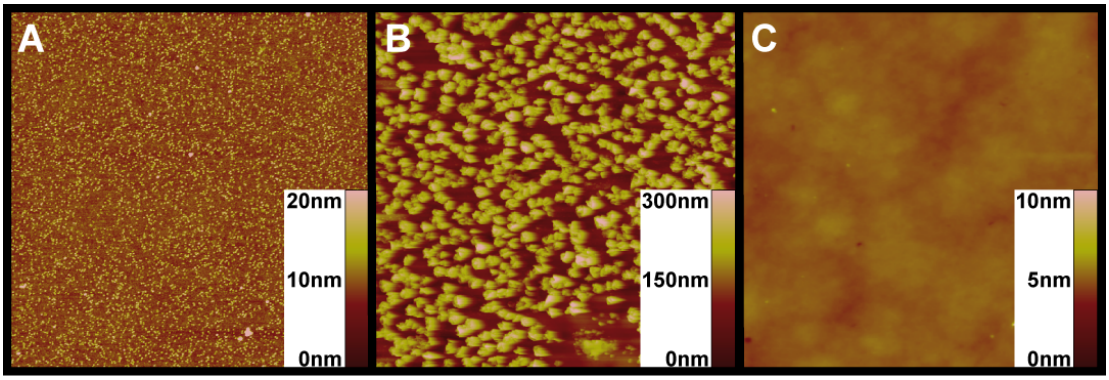


Figure 3.

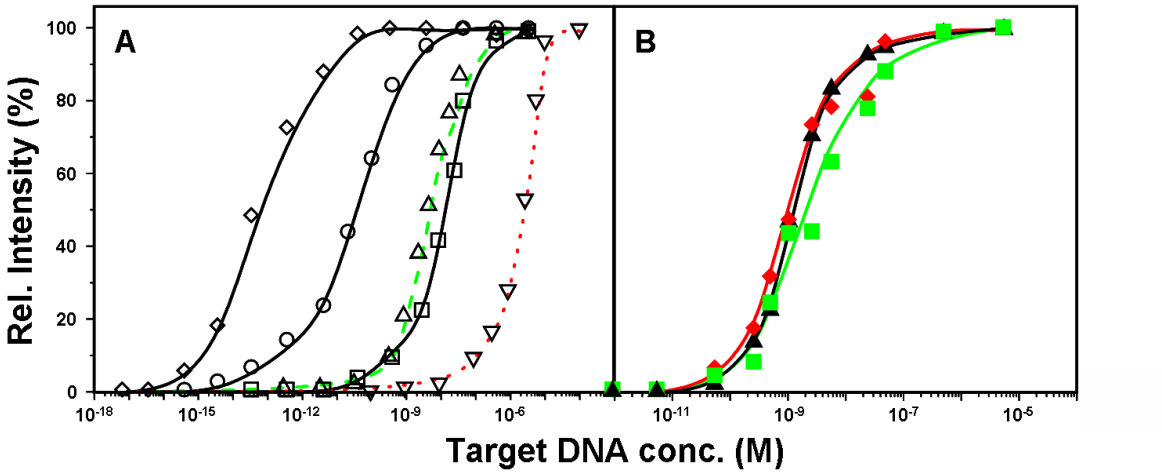


Figure 4.

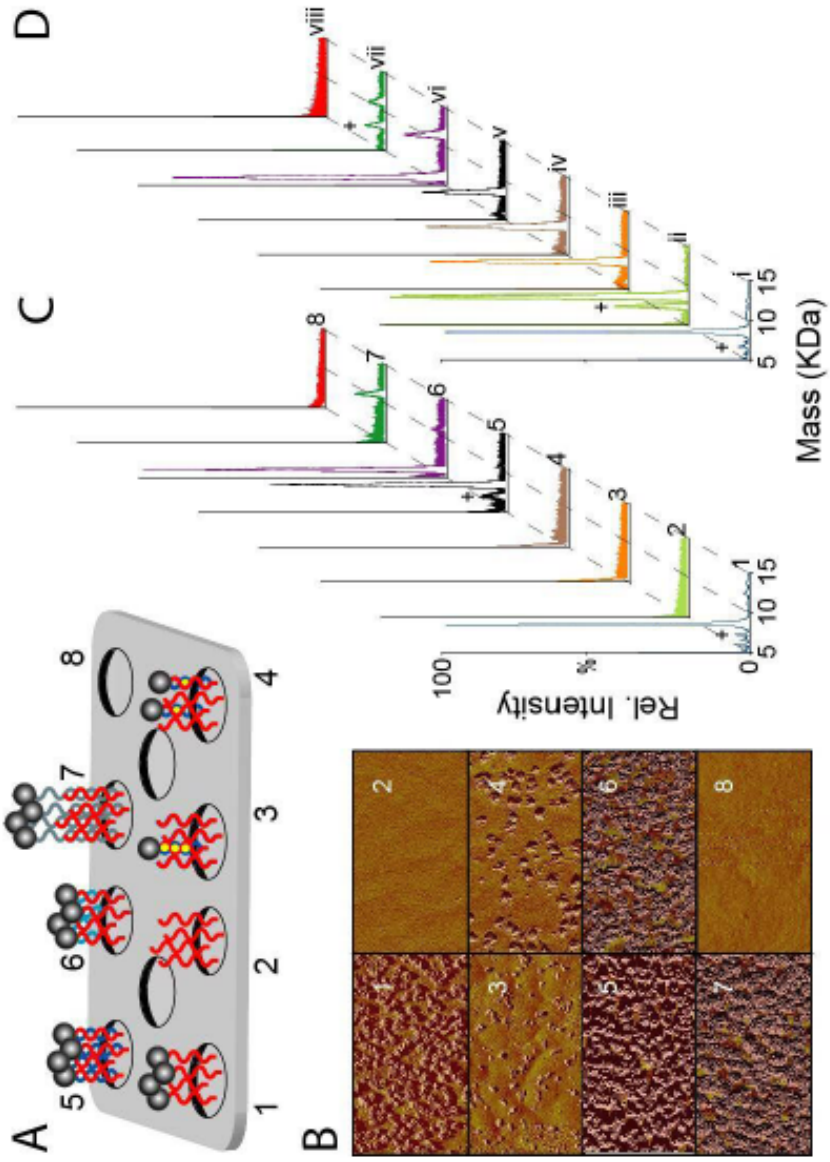


Figure 5.

2.6 Manuscript VI

A dual fluorescent/MALDI chip platform for the rapid and specific analysis of enzymatic activity and protein profiles

Vincentius A. Halim, Alexander Muck, Markus Hartl, Alfredo J. Ibáñez, Ashok Giri, Florian Erfuth, Ian T. Baldwin, and Aleš Svatoš

Molecular & Cellular Proteomics, **submitted**

The complete mapping of several genomes has given us a major tool with which to study the processes occurring inside of the organism. Nevertheless, our understanding of these processes is far from complete. For example, proteases have been identified in organisms whose genomes have been fully sequenced; however, knowledge about their activity is lacking, making their categorization very difficult if not impossible. Therefore, the ability to perform analysis of protein activity quickly and easily is becoming increasingly important.

The workhorse in industry for the identification of enzyme inhibitors as well as other screening assays has been fluorescence detection, due to its high-throughput (protein array format), excellent reproducibility, and sensitivity. However, the concentration of some enzyme inhibitors is lower than the detection limit offered by fluorescence, thus, new methods have to be developed using the original assay conditions for fluorescence.

This manuscript describes the development of a dual fluorescent/MALDI-MS read-out system for the analysis of plant protease inhibitors (PIs), which are part of the plant defense mechanism of *Solanum nigrum* against herbivores.

Dr. Vincentius A. Halim performed the majority of the presented experiments. Under the supervision of Dr. Aleš Svatoš I contributed by assisting in chip fabrication. Together, we shared information in the development of the digestion system. Dr. Alexander Muck provided information about the MALDI instrument. Quantification of protein adsorption on the chip was done using a fluorescence scanner that was assembled by Dr. Florian Erfurt. Markus Hartl and Dr. Ashok Giri under the supervision of Prof. Dr. Ian T. Baldwin were our collaborators; they established the biological system and supplied the extracted samples, providing valuable information on protein inhibitors processing and statistic analysis.

A Dual Fluorescent/MALDI Chip Platform for the Rapid and Specific Analysis of Enzymatic Activity and Protein Profiles

Vincentius A. Halim+, Alexander Muck+, Markus Hartl‡, Alfredo J. Ibáñez+, Ashok Giri‡,#, Florian Erfurth±, Ian T. Baldwin‡, and Aleš Svatoš +*

Mass Spectrometry Research Group+ and Department of Molecular Ecology‡, Max Planck Institute for Chemical Ecology, Hans-Knöll-Str. 8, D-07745 Jena, Germany; Association for Medical, Biological and Environmental Technologies, Department of Photonics and Sensors±, Felsbachstraße 7, D-07745 Jena, Germany; #present address: Division of Biochemical Sciences, National Chemical Laboratory, Pune, 411008, MS, India

Running Title: Fluorescence/pMALDI-TOF-MS Chip for Protein Function Analysis

* Corresponding Author, E-mail: svatos@ice.mpg.de
Tel. + 49 3641 571700, Fax. + 49 3641 571701

Abbreviations
MALDI: matrix-assisted laser desorption/ionization
ESI: electrospray ionization
TOF: time of flight
MS: mass spectrometer/ spectrometry
pMALDI: polymer MALDI target
SELDI: surface-enhanced laser desorption/ionization
BSA: bovine serum albumin
CAM-BSA: carbamidomethylated bovine serum albumin
PMF: peptide mass fingerprint
PIs: protease inhibitors
PIN2: protease inhibitor 2
MeJA: methyl jasmonate
*Sn*MeJA: extract from MeJA-treated *Solanum nigrum*
*Sn*irPIN2: transgenic *Solanum nigrum* expressing inverted repeat of the *PIN2b* gene
*Sn*irPIN2MeJA: extract from MeJA-treated transgenic *Solanum nigrum* expressing inverted repeat of the *PIN2b* gene
NCBI: National Center for Biotechnology Information
Kd: dissociation constant

SUMMARY

A fast and specific method of analyzing enzymatic activity using fluorescence to detect enzymatic products on copolymer pMALDI target array chips combined with analysis of specific products by MALDI-TOF-MS has been developed. The method has been used to analyze the activity of low amounts of plant protease inhibitor, components of plant defense against herbivores and pathogens, and their profiles. Extracts of PIs from *Solanum nigrum* and trypsin were co-applied to a pMALDI chip on which carbamidomethylated bovine serum albumin was adsorbed as substrate. Fluorescamine, which reacts with proteolytically released primary amine groups, was subsequently applied to rapidly detect enzyme activity by fluorescence. Reflectron MALDI-TOF-MS measurements yield information about the sequence specificity of various proteases. To analyze protein profiles, the same extracts were applied to the hydrophobic surface of the array without BSA substrate and analyzed by linear MALDI-TOF-MS. Comparing protein profiles and their activity deepens our understanding of the protease inhibition processes of *S. nigrum* on the expression level.

Keywords: enzyme activity assay, protein profiling, pMALDI chip, fluorescamine, *Solanum nigrum,* fluorescence screening, potato inhibitor II, PI-II

INTRODUCTION

Activity assays of proteins. Currently, the identification of proteins depends heavily on databases that contain mostly genomic data (1). Homology-based searches have enabled proteins from non-sequenced organisms (2) to be identified; however, accurate identification still depends on biochemical confirmation of proteins, their localization in cells, and biochemical activity (3). Biochemical activity can be measured using 1) a library of active site specific suicide inhibitors combined with LC/MS/MS analysis (4), 2) a combination of microtiter-plate enzymatic assays with fluorescence read-outs (5) for enzymatic product detection, or 3) mass spectrometry (MS) analysis (6), which is especially suitable for protease assays (7). Recently, the fluorescence-based detection of renin and angiotensins using high density antibody arrays and the mass spectrometric analysis of the bound peptides were integrated on one platform (8). Our method builds on this concept, combining fluorescence detection with MALDI-TOF-MS detection of enzymatic products on the polymeric array chip bearing protein adsorption properties using native substrates, which do not need to be labelled with fluorophore groups. The assay developed for detecting protease activity and plant derived protease inhibitors (PIs) can be readily extended for a broad range of enzymatic reactions. The multi-well platform enables us to perform more enzymatic reactions as well as more replicates, as with it both the fluorescence detection analysis and the MALDI-TOF-MS analyses can be conducted rapidly.

pMALDI chips. Matrix-assisted laser desorption/ionization (MALDI) is a soft ionization technique in mass spectrometry that enables intact macromolecules to be analyzed. Combined with time-of-flight (TOF) analyzers, which provide a high degree of mass accuracy and permit large-scale analyses, MALDI-TOF-MS is a powerful tool for analyzing proteins and peptides (9). Moreover, compared to electrospray ionization (ESI), MALDI is more tolerant of the presence of salt, which is often found in protein samples due to the buffers commonly used during protein extraction. These favorable properties have made MALDI TOF-MS platforms increasingly interesting to researchers. Important

developments such as surface-enhanced laser desorption/ionization (SELDI) (10, 11) and the use of hydrophilic anchors on hydrophobic surfaces (Anchor Chips) (12) to enhance signals or desalt samples highlight the importance of MALDI target materials for such mass spectral analysis. Our group has recently introduced new disposable poly(alkyl methacrylate-co methyl methacrylate) copolymer MALDI targets (pMALDI chips) with tunable hydrophobicity that are prepared according to a fast atmospheric pressure molding protocol (13). In the facile fabrication procedures of these chips, material properties can be modified by introducing different physicochemical characteristics to monomers of choice, rather than using multistep surface modification protocols. The versatility of this approach has been further demonstrated in ionic (14, 15) and enzyme-linked pMALDI chip arrays (16).

Protease activity assay. Like the activity of other enzymes, the activity of proteases can be analyzed by measuring substrate depletion and/or product formation. Spectrophotometric and titrometric approaches are used to quantify protease activity (17). Fluorescamine has been used to detect primary amines, amino acids, peptides, and proteins (18-21), offering fast reactivity, high fluorescence upon binding, and high sensitivity. Its fluorescence can be measured with particular excitation (λex) and emission (λem) wavelengths (18). Because this reagent can covalently bind the primary amines of proteins as well as of peptides and amino acids, it can also be used to detect protease activity. Theoretically, complete digestion of a single carbamidomethylated bovine serum albumin (CAM-BSA) molecule by trypsin results in an accumulation of 82 peptides and amino acids that leads to a corresponding increase of fluorescence signals. As fluorescent compounds also absorb UV light, UV detection can also theoretically be used in such an assay. Although spectrophotometric detection is an established method for conducting enzyme activity assays, some errors related to the use of artificial substrates and the presence of unwanted contaminants have been reported (22). Mass spectrometry offers specificity by providing the molecular ion masses of substrates or products in enzymatic reactions, and some enzymatic assays using ESI-MS and MALDI TOF-MS have been reported (23, 24).

Plant protease inhibitors and their analysis. Inhibitors of proteolytic activity (PIs) play an important role in plant metabolism and defense mechanisms against herbivores and pathogens (25). These typically small proteins inhibit various proteolytic enzymes of the plant itself and of herbivores and invading bacteria. Solanaceous plants are particularly rich in PIs and a large number of them have been found in important model plant systems such as potato, tomato, and tobacco (25, 26). PIs are constitutively present in various organs and tissues, e.g. flowers, vascular bundles, and tubers, and their production can also be induced by wounding or herbivore and pathogen challenge in leaves (25, 27, 28). The best-studied example of such induced PIs are the members of the potato-inhibitor-II class (PI-II or PIN2). Many PIN2 inhibitors are generated after cleavage of a circular multidomain pre-protein (29) as a single gene codes for a number of structurally very similar, active PIs, that can differ in their biochemical and inhibitory properties. Additionally, the processing pattern of the preprotein can change under certain circumstances (e.g. elicitation by methyl jasmonate - MeJA), which might lead to an even higher number of iso-inhibitors (30). To better understand the role of PIs and their various isoforms for the physiology and ecology of a plant, powerful qualitative and quantitative methods are needed. Several methods for analyzing PIs are already available (17). Direct analysis, such as radial immunoassay, has been performed with specific antibodies against

protease inhibitors (31). The co-application of a protease with crude or gel-separated PI-containing protein extracts with a suitable substrate offers a way to carry out indirect analyses (17, 32). However, both approaches suffer from either a long analysis time or low specificity.

Here we present a rapid, specific activity assay for plant PIs using a general sample pretreatment/enzyme reaction platform that enables direct fluorescence and MALDI-TOF MS analysis. A Solanaceous relative of potato and tomato, *Solanum nigrum*, is used as a model plant ecological expression system (33). As demonstrated, our assay platform can be used for rapid activity screening by fluorescence which is readily performed by a simple UV transluminator; meanwhile the samples of particular interest can be further analyzed using MALDI-TOF-MS. The small assay platform greatly reduces sample consumption.

EXPERIMENTAL PROCEDURES

Materials. The copolymeric matrix-assisted laser desorption/ionization array chip (pMALDI chip) was prepared from butyl- and methyl methacrylate (Polysciences, Warrington, PA, USA). This copolymer had a monomer ratio of 9:1 v/v, respectively. The chips were prepared by the atmospheric molding fabrication protocol. Details of chip fabrication were provided earlier (13). The substrate was prepared as follows. Approximately 10 nmol BSA fraction V (Sigma, St. Louis, MO, USA) were dissolved in 100 µl of 25 mM ammonium bicarbonate buffer and reduced by adding 100 µl of 10 mM dithiothreitol (Carl Roth, Karlsruhe, Germany). The solution was incubated for 1 h at 56°C at 350 rpm. The reduced BSA was then alkylated by adding 400 µl of 55 mM iodoacetamide (Sigma). The solution was incubated at 25°C and 350 rpm in the dark. After 45 min, the reaction was terminated and the protein was precipitated by adding 600 µl of 3.05 N trichloroacetic acid (TCA) (Sigma). This solution was mixed and kept on ice for 2 h. After centrifugation at 16000 × g for 15 min to collect the precipitated protein, the pellet was washed with 1 ml water. The clean pellet of carbamidomethylated BSA (CAM-BSA) was resuspended in 100 µl water or 100 µl 25% HPLC grade acetonitrile (Sigma). Half pmol sequencing grade trypsin (Promega, Madison, WI, USA) was dissolved in 50 mM ammonium bicarbonate buffer for digestion. Soybean trypsin inhibitor (M.W. 20000) and aprotinin (M.W. 6512) (both Merck-Calbiochem, Darmstadt, Germany) were also prepared in 50 mM ammonium bicarbonate buffer. Fluorescamine [4-phenylspiro(furan-2(3H),1-phthalan)-3,3'-dion] was purchased from Fluka under the commercial name Fluram. Fluorescamine is not soluble in water but can be dissolved in ethanol. For the chip application, fluorescamine was dissolved in ethanol (1:100 w/v) with identical amounts (1:1 v/v) of glycerol added to increase viscosity.

Protein adsorption and quantification. Two microliters of CAM-BSA were applied in 2×2 mm square, 50 µm deep wells by 25× expiring and aspiring the protein solution from a 10 µl plastic pipette tip to enhance protein contact with the chip surface. The CAM-BSA solution was incubated at room temperature for 3 minutes and then removed. The well was overlaid by 3 µl water, followed by 3× expiration and aspiration to remove the non-adsorbed CAM-BSA and other possible contaminants. A further aliquot of 3 µl water was applied in the well and left for 3 minutes at room temperature. The water was removed before quantification or digestion. To quantify the adsorbed protein, 1 µl fluorescamine solution 0.5% (w/v) was applied and incubated for 10 minutes at room temperature in a closed plastic box. The sample was then analyzed by a fluorescence scanner consist of the imaging spectrograph ImSpector (Specim, Oulu, Finland) and the high-sensitivity iXon

camera (Andor, Belfast, Northern Ireland) (34, 35) (λex=376 nm, λem= 490 nm). Calibration curves were measured from serial dilutions of 0.1 pmol to 100 pmol BSA.

Protein digestion and inhibition. The pMALDI chip platform was further used for *in situ* digestion. One pmol trypsin in 2 µl 50 mM ammonium bicarbonate (pH 7.8) was applied to the adsorbed CAM-BSA. The reaction was performed inside a small plastic humidity chamber which was kept at 37 °C in an incubator for 18 h (16). Efficient digestion could be achieved in just 1h by adding 1 mM CaCl2 to the digestion buffer. Complete inhibition was achieved by adding 20 pmol standard inhibitor to 1 pmol trypsin solution, incubating the mixture for 1 min. and applying it to the substrates on the chip.

Plant growth and treatments. We used the *Solanum nigrum* L. inbred line *Sn*30 (33) in all our experiments as a wild-type control. Transgenic lines silenced in PIN2-expression were generated through *Agrobacterium tumefaciens*-mediated stable transformation with an inverted-repeat construct of the *SnPIN2b*-gene (36). Plants were grown as described before (37) and used for experiments 4-5 weeks after planting. For methyl jasmonate treatment 150 µg of MeJA in 20 µL lanolin (both Sigma-Aldrich) were applied to the base of the leaf and petiole of the youngest fully expanded leaf. Leaves of control plants were treated with 20 µL lanolin only. The treated leaves were harvested three days after MeJA application, the parts carrying lanolin were removed and the remaining tissue immediately frozen in liquid nitrogen.

Plant protease inhibitor extraction and purification. A plant protease inhibitor extraction procedure was adapted from the literature (38). Leaf samples were ground in liquid nitrogen, weighed, and transferred to Eppendorf tubes. For each experiment and treatment equal amounts of leaf tissue from three plants were pooled into one replicate. Two hundred mg of this pooled ground leaf material was extracted using 0.4 ml extraction buffer consisting of 0.1 M Tris-Cl buffer (pH 7.6) containing polyvinylpolypyrrolidone, 2 mg.ml-1 phenyl thiourea, 5 mg.ml-1 diethyldithiocarbamate, and 0.05 M Na2EDTA (all from Sigma). The extraction buffer partially prevented the phenolic oxidation of the proteins (38). The sample was centrifuged immediately at 16000×g for 30 min. at 4°C, and the supernatant was transferred to a new plastic tube. The extract was then heat-treated for 30 min. at 60°C to denature and precipitate heat-labile proteins. After centrifugation at maximum speed (16000×g) at 4°C, the supernatant was dialyzed in benzoylated cellulose tubing with 1.2 kDa cut off (Sigma) overnight in 0.2 % TFA at 4°C. Protein concentration of the extract was measured by the Bradford assay (39). For the fluorescence-based activity assays, 1 µl of crude extract was applied to the well of the pMALDI chip and rinsed with 2 µl deionized (Milli-Q grade) water. The adsorbed proteins were then recovered from the surface by 2 µl solution of 0.1% (v/v) TFA and acetonitrile (3:2, v/v). The PIs from 3 µl crude extract were combined, vacuum dried, mixed with trypsin in the digestion buffer, incubated for 1 min, and applied to the substrate. This additional step was not needed for the MALDI-TOF-MS based activity assay.

Fluorescence and MALDI-TOF-MS analysis. Fluorescamine was applied after digestion was terminated. 0.5% (w/v) fluorescamine was dissolved in a mixture of ethanol and glycerol (1/1, v/v) and 1 µl was applied on the chip well. The sample was analyzed 10 min. after application by a transluminator with a UV lamp and CCD camera (Biometra, Goettingen, Germany). Data were processed with BioDoc Analyze 1.0 software. The peptide spectra were acquired using a MALDI-TOF mass spectrometer (TofSpec2E;

Micromass, Manchester, UK) operated in reflectron mode. Linear mode measurements were used for protein analysis. The desorption/ionization was accomplished by a nitrogen UV laser (337 nm, 4 ns pulses of max. 180 μJ). The positive ions were subjected to 20 kV accelerating potential and measured by a micro channel plate (MCP) detector at 1900 V, and 3600 V for reflectron and linear mode, respectively. Matrix ions were suppressed using low-mass cut-off *m/z* 500 for peptides and *m/z* 2000 for proteins analysis. For each mass spectrum, 15 laser shots were accumulated; the displayed spectra are averages from 17 consecutive automatic acquisitions with an octagonal pattern covering the whole area of the chip wells. The absolute signal intensity (total ion count) was used after smoothing and baseline subtraction for quantitative evaluations. The spectra were calibrated using calculated mono-isotopic masses of [M+H]+ peaks where needed. The data were recorded on a PC workstation running MassLynx 3.4 software (Micromass, Manchester, U.K.).

Statistic analysis. SPSS 15.0 was used to apply a mixed-effect model to the fluorescence assay data in Figure 5 with "treatment" as a fixed and "experiment" as random factor. The Tukey's test was used as a post-hoc analysis.

RESULTS

Reduction and alkylation reactions of BSA were performed in order to open the protein structure and facilitate the substrate's adsorption and digestion. The degree of reduction/ alkylation was confirmed by MALDI-TOF-MS and Q-TOF-MS analysis of BSA and CAM-BSA. As shown in the insert of Figure 1, there is a 1.96±0.03 kDa mass difference between CAM-BSA and BSA, which correlates with all 35 residues of cysteine in BSA. Importantly, peptide mass fingerprint (PMF) analysis of trypsin-digested CAM-BSA showed increased peptide coverage: 31 tryptic peptides compared to 8 for trypsin-digested BSA. Figure 1 also shows the possible increase of adsorption after alkylation. That the MS signal intensity of CAM-BSA is more than the intensity of BSA on the chip after adsorption indicates more adsorption. To quantify the actual adsorption of CAM-BSA on the surface, fluorescence measurements using fluorescamine were used. This approach modified an existing method (20) that permits proteins in the nanogram range to be analyzed. Upon application, non-fluorescent fluorescamine covalently binds to primary amines and acquires fluorescence properties. This protein analysis method has pmol sensitivity and a wide dynamic range. Analysis of BSA protein on the chip ranging from 0.1 to 100 pmol demonstrated the linearity of the method (Fig. 2, top). Figure 2 (bottom) shows that 34 pmol CAM-BSA was recovered after reduction, alkylation, and purification of 100 pmol of BSA. The low recovery after alkylation could be attributed to the difficulty of resuspending CAM BSA after TCA precipitation. Adding 25% acetonitrile improved the resuspension of CAM BSA. When 34 pmol of CAM-BSA is applied on the surface, approximately 5 pmol is adsorbed. In spite of the overall adsorption capacity of proteins, the reproducible adsorption of the CAM-BSA on the surface enables consistent experiments to be performed.

We examined the use of fluorescamine for detecting protease activity due to its reactivity with primary amines. Theoretically, a single CAM-BSA molecule should be cut by trypsin at 81 sites, resulting in 82 peptides and amino acids. Therefore protease activity increases the amount of primary amines available and increases the fluorescence signal. Considering the need for fast and continuous activity analysis, a UV transluminator instead of the fluorescence scanner was used. Despite its lower sensitivity and selectivity, the UV transluminator was able to screen for enzyme activity. Higher sensitivity and selectivity of analysis can be attained by equipping the CCD camera with a filter transmitting specific

wavelengths. Figure 3 (top) shows that the trypsin activity on CAM-BSA on the surface can be detected after adding fluorescamine and exposing the chip to UV radiation. To confirm the activity, a similar set of digestion experiments was performed and analyzed using MALDI TOF-MS in reflectron mode. Peptide accumulation in MS analysis as shown in Figure 3 (bottom) indicates the process of enzymatic digestion. The specific peptides derived from CAM-BSA were confirmed by a BLAST PMF comparison of the spectrum list to Swiss-Prot database (v 51.3) using MASCOT software (http://www.matrixscience.com/, Matrix Science, Boston, MA, USA). Significant matching to bovine BSA (*Bos taurus*) was achieved as the first hit had a MOWSE score 75 ($p=0.0017$). Both fluorescence and MALDI-TOF-MS approaches can thus reveal enzymatic digestion, and each has its advantages. While fluorescence analysis can be done very rapidly, MS analysis can provide profiles of peptides. Therefore, such a combination of fluorescence and MS analysis offers a useful approach for obtaining information about the activity of the enzymes studied together with detailed information about enzyme reaction products and specificity.

The technique was also used to study model PIs, specifically, soybean trypsin inhibitor and aprotinin. Soybean trypsin inhibitor is a trypsin inhibitor with Kd = 0.5 μM and specific activity ≥ 5000 units/mg protein. Aprotinin is a serine protease inhibitor which inhibits trypsin with Kd = 0.05 pM. Fluorescent signal intensity after the co-application of 20 pmol of either trypsin soybean inhibitor or aprotinin was lower than the intensity that characterizes trypsin digestion without protease inhibitor (Fig. 4, top panel). This was confirmed with MALDI-TOF-MS analysis as shown in Figure 4 (bottom). Additionally, at lower concentrations, namely 2, 5, and 10 pmol, we observed different levels of inhibition by trypsin soybean inhibitor and aprotinin, indicated by the lower fluorescent signal that characterizes digestion when the inhibitor is co-applied (data not shown).

The following experiments demonstrated the utility of this technique to answer biological questions. We used leaf tissue from two genotypes of *S. nigrum*: a wild-type line and a transgenic line, silenced in PIN2 expression. While untreated leaves show very low amounts of PIN2, the application of MeJA dramatically increases amounts of PIN2, this increase is suppressed in the transgenic line. Simple extraction procedures followed by heat treatment enrich the PIs in the protein extract because of their small size and relative heat resistance compared to other proteins. Dialysis was performed to remove excess salts and small-molecule contaminants. However, the dialysis step is optional since similar results could be obtained without it (data not shown). Further clean-up was done on the surface of the chip: the relatively clean sample on the chip surface was recovered and coapplied with trypsin to the adsorbed CAM-BSA. Figure 5 shows reduced fluorescent signal intensity compared to the intensity seen during standard digestion, when extracts from MeJA induced wild-type and transgenic plants were co-applied with trypsin before digestion. To obtain more information on protease inhibitors, we conducted a larger experiment with control and MeJA-induced samples from both genotypes. Crude extracts that had not been cleaned up further were co-applied with the trypsin-mediated digestion of CAM-BSA and analyzed using MALDI-TOF-MS in reflectron mode. No specific peptides were detected in extracts of MeJA-treated wild-type plants (Fig. 6B) or in MeJA-treated transgenic plants (Fig. 6D). Finally, the highest accumulation of peptides was found in controls, non-induced transgenic plants (Fig. 6C), that contain very low level of PIs. To determine if the inhibition of digestion correlated with PI accumulation, protein profiles in the extracts of plant samples were analyzed. The protein extracts were mixed with sinapinic acid matrix, dried, and analyzed with MALDI-TOF-MS in linear mode. Figure 7 shows the PI protein profile. MeJA was able to induce wild-type plants to accumulate

some inhibitory proteins (Fig. 7B). Small amounts of those proteins also accumulated in MeJA-treated transgenic plants (Fig. 7D). Higher accumulations of these inhibitor proteins (Fig. 7) negatively correlated with lower levels of digestion (Fig. 6), using an exponential decay of correlation function (y=3442.1e**-0.0085x**, R2 = 0.9734). The ability to provide information about the molecular mass of these proteins (with ca 50 Da accuracy) is a valuable feature of our system. The cDNA derived protein sequence of the *Sn*PIN2b precursor is available in the NCBI database (NCBI accession number AAR37362) (Fig. 8C). This sequence is highly similar to the SlPIN2 sequence from tomato (NCBI accession number BAC76901). Based on available sequence information and its possible post-translational processing, PI profiles of MeJA-treated wild type plant were analyzed (Fig. 8A, B). Using the molecular mass information from these databases, we found native as well as processed proteins of PI precursors in the profiles. Further protein sequencing is needed to confirm these findings.

DISCUSSION

Assigning protein function to poorly annotated gene products and correlating them with MS sequence data could fill the gap between sequence information in the protein database and protein function. However, the functional analysis of proteins with enzymatic activity or with inhibitor activity requires several steps, including purification, activity testing, and identification. In this study, we developed a rapid and specific method to analyze the effect of inhibitors on a protease, using PIs from *Solanum nigrum* as a model. Trypsin and BSA were chosen as the enzyme-substrate system in our experiment due to their frequent use in studies of enzymatic digestion and their commercial availability. Moreover, trypsin is a serine-type protease which occurs naturally in the insect gut (25, 40).

Several advantages of using MALDI-TOF-MS for an enzymatic activity assay include accuracy, sensitivity, and high throughput. The soft ionization of MALDI combined with the ability to perform wide-ranging mass analyses that TOF offers enable substrates, products, as well as intact proteins to be investigated. MALDI-TOF-MS is also relatively tolerant of salts, which often appear in protein extracts after sample preparation and purification. The pMALDI chip previously developed in our group (13) was used as an enzyme assay platform. The chip is a MALDI target from copolymer material (13, 15) enabling simple sample clean-up based on a tailored composition of the polymer without the need for surface modification such as in SELDI technologies (10, 11). Such pMALDI chips, which are transparent to UV light, allow us to combine the pMALDI-TOF-MS assay with fluorescence assay (Fig. 3) and permit rapid, quantitative analyses of enzymatic reactions and the ability to measure enzymatic reactions in the presence of inhibitors (Fig. 4).

It is interesting to compare this method with common practices in PIs analysis. Radial immunoassay (31) and spectrophotometry assay (17) are commonly used to analyze PI activity. For every radial immunoassay, optimal antibodies must be produced. Possible errors and artifacts in spectrophotometer-based activity tests due to the use of artificial substrates, as well as unwanted contaminants in complex matrices, have been reported (22, 23). The newly developed method is very amenable to high-throughput analysis because an optimal digestion in a small volume could be achieved within 1 h on the pMALDI chip, due to the short fluorescence reactions, the analysis could be conducted in only a few minutes. Moreover, the use of MALDI-TOF-MS to analyze specific reaction products (Fig. 6) prevented erroneous conclusions from being drawn. Consecutive automatic acquisition

of MALDI spectra with an octagonal pattern covering the entire well area of the chip allowed the data to be analyzed in a semi-quantitative way. The lack of specific peptides in the MALDI well after co-applying MeJA-treated wild-type plant extracts to the trypsin digestion demonstrate that MeJA elicited PIs in wild-type plants inhibit the activity of trypsin. The low amounts of specific peptides detected after co-applying MeJA-treated transgenic plant extracts to the trypsin digestion suggest that the transgenic plants were still able to produce serine-PIs, despite being silenced in their expression of the *PIN2* gene. This can be explained by the great diversity of PIs produced in Solanaceous plants (25, 41), which would not be reduced if only a single *PIN2* gene was silenced. Moreover, RNAi-based silencing usually does not completely silence gene expression, so that a residual amount of PIN2 inhibitors in the transgenic plant is to be expected. Given the high sensitivity of MALDI-TOF-MS, this residual activity would likely be detected. Inhibiting digestion by introducing extracts from MeJA-treated plants correlated with the presence of small proteins in the extract. Such correlations are normally difficult to make due to the low sensitivity (e.g. coomassie blue) and staining discrimination (e.g. silver staining) of protein analysis methods. We inferred that these small proteins were PIs from their inducibility with MeJA and their disappearance or reduction in transgenic plants silenced in *PIN2* expression. The measured molecular masses of these small proteins (Figure 8A,B) roughly matched to masses of pre-protein and different fragments of the *Sn*PIN2b calculated from cDNA-derived inhibitor protein sequence (Figure 8C), assuming a post-translational processing similar to a previous report (29). However, as the details of the actual processing of *Sn*PIN2 are not known, the discrepancies between calculated and observed masses are not surprising. Thus further *de novo* sequencing is needed to confirm the actual structure of the proteins that are elicited by MeJA treatment and do not occur in extracts of PIN2-silenced plants. Together, these results demonstrate that the rapid, specific enzymatic assay on the pMALDI chip platform is a powerful tool for functionally analyzing plant protease inhibitors. This technique could be easily adapted to analyze different enzymes or different substrates.

Acknowledgments

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References

(1) Aebersold, R., and Mann, M. (2003) Mass spectrometry-based proteomics. *Nature* 422, 198-207.

(2) Shevchenko, A., Sunyaev, S., Loboda, A., Shevchenko, A., Bork, P., Ens, W., and Standing, K. G. (2001) Charting the proteomes of organisms with unsequenced genomes by MALDI-quadrupole time-of-flight mass spectrometry and BLAST homology searching. *Anal Chem* 73, 1917-26.

(3) Saghatelian, A., and Cravatt, B. F. (2005) Assignment of protein function in the postgenomic era. *Nat Chem Biol* 1, 130-42.

(4) Sieber, S. A., Niessen, S., Hoover, H. S., and Cravatt, B. F. (2006) Proteomic profiling of metalloprotease activities with cocktails of active-site probes. *Nat Chem Biol* 2, 274-81.

(5) Babiak, P., and Reymond, J. L. (2005) A high-throughput, low-volume enzyme assay on solid support. *Anal Chem* 77, 373-7.

(6) Min, D. H., Yeo, W. S., and Mrksich, M. (2004) A method for connecting solution phase enzyme activity assays with immobilized format analysis by mass spectrometry. *Anal Chem* 76, 3923-9.
(7) Schluter, H., Jankowski, J., Rykl, J., Thiemann, J., Belgardt, S., Zidek, W., Wittmann, B., and Pohl, T. (2003) Detection of protease activities with the mass-spectrometry assisted enzyme-screening (MES) system. *Anal Bioanal Chem* 377, 1102-7.
(8) Finnskog, D., Ressine, A., Laurell, T., and Marko-Varga, G. (2004) Integrated protein microchip assay with dual fluorescent- and MALDI read-out. *J Proteome Res* 3, 988-94.
(9) Karas, M., and Hillenkamp, F. (1988) Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. *Anal Chem* 60, 2299-301.
(10) Hutchens, T. a. Y., TT. (2002), Retentate Chromatography and Protein Chip Arrays with Application in Biology and Medicine. Patent 0123043 A1.
(11) Weinberger, S. R., Dalmasso, E. A., and Fung, E. T. (2002) Current achievements using ProteinChip Array technology. *Current Opinion in Chemical Biology* 6, 86-91.
(12) Schurenberg, M. a. F., J. (2002), Structured Biosample Support Plates for Mass Spectroscopic Analyses and Procedures for Manufacturing and Use. Patent 0045270 A1.
(13) Muck, A., Nesnerova, P., Pichova, I., and Svatos, A. (2005) Fast prototyping of hydrophobic disposable polymer support arrays for matrix-assisted laser desorption/ionization-time of flight-mass spectrometry of proteins by atmospheric molding. *Electrophoresis* 26, 2835-42.
(14) Muck, A., Ibanez, A., Halim, V., Stauber, E., Mansourova, M., and Svatos, A. (2006) MALDI-TOFMS high-density polymer micro array for direct protein analysis from complex biological samples. *Molecular & Cellular Proteomics* 5, S151-S151.
(15) Muck, A., Ibanez, A. J., Stauber, E. J., Mansourova, M., and Svatos, A. (2006) Atmospheric molding of ionic copolymer MALDI-TOF/MS arrays: a new tool for protein identification/profiling. *Electrophoresis* 27, 4952-9.
(16) Ibanez, A. J., Muck, A., Halim, V., and Svatos, A. (2007) Trypsin-Linked Copolymer MALDI Chips for Fast Protein Identification. *J Proteome Res* 6, 1183-9.
(17) Richardson, M. (1991) Seed Storage Proteins: The Enzyme Inhibitors. *Methods in Plant Biochemistry* 5, 259-305.
(18) Bohlen, P., Stein, S., Dairman, W., and Udenfriend, S. (1973) Fluorometric assay of proteins in the nanogram range. *Arch Biochem Biophys* 155, 213-20.
(19) Stein, S., Bohlen, P., Stone, J., Dairman, W., and Udenfriend, S. (1973) Amino acid analysis with fluorescamine at the picomole level. *Arch Biochem Biophys* 155, 202-12.
(20) Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Leimgruber, W., and Weigele, M. (1972) Fluorescamine: a reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. *Science* 178, 871-2.
(21) Weigele, M., Tengi, J. P., Leimgrub.W, and Debernar.Sl. (1972) Novel Reagent for Fluorometric Assay of Primary Amines. *Journal of the American Chemical Society* 94, 5927-5928.
(22) Kruger, N. J. (1995) Errors and artifacts in coupled spectrophotometric assays of enzyme activity. *Phytochemistry* 38, 1065-71.

(23) Bungert, D., Heinzle, E., and Tholey, A. (2004) Quantitative matrix-assisted laser desorption/ionization mass spectrometry for the determination of enzyme activities. *Anal Biochem* 326, 167-75.
(24) Bothner, B., Chavez, R., Wei, J., Strupp, C., Phung, Q., Schneemann, A., and Siuzdak, G. (2000) Monitoring enzyme catalysis with mass spectrometry. *J Biol Chem* 275, 13455-9.
(25) Ryan, C. (1990) Protease Inhibitors in Plants: Genes for Improving Defenses Against Insects and Pathogens. *Annual Reviews Phytopathology* 28, 425-449.
(26) Pearce, G., Johnson, S., and Ryan, C. A. (1993) Purification and characterization from tobacco (Nicotiana tabacum) leaves of six small, wound-inducible, proteinase isoinhibitors of the potato inhibitor II family. *Plant Physiol* 102, 639-44.
(27) Atkinson, A. H., Heath, R. L., Simpson, R. J., Clarke, A. E., and Anderson, M. A. (1993) Proteinase inhibitors in Nicotiana alata stigmas are derived from a precursor protein which is processed into five homologous inhibitors. *Plant Cell* 5, 203-13.
(28) Xu, Z. F., Qi, W. Q., Ouyang, X. Z., Yeung, E., and Chye, M. L. (2001) A proteinase inhibitor II of Solanum americanum is expressed in phloem. *Plant Mol Biol* 47, 727-38.
(29) Lee, M. C., Scanlon, M. J., Craik, D. J., and Anderson, M. A. (1999) A novel two chain proteinase inhibitor generated by circularization of a multidomain precursor protein. *Nat Struct Biol* 6, 526-30.
(30) Horn, M., Patankar, A. G., Zavala, J. A., Wu, J., Doleckova-Maresova, L., Vujtechova, M., Mares, M., and Baldwin, I. T. (2005) Differential elicitation of two processing proteases controls the processing pattern of the trypsin proteinase inhibitor precursor in Nicotiana attenuata. *Plant Physiol* 139, 375-88.
(31) Ryan, C. A. (1967) Quantitative determination of soluble cellular proteins by radial diffusion in agar gels containing antibodies. *Anal Biochem* 19, 434-40.
(32) Jongsma, M. A., Bakker, P. L., and Stiekema, W. J. (1993) Quantitative determination of serine proteinase inhibitor activity using a radial diffusion assay. *Anal Biochem* 212, 79-84.
(33) Schmidt, D. D., Kessler, A., Kessler, D., Schmidt, S., Lim, M., Gase, K., and Baldwin, I. T. (2004) Solanum nigrum: a model ecological expression system and its tools. *Mol Ecol* 13, 981-95.
(34) Erfurth, F., Schmidt, W.-D., Nyuyki, B., Fassler, D. (2006) *13th Heiligenstädter Kolloquium*, Heiligenstadt.
(35) Schmidt, W.-D., Erfurth, F. Tretyakov, A., Nyuyki, B., Mrotzek, G., Saluz, HP, Fassler, D. (2006) in *3rd International Workshop on Spectral Imaging (ECCV 2006).* 33-39, Graz / Österreich.
(36) Hartl, M., Giri, A.P., Kaur, H., Baldwin, I.T. (*manuscript in preparation*)
(37) Schmidt, S., and Baldwin, I. T. (2006) Systemin in Solanum nigrum. The tomato494 homologous polypeptide does not mediate direct defense responses. *Plant Physiol* 142, 1751-8.
(38) Jongsma, M. A. B., P.L.; Visser, B. and Stiekema, W.J. (1994) Trypsin inhibitor activity in mature tobacco and tomato plants is mainly induced locally in response to insect attact, wounding and virus infection. *Planta* 195, 29-35.
(39) Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72, 248-54.

(40) Zhu, Y. C., Liu, X., Maddur, A. A., Oppert, B., and Chen, M. S. (2005) Cloning and characterization of chymotrypsin- and trypsin-like cDNAs from the gut of the Hessian fly [Mayetiola destructor (Say)]. *Insect Biochem Mol Biol* 35, 23-32.
(41) Rawlings, N. D., Tolle, D. P., and Barrett, A. J. (2004) MEROPS: the peptidase database. *Nucleic Acids Res* 32, D160-4.

Figure Captions

Fig. 1 Effect of alkylation on the adsorption of BSA on pMALDI BMA protein chips. 100 pmol BSA was alkylated and adsorbed on the surface (CAM-BSA). For control, 100 pmol unalkylated BSA was adsorbed and signals of adsorbed BSA and CAM-BSA were compared using MALDI-TOF-MS. The error bars represent the standard deviation of the mean (n = 3).

Fig. 2 Linear CAM-BSA fluorescence assay signals on the pMALDI chip. Different amounts of BSA were spotted on the chip and fluorescamine was added. The emission fluorescence was measured at 490 nm (top) with an excitation wavelength 376 nm. The assay was used to quantify protein recovery after alkylation and adsorption (bottom). The error bars represent the standard deviation of the mean (n = 2).

Fig. 3 On-chip digestion of adsorbed CAM-BSA rapidly analyzed by fluorescent analysis (top) and by a peptide mass fingerprint using a MALDI-TOF-MS (bottom). Low signals are observed for CAM-BSA and trypsin (A and B, respectively), whereas application of trypsin to CAM-BSA for 18 h results in an increased signal (C). After optimization, 1h digestion times produced similar signal intensities (D). Numbers on the spectrum indicate typical tryptic peptides of CAM-BSA. The error bars represent the standard deviation of the mean (n = 2). The experiment was repeated with a similar result.

Fig. 4 Inhibition of protease activity by the soybean trypsin inhibitor and aprotinin analyzed by fluorescent analysis (A) and MALDI-TOF-MS (B) assays using typical tryptic peptides of CAM-BSA (*).The error bars represent the standard deviation of the mean (n = 2). The experiment was repeated with a similar result.

Fig. 5 Testing plant protease inhibitor activity by a fluorescence assay. The crude extract from plants was cleaned up and enriched by adsorption on the pMALDI chip. CAM + buffer (A), CAM-BSA + trypsin (B), CAM-BSA + trypsin + *Sn*MeJA (C), CAM-BSA + trypsin + *Sn*irPIN2MeJA (D). Eight replicates from two different experiments were analyzed using univariate ANOVA. Different letters indicate significant differences (Tukey's post hoc test; $p < 0.05$, n = 8).

Fig. 6 Protease inhibitor activity assays of wild-type (A, B) and transgenic *S. nigrum* plants silenced in their *PIN2* expression (C, D) induced with MeJA (B, D) and untreated controls (A, C) analyzed by MALDI-TOF-MS in reflectron mode. Typical tryptic peptides of the digested CAM-BSA are marked (*).

Fig. 7 Protease inhibitor assays of wild-type (A, B) and transgenic *S. nigrum* plants silenced in their *PIN2* expression (C, D) induced with MeJA (B, D) and control treatments (A,C) analyzed by MALDI-TOF-MS (linear mode).

Fig. 8 MeJA-treated wild-type *S. nigrum* plant protein extracts analyzed by MALDI-TOF MS (linear mode) using sinapinic (A) and a mixture of sinapinic and alpha matrices (1:1 v/v, B). Protein sequences of *Sn*PIN2b precursor and *Sl*PIN2 derived from DNA sequences were aligned to demonstrate the ability of such assays to identify molecular weights of proteins (C). MW was given as average mass of the processed protein in reduced form. The location of putative protease processing sites leading to mature *Sn*PIN2B proteins is indicated in red (A: signal peptide; B1,B2: two peptides forming one functional domain linked by sulfur bridges after processing; C: functional domain). The location of cysteins is indicated in green.

Sup. 1 Repeated experiment of plant protease inhibitor activity analysis using a fluorescence assay. The crude extract from plants was cleaned up and enriched by dialysis and adsorption on the pMALDI chip. CAM-BSA + buffer (1), CAM-BSA + trypsin (2), CAM-BSA + trypsin + *Sn*MeJA (3), CAM-BSA + trypsin + *Sn*irPIN2MeJA (4). Each bar represents the mean of two technical replicates.

Sup. 2 Repeated experiment of plant protease inhibitor activity analysis using a fluorescence assay. The crude extract from plants was cleaned up and enriched by adsorption on the pMALDI chip. CAM-BSA + buffer (1), CAM-BSA + trypsin (2), CAM-BSA + trypsin + *Sn*MeJA (3), CAM-BSA + trypsin + *Sn*irPIN2MeJA (4). Error bars represent standard deviation of the mean (n = 3).

Sup. 3 On-chip digestion of adsorbed CAM-Rubisco rapidly analyzed by fluorescence assay (top) and its peptide mass fingerprint using a MALDI-TOF-MS (bottom). CAM Rubisco + buffer (A). Trypsin + buffer (B) CAM-Rubisco + Trypsin (C). Error bars represent standard deviation of the mean (n = 3).

Figures

Fig.1

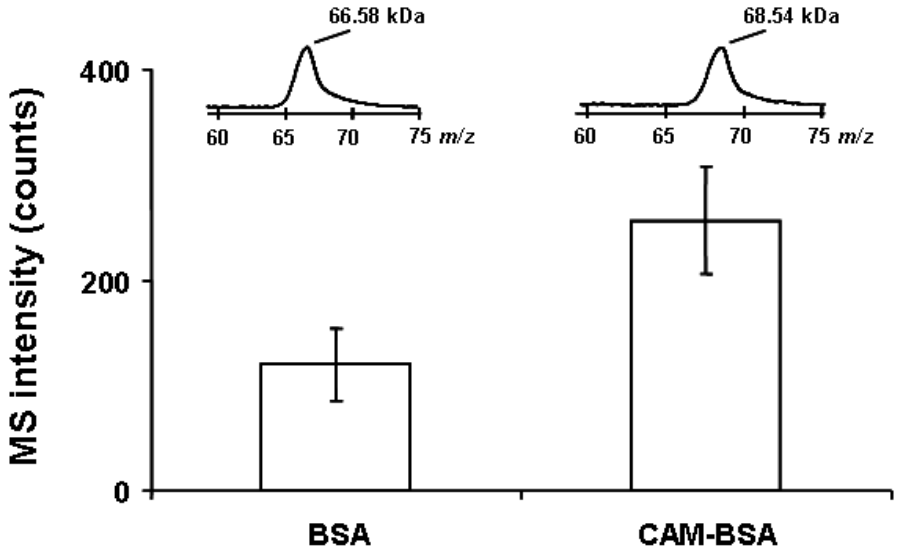
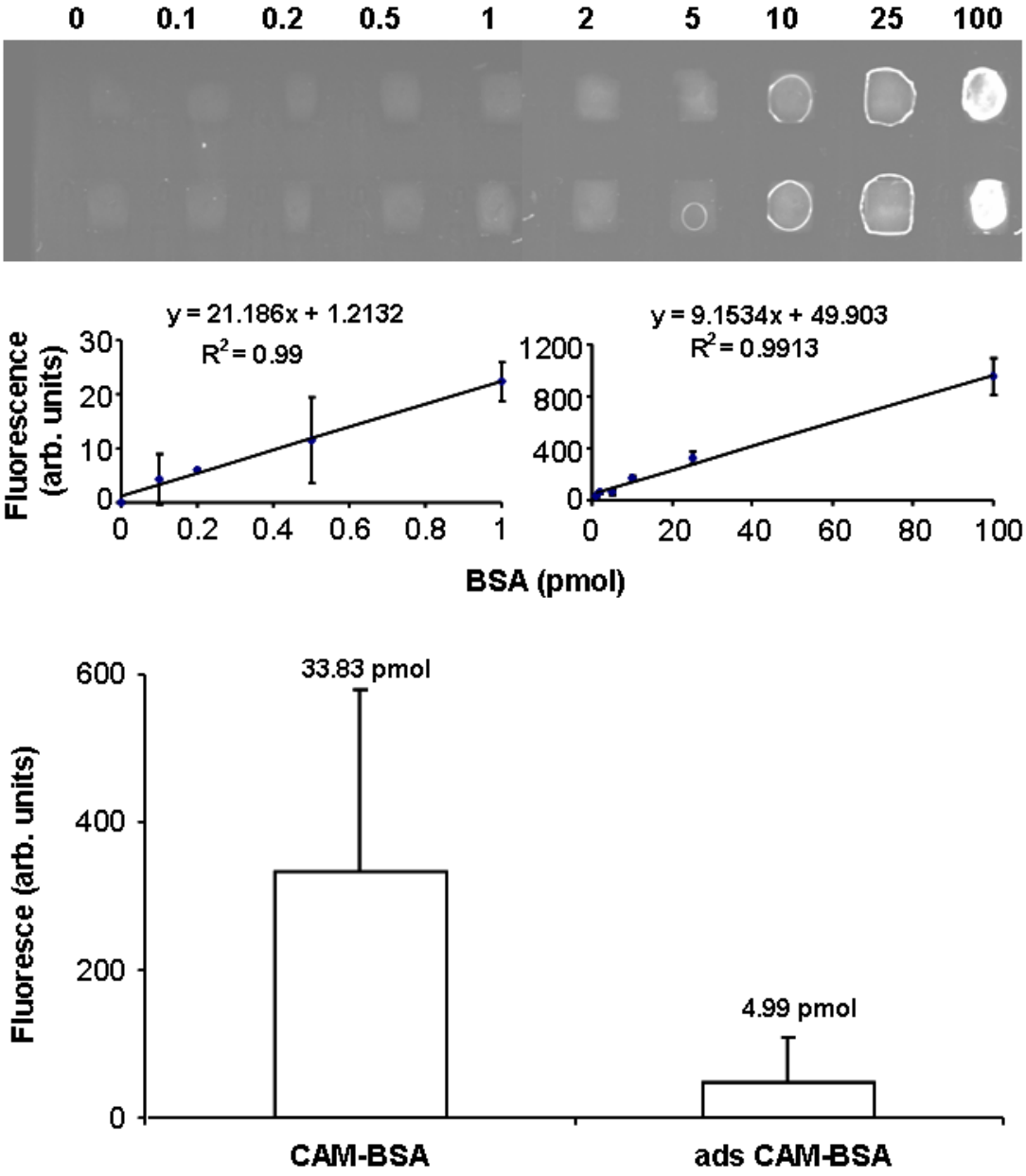


Fig. 2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R2 = 0.9913
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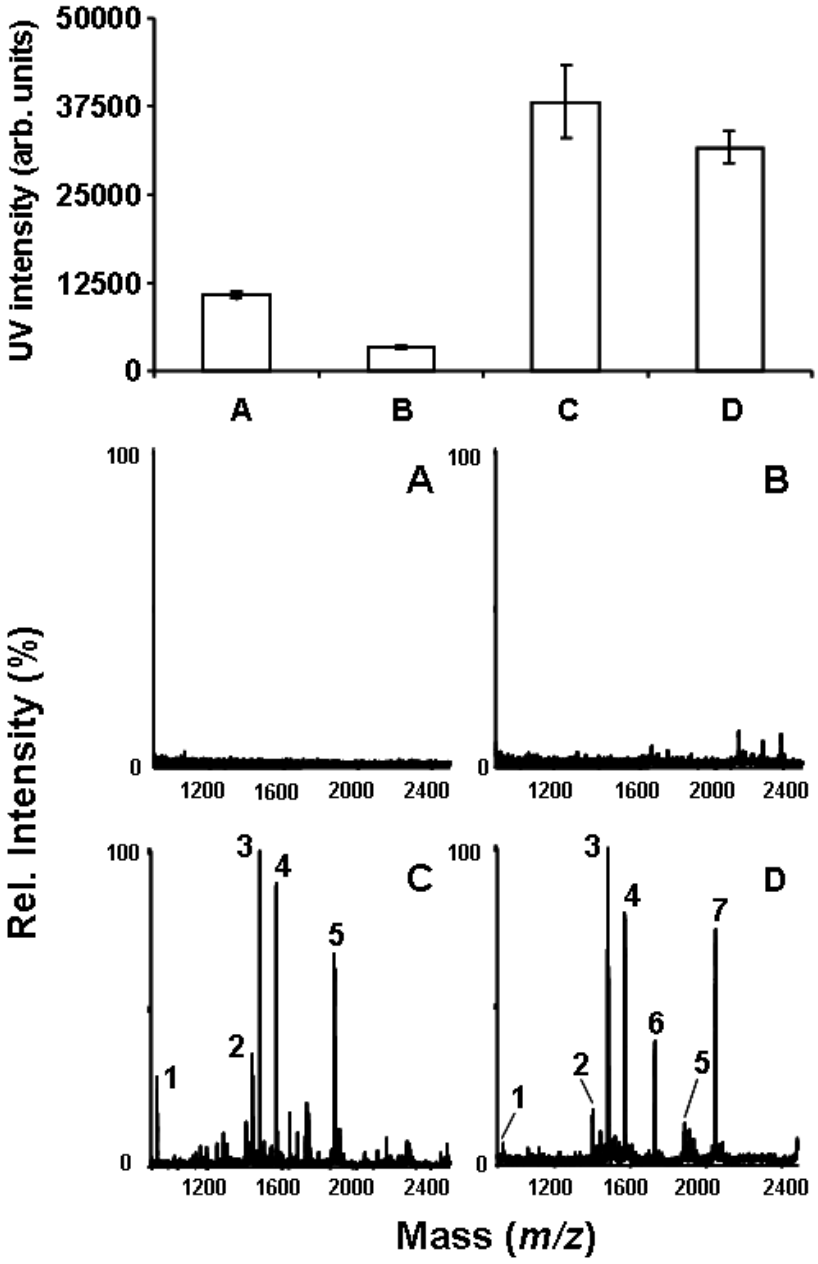
Fluoresce (arb. units)
CAM-BSA
ads CAM-BSA

Fig. 3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B
C
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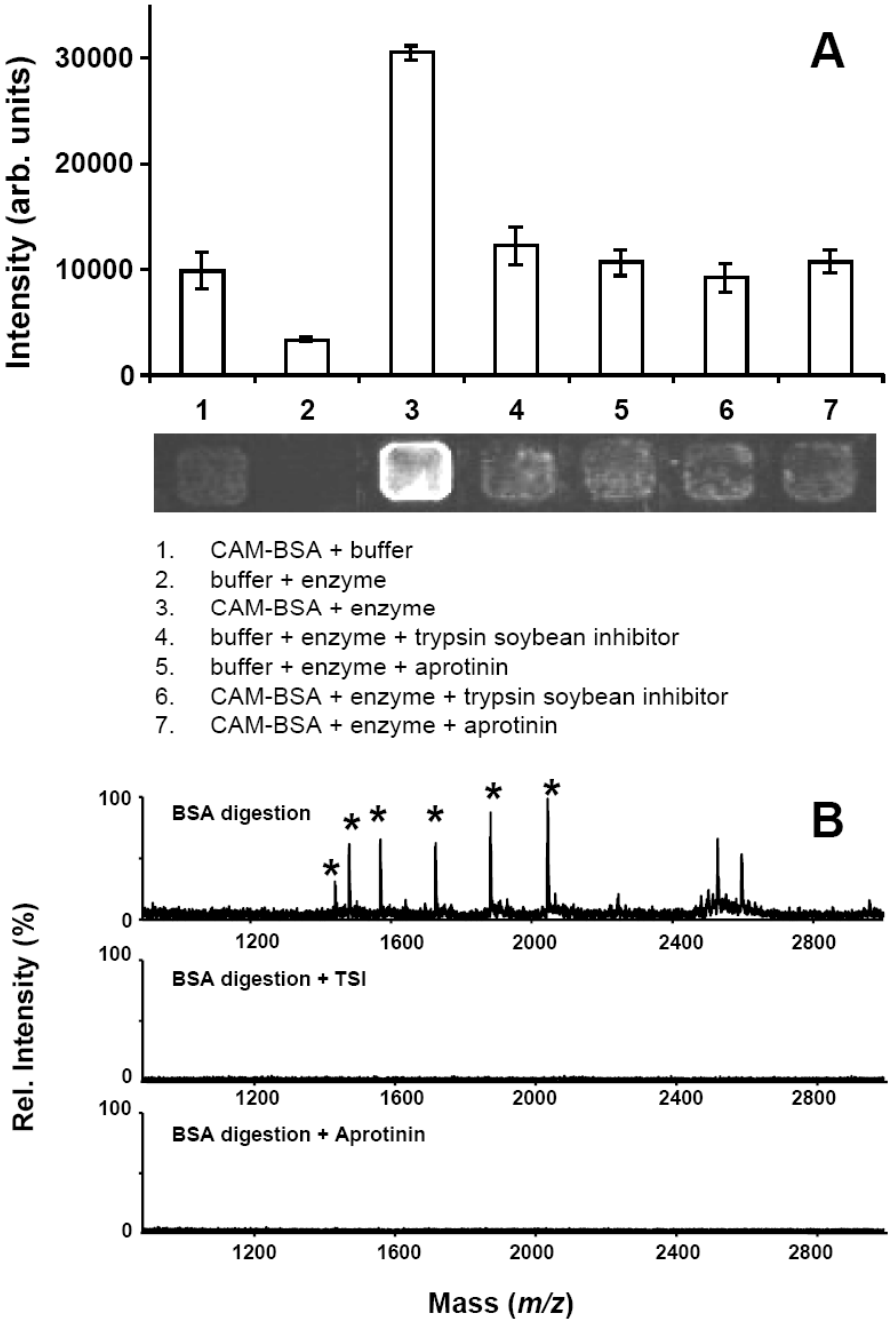
4
5
6
7
Mass (m/z)

Fig. 4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2
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4
5
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7
1. CAM-BSA + buffer
2. buffer + enzyme
3. CAM-BSA + enzyme
4. buffer + enzyme + trypsin soybean inhibitor
5. buffer + enzyme + aprotinin
6. CAM-BSA + enzyme + trypsin soybean inhibitor
7. CAM-BSA + enzyme + aprotinin
B
Rel. Intensity (%)
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BSA digestion + TSI
BSA digestion + Aprotinin
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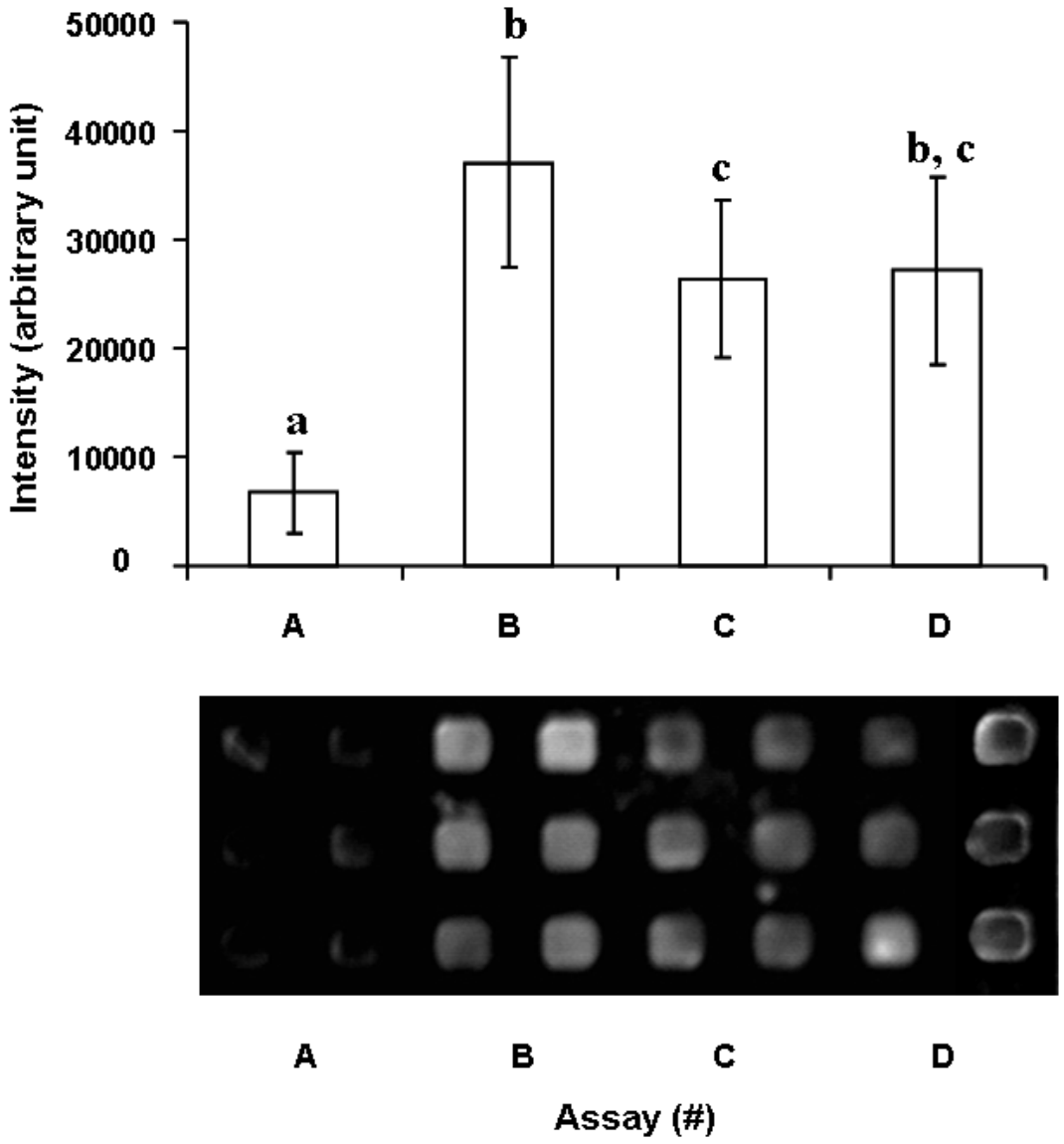
1600
2000
2400
2800
Mass (m/z)

Fig. 5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A
B
C
D
Assay (#)

Fig. 6

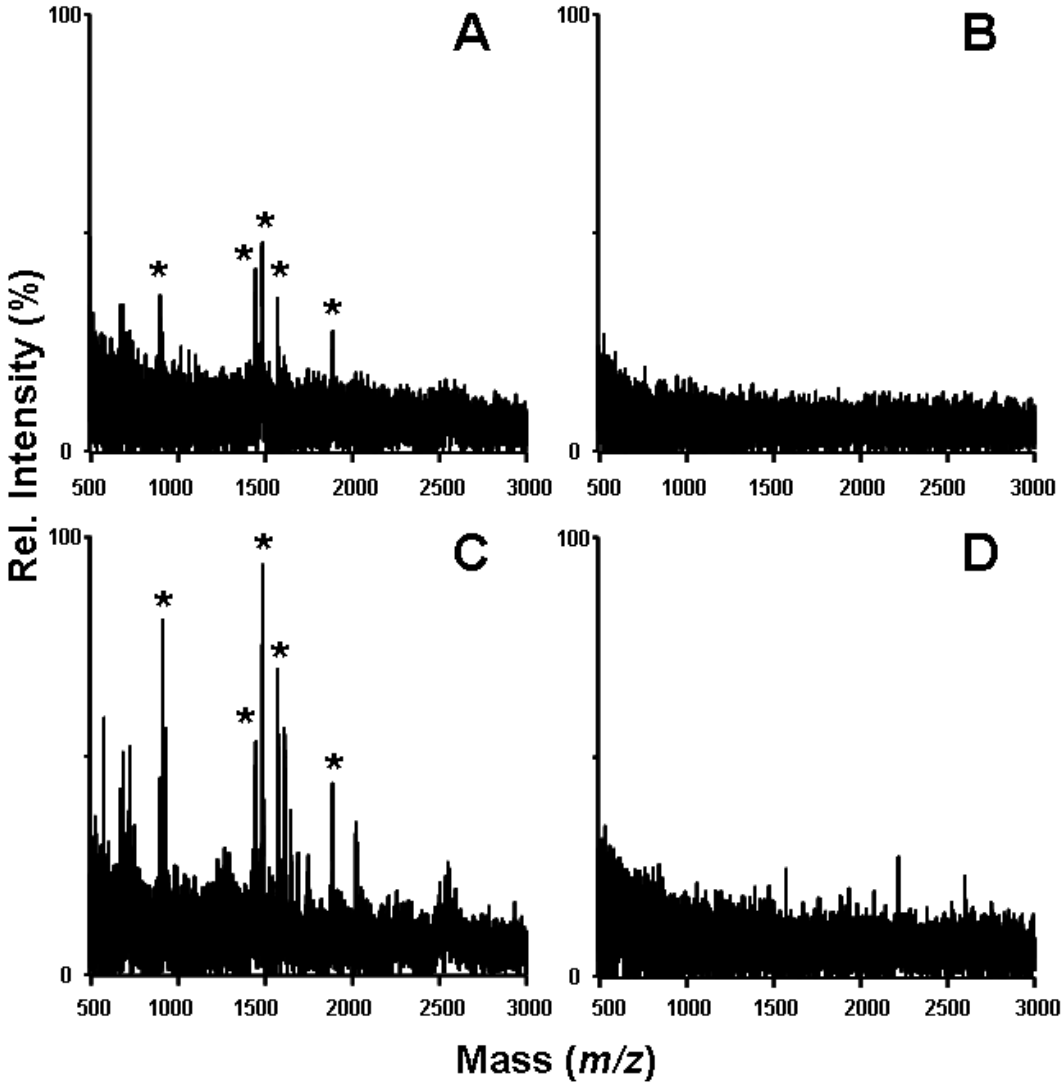
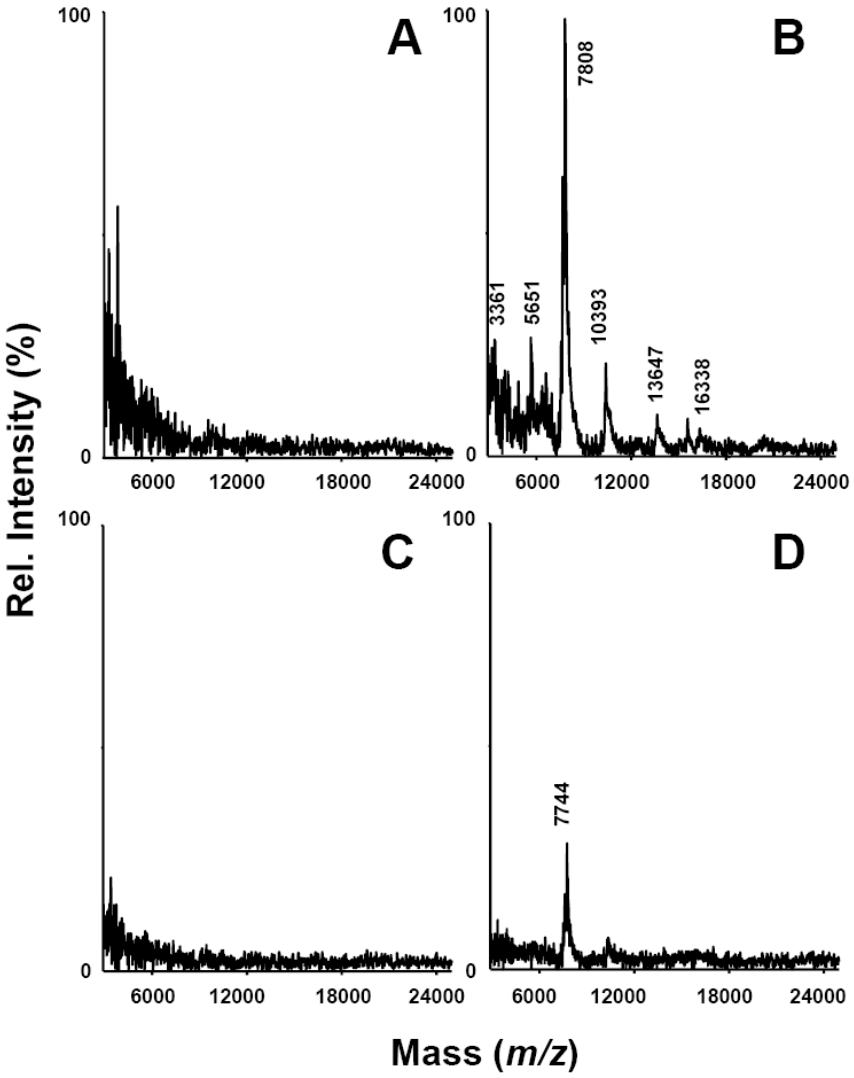


Fig. 7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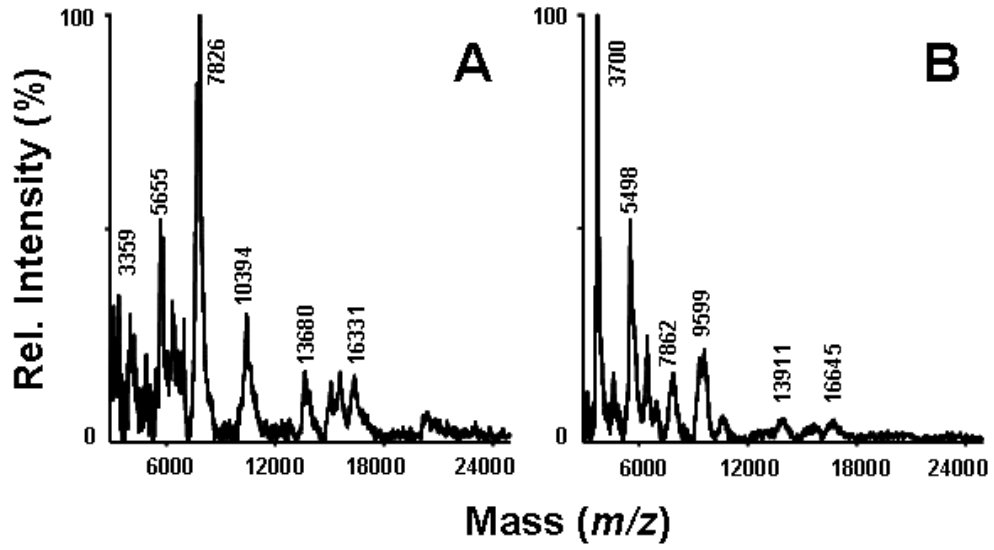
Mass (m/z)

Fig. 8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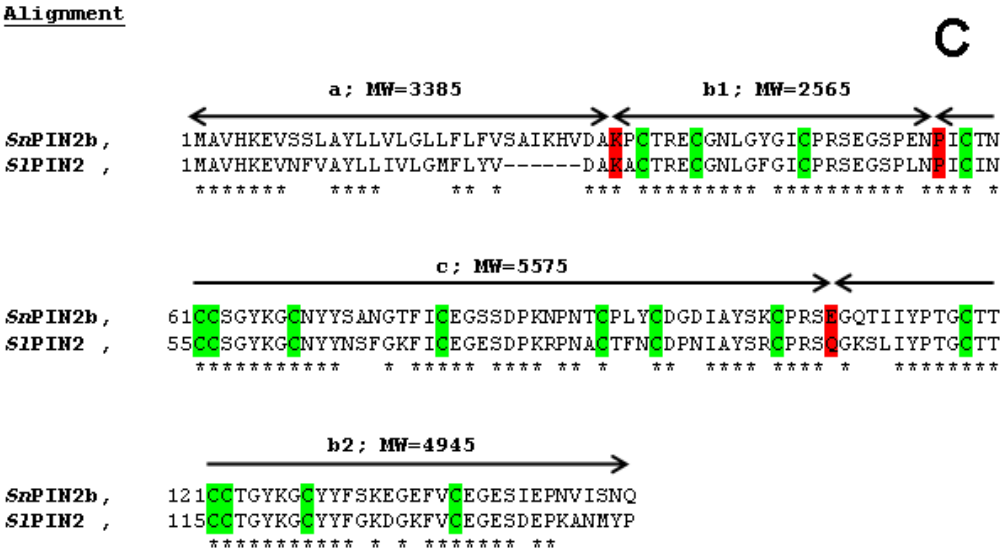
Mass (m/z)


Alignment
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a; MW=3385
b1; MW=2565
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SlPIN2 , 1MAVHKEVNFVAYLLIVLGMFLYV------DAKACTRECGNLGFGICPRSEGSPLNPICIN
c; MW=5575
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SlPIN2 , 55CCSGYKGCNYYNSFGKFICEGESDPKRPNACTFNCDPNIAYSRCPRSQGKSLIYPTGCTT
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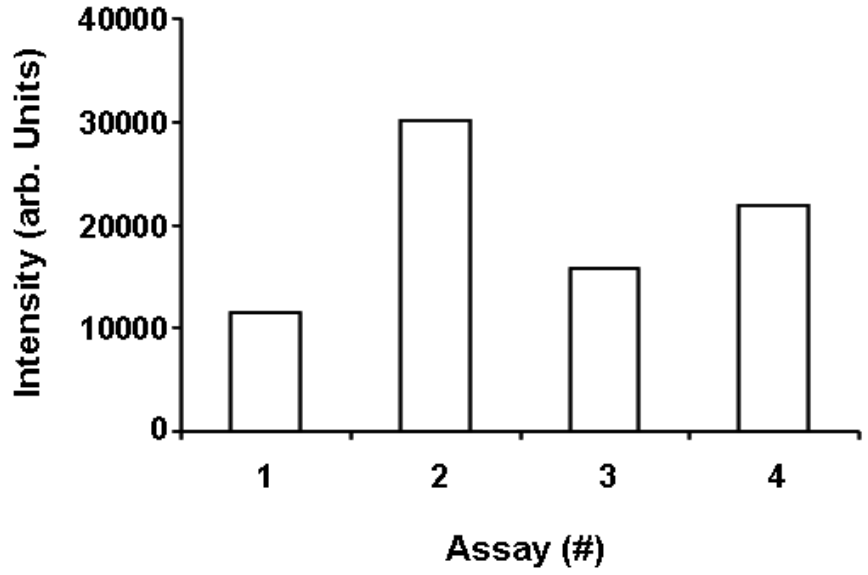
Sup. 1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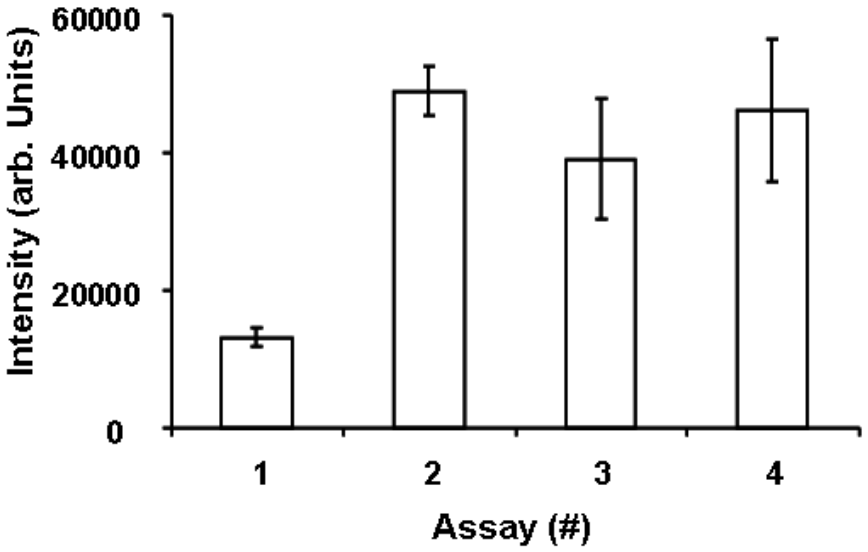
Assay (#)

Sup. 2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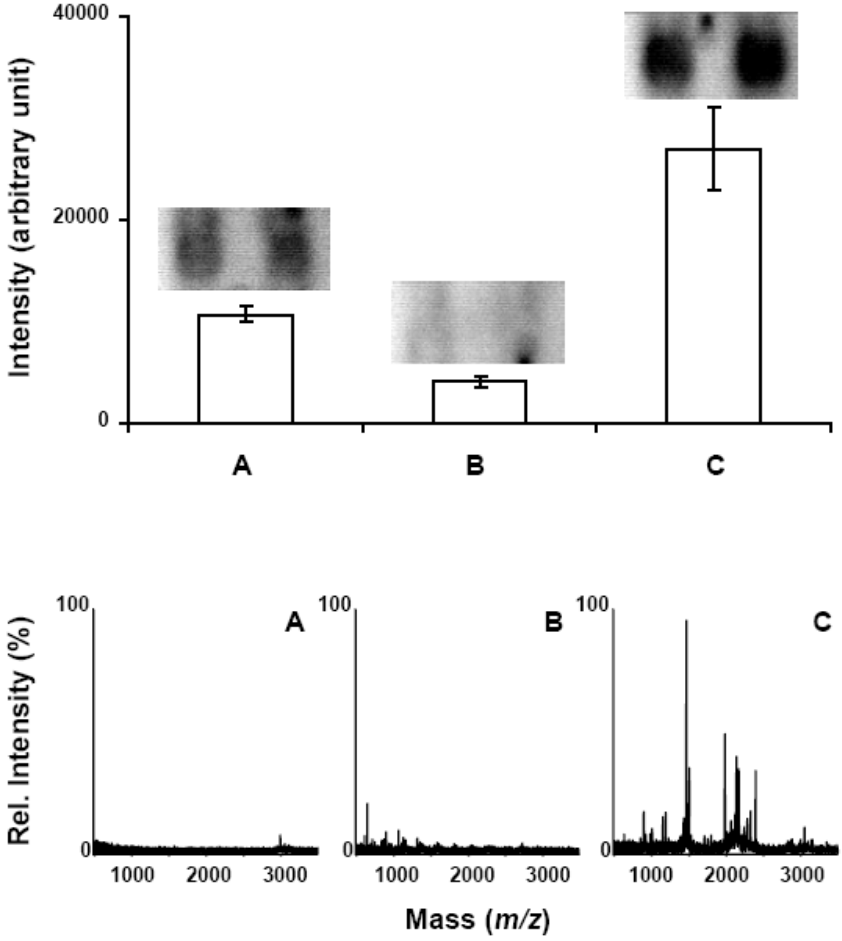
Assay (#)

Sup. 3


40000
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Intensity (arbitrary unit)
A
B
C
100
Rel. Intensity (%)
0
1000
2000
3000
Mass (m/z)

2.7 Declaration of author contributions:

I, Dr. Aleš Svatoš, agreed to what is written here as my contribution in the manuscripts included in the thesis.

I, Prof. Dr. Hans-Peter Saluz, agreed to what is written here as my contribution in the manuscripts included in the thesis.

I, Prof. Dr. Ian T. Baldwin, agreed to what is written here as my contribution in the manuscripts included in the thesis.

I, Dr. Alexander Muck, agreed to what is written here as my contribution in the manuscripts included in the thesis.

I, Dr. Vincentius A. Halim, agreed to what is written here as my contribution in the manuscripts included in the thesis.

I, Dr. Madina Mansourova, agreed to what is written here as my contribution in the manuscripts included in the thesis.

I, Mr. Einar J. Stauber, agreed to what is written here as my contribution in the manuscripts included in the thesis.

I, Mr. Thomas Schüler, agreed to what is written here as my contribution in the manuscripts included in the thesis.

I, Dr. Robert Möller, agreed to what is written here as my contribution in the manuscripts included in the thesis.

I, Dr. Wolfgang Fritzsche, agreed to what is written here as my contribution in the manuscripts included in the thesis.

I, Mr. Markus Hartl, agreed to what is written here as my contribution in the manuscripts included in the thesis.

I, Dr. Ashok Giri, agreed to what is written here as my contribution in the manuscripts included in the thesis.

I, Dr. Florian Erfurth, agreed to what is written here as my contribution in the manuscripts included in the thesis.

3. Results & discussion

The proteome refers to the entire protein complement expressed based on a genome, transcriptome, and post-translational modified by cellular events; and proteomics is the study of the proteome.[2] Since the proteome is dynamic, proteomics involves the identification, characterization, and quantification of proteins expressed by an organism under specific conditions. Proteomics research integrates several technological advances in the field of high-throughput protein separation, mass spectrometry, genomic database, and bioinformatics.

MALDI-MS and in particular SEAC-MS are ionization techniques for mass spectrometry which are useful in the field of proteomics research.[8,18] In the proteomic era, the number of measurements carried out by laboratories using these techniques has increased exponentially; thus, the introduction of disposable polymer-based MALDI probes by the Swedish collaboration of the research groups of Marko-Varga and Laurell[47,48] was a great breakthrough for scientists.

Although the initial fabrication method was crude and required optimization, it gave insight into the advantages of using low-cost disposable MALDI-MS targets, compared to the use of traditional metallic probes. Consequentially, chemical/physical surface modifications have made plastic surfaces similar to those used for SEAC-MS available at lower costs.

While advances in microfabrication and surface chemistry have clearly increased our knowledge about the use of such devices, fabrication methods are still complex and time consuming. The aim of this thesis is to further develop the initial work done in Dr. Svatoš' group in the field of disposable MALDI-MS substrates produced by replica molding (using a combination of reactive-injection and UV-polymerization techniques), by exploring new fabrication methods and concrete applications in the context of proteomic studies.

3.1 How to have your cake and eat it too: The charging effect challenge

Non-metallic sample supports for MALDI MS can increase measurement sensitivity when compared to metallic MALDI probes, because interference is removed after the adsorption of the target proteins/peptide onto the surface, or the formation of crystal spots is enhanced due to surface wettability properties (manuscript I).

Irradiation of peptides and protein samples on non-conductive polymeric substrates with high laser fluencies does not result in any significant charging effects, although it depletes the sample from the plate extremely fast. However, low laser fluencies in combination with longer laser irradiations display a pronounced mass shift (a 2000–5000 ppm shift) to higher *m/z* values when compared to a metallic target. As a result, the mass resolution decreases due to the accumulation of multiple scans, where each scan presents a gradually increasing m/z value (manuscript I).

The loss in resolution is linked experimentally to the accumulation of photo-electrons from matrix molecular orbitals by the laser during the desorption/ionization step (manuscript I).

In order to suppress the charging effects for long laser irradiations on a butyl:methyl methacrylate (90:10 molar ratio, BMA-pMALDI chip):

- The probe itself can be made of or contain a conductive material. Due to our interest in keeping the plastic active surface accessible for protein/peptide interactions, we discouraged the use of a thin gold nanolayer on the surface. Instead, an electron acceptor, methyl viologen dichloride hydrate (MV), was dissolved in the monomeric mixture of prior polymerization of the BMA-pMALDI chips (see Figure 8). Although the final result was a chip containing a functional group with the ability to trap photo-electrons, the BMA-pMALDI chip was extremely brittle due to the quenching effect of MV during the radical UV-polymerization.
- The electron capture substance can be added with the matrix and/or sample. MV was dissolved with the matrix and co-crystallized with the protein sample on the surface of the BMA-pMALDI chip (manuscript I). The result was that the charging effect was kept under control (see Figure 8); meanwhile, the electron capture material was not saturated (manuscript I). Unfortunately, the addition of a substance in a higher concentration than the sample (other than the matrix) led to ion suppression effects, hence the compromise between sensitivity and the resolution for certain proteins (manuscript I). Moreover, the electron capture substance, which must be compatible, is not universally in use, and it must be optimized according to the matrix used for each experiment (manuscript I).

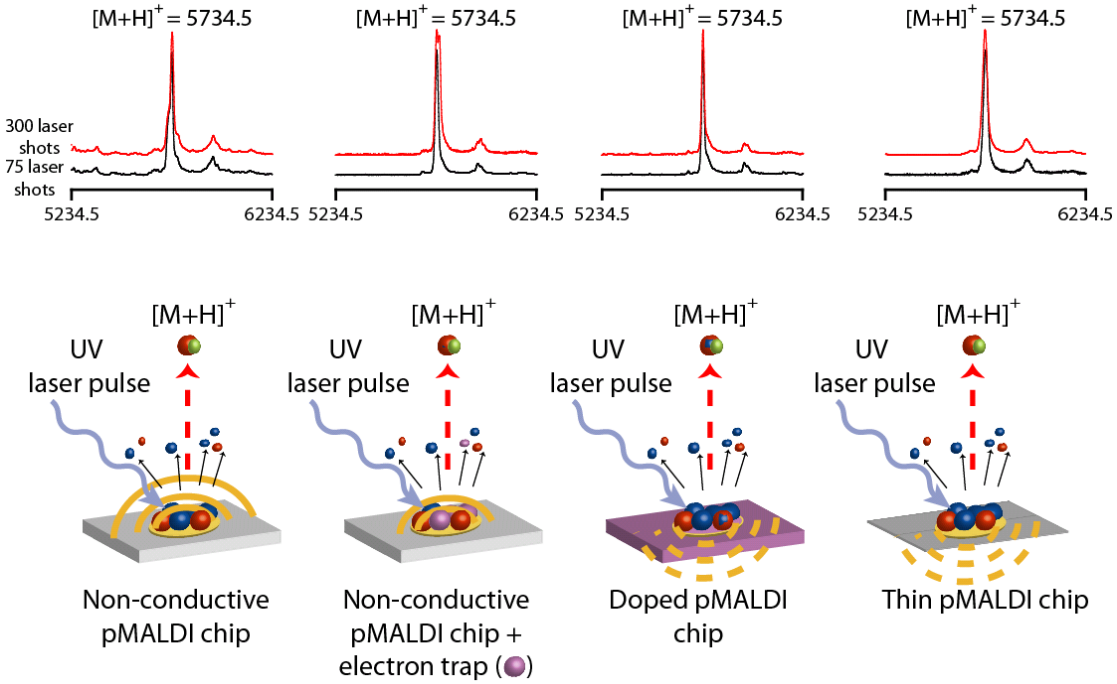

Figure 8. Different methods for reducing the charging effect on plastic chips. (a) Red spectra are combined from 75 consecutive laser shots. (b) Black spectra are combined from 300 laser shots.

- Physically modifying the BMA-pMALDI chip to make it conductive. By following electric engineering principles:

$$\text{Flow of electrons: } I = \frac{\partial Q}{\partial t} = C * \frac{\partial V}{\partial t} = \left(\varepsilon A / d\right) * \frac{\partial V}{\partial t} \quad \text{(eq. 3),}$$

where I = current, Q = electrical charge, C = capacitance, V = electric field, ε = permittivity, A = transversal area, d = thickness, the BMA-pMALDI chip was made thinner using a smaller aluminum spacer during the polymerization of the chip (manuscript VI). The reduction of the nonconductive thickness (d) increases the flow of electrons through the polymer to the base plate, hence decreasing the charging effect (see Figure 8). The deficiency in this fabrication method was the lack of mechanical stability for the pMALDI chip; hence the chips were re-strengthened by immobilizing them on solid supports prior to analysis (see Figure 8).

3.2 Laws of attraction: Optimization of protein-surface interactions for pMALDI chips

The development of efficient methods for assessing peptide/protein adsorption to surfaces is of great importance for the characterization of new biomaterials and for giving deeper insight into protein activity when immobilized for protein microarray or biosensor applications.[53-56]

The interactions between a peptide/protein and a surface can be divided into nonspecific and specific. The term "non-specific" refers to general Van der Waals forces, as well as for loose hydrophobic and electrostatic interactions; on the other hand, the term "specific" refers to those interactions which rely highly on a close complementarity between protein and surface.[7, 75, 97, 98]

In the case of the nonspecific interaction between a protein and a hydrophobic surface (manuscripts I and VI), the agreed mechanism is entropically driven[75,97] and it may be described as follows:

1) Diffusion of the protein to the surface.
2) Reversible adsorption on the surface.
    a. Removal of the solvation layer.
    b. Removal of water/ion molecules from the surface.
    c. Formation of hydrophobic interactions between the protein and surface.
    d. Structural rearrangements of the protein.
    e. Structural rearrangements of the excluded molecules.
3) Irreversible denaturation of the protein on surface.

In manuscripts II and VI, highly ionic media help the adsorption of proteins to the hydrophobic surface of the pMALDI chip by stabilizing the structure that proteins adopt on the surface; or by increasing the strength of the hydrophobic interactions, which try to repel the highly ionic surroundings.

In the case of the non-specific interaction between a protein and a polar/charged surface (manuscript II), the agreed adsorption mechanism is mainly enthalpy driven.[71,97] In this

case, the previous mechanism is extended to consider the formation of weak and strong ionic couples:

1) Diffusion of the protein to the surface.
2) Reversible adsorption on the surface.
    a. Removal of the solvation layer.
    b. Removal of water/ion molecules from the surface.
    c. Formation of hydrophobic, dipole, ionic, hydrogen bridge interactions between the protein and the surface.
    d. Structural rearrangements of the protein.
    e. Structural rearrangements of the excluded molecules.
3) Irreversible de-naturalization of the protein on surface.

Therefore, the overall effect on the change of Gibbs's free energy depends on a balance between electrostatic attraction and repulsion forces (enthalpy), making possible the separation of proteins at different pH or different ionic strength concentrations as shown in manuscript II.

In the case of a specific interaction between a protein and affinity-modified surface (manuscript III), the agreed mechanism is also enthalpy driven. Similar to the previous mechanism, the formation of closely complementary interactions at the molecular level is what allows the selective binding of one peptide/protein over the rest.

Additional evidence for the mechanism, in which the proteins were adsorbed onto the surface of a hydrophobic/ionic chip, was partially given in manuscripts II, IV and VI. When the peptide fragments from known proteins (i.e. sequenced proteins) were digested under different conditions, such as (a) in-solution (free enzyme; free protein), (b) on a trypsin-pMALDI chip (bound enzyme; free protein), and (c) after adsorption of the protein onto a hydrophobic or ionic pMALDI chip (free enzyme; bound protein), it is possible to determine which were the binding sites of the protein toward the surface (in a similar way that epitope profiling is currently done in immunochemical studies). Hence, the combination of these types of digestions can provide information about how a protein molecule folds on a 2-D surface and what the influence of the local surface chemical environment on the folding process.

3.3 How to avoid undesirable guests: Preconcentrating/fractionating of samples on pMALDI chips

MALDI-MS techniques rely on an ion source (MALDI ionization process); a mass analyzer (usually TOF/MS) that measures current and time, which are later converted into mass to charge ratio (*m/z*) units; and a detector to count the number of ions of particular *m/z* (see section 1.2.1, p.6). Due to the mechanisms behind their ionization and mass separation, MALDI-TOF/MS instruments can reach very low limits of detection (femto molar range) using low volume samples (pL). Accordingly various forms of interference such as salts/surfactants or high abundant proteins must be removed prior to MALDI-TOF/MS measurements.

3.3.1 Salts and surfactants as contaminants

At present, there are two kinds of strategies for alleviating both ion suppression effects and mass resolution effects associated with the presence of salts and surfactants in MALDI-TOF/MS samples. For example, when a protein is measured in the presence of salts, the formation of not only $[M+H]^+$, but also $[M+Na]^+$, $[M+K]^+$, etc., can affect the Gaussian shape of the protein signal (i.e. it becomes more dispersed, hence overlapping with signals of different ion species).

State-of-the-art separation/purification steps prior to MALDI-TOF/MS measurements will make the samples more compatible for use in the traditional organic matrix MALDI-MS. These systems can be classified as

- off-probe and off-line approaches such as solid-phase extraction, microdialysis membranes and Millipore ZipTip, etc.;
- off-probe and on-line approaches such as liquid chromatography or electrophoresis separation devices coupled to a MALDI plate using a microdispenser;
- on-probe or *in situ* approaches such as use of self-assembled monolayers, polymer-coatings/membranes/films, porous monolithic materials on MALDI plates.

Manuscripts I, II, and III report how the microfabricated pMALDI chips are successful in desalting and removing other difficult MALDI contaminants (such as surfactants). In these manuscripts, polymeric surfaces are used as the affinity substrate (similar to solid-phase extraction substrates), onto which the peptides/proteins are extracted, allowing various kinds of interference such as salts (manuscripts I and III) and surfactants (manuscript II) to be removed during a subsequent flushing step.

3.3.2 Peptides and proteins as contaminants

One major challenge associated with peptide/protein analysis using MALDI-TOF/MS is the lack of selectivity during the ionization process, due to the various amounts of peptides/proteins which can be present in the same sample, and their different ionization efficiencies. This lack of selectivity in MALDI-MS instruments is the main reason for the predominance of SEAC substrates in scientific applications, such as diagnostic studies, biomarker discovery, drug testing, etc.[40, 99,100]

The selectivity of the interaction between a peptide or a protein and a given surface can be measured in terms of the enthalpy and entropy energies (i.e. Gibb's free energy, section 3.2, p.95). The selectivity of peptides/proteins toward different surfaces was studied, such as hydrophobic (manuscripts I, II, & V), ionic (manuscript II), and with affinity probes (manuscript III).

Because of the non-specific interactions involved during the peptide/protein adsorption onto hydrophobic and ionic pMALDI chips, the selectivity of both is rather poor and will depend strongly on the intrinsic characteristics of the peptide and/or protein such as hydrophobicity (GRAVY) and/or isoelectric point (pI) value, in combination with the properties of the media in which they are adsorbed (such as pH and ionic strength).

On the other hand, affinity-based pMALDI chips, in particular ion metal affinity-pMALDI chips (manuscript III), have proved to be strongly selective toward phosphorylated residues present in peptides and proteins. Based on the Pearson metal ion classification, a Ni^{2+}-pMALDI chip was used to selectively retain α-casein in the presence of two other proteins, both of which have stronger ionization efficiencies; and a Ga^{3+}-pMALDI chip was used to selectively retain phosphorylated-peptides in a salt-rich complex digestion mixture.

3.4 Building a micro-production plant: Bioreactor-pMALDI chips

Section 3.2 covers the preconcentration of the sample (analyte of interest) directly on the surface of MALDI target by direct adsorption of the sample onto a hydrophobic, ionic or similar affinity probe element on the surface of the pMALDI chip. In this section, the preconcentration of an analyte is achieved by avoiding the dilution effects and the losses associated with carrying out (bio)chemical reactions prior to the MALDI-TOF/MS analysis.

In manuscript IV, we fabricate an amine-functionalized polymeric material that can be readily modified via a crosslinker to different enzymes (see Figure 9). In this manuscript, we immobilize as an example two enzymes on the surface of the pMALDI chip (i.e. trypsin and alkaline phosphatase). The optimized size cross-linker enhanced the enzymatic activity of the immobilized enzyme when compared to the same enzyme in-solution (free enzyme).

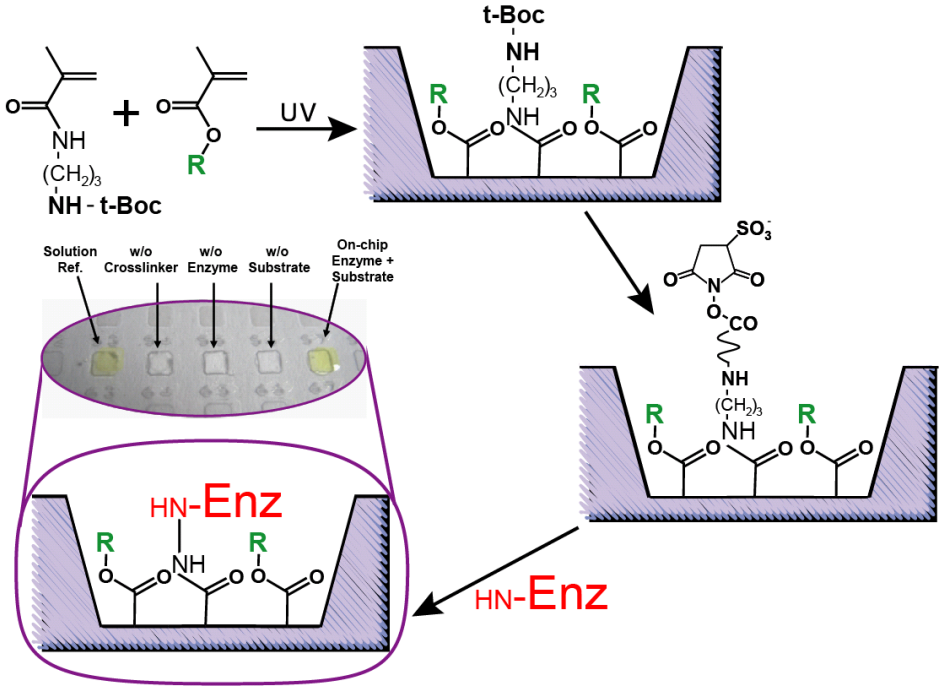

Figure 9. Modification of a pMALDI chip with an enzyme.

The application of the trypsin-linked pMALDI chip can be used to further identify isolated proteins from either pMALDI chips or from other sources, such as nano-LC or electrophoretic separation systems. In all cases, the method of identification is as follows: the protein is prefractionated (ideally, it is completely isolated) by one of the previously mentioned methods. Then, it is transferred to the trypsin-pMALDI chip where it is identified by comparing the tryptic peptide *m/z* values with *in silico* values from a database (see PMF definition in section 1.2.1, p.7).

In manuscript IV, the information obtained from hydrophobic-pMALDI chips, such as *m/z* and relative hydrophobicity, is used to limit the number of possible candidates from the database search, hence, significantly increasing the identification score (identification probability) for the protein that is analyzed.

3.5 What is this peptide/protein for?

3.5.1 DNA microarray studies on pMALDI chips

The proteome is the set of proteins produced by a living organism during its life. The new field of proteomics encompasses not only efforts to identify proteins but also efforts to catalog and determine the functions of all the proteins in a proteome.

Current novel technologies, DNA libraries, PCR, and hybridization have come together in the development of DNA microarrays that allow the rapid and simultaneous screening of several DNA sequences. These microarrays can tell researchers which genes are being expressed at a given stage in the development of an organism, or what changes (up/down regulations) took place when organisms were exposed to external stimuli. Currently, one of the most effective ways to determine the function of a protein is comparative genomics.[101] Homology-based searches (the search for genes that are paralogs and orthologs) have enabled proteins and their possible function in non-sequenced organisms to be identified.

Many DNA microarrays which are currently commercially available are not suitable for diagnosing short DNA/RNA sequences and low numbers of single base variation in potentially varying DNA samples. Moreover, false positive errors may arise when fluorescent-labelled DNA microarray are used, if DNA is indirectly detected on the surface (DNA is not natively fluorescent). In contrast, MALDI-MS directly measures sample molecules, making it a powerful and cost-efficient tool for DNA diagnoses (manuscript V).

In manuscript V, we combine the properties of an optically-labelled DNA microarray with those of MALDI-MS DNA microarray.

3.5.2 Peptide/Protein interaction (activity) studies on pMALDI chips

As mentioned above, DNA microarrays can be used to determine the function of proteins in organisms by comparing the cDNA libraries of target and known organisms (i.e. that have already been sequenced). Nevertheless, accurate identification still depends on the

biochemical confirmation of proteins, and their localization in cells, and on biochemical activity.

Biochemical activity using pMALDI chip can be measured in two different ways, either by measuring the protein-protein interaction when (1) the known protein is bound to the surface, while the unknown protein is in solution or (2) the unknown protein is bound to the surface, while the known protein is in solution.

In the first method, a known protein is adsorbed onto a surface such as trypsin (manuscript IV), and later exposed to an individual protein or cocktails of proteins to see how their activity differs. We used this method to study the activity of protease inhibitors when trypsin was immobilized on the surface of the pMALDI chip. In the second method, an individual or mixture of proteins is adsorbed onto the surface, and they are later exposed to proteins with known activity (manuscript VI). Although both methods seem to be similar, the environments in which the samples are run differ; the first method can be used to study protein:protein interactions using the immobilized protein on the surface as an affinity binding material. In the second method, the unknown protein(s) is(are) first applied onto a hydrophobic or ionic pMALDI chip, taking advantage of the pMALDI preconcentration/fractionation properties, which removes interference that hinders the consecutive enzymatic reaction (manuscript VI).

3.6 Conclusion

Novel disposable plastic substrates for MALDI-MS and SEAC-MS applications were studied and developed (manuscripts I-VI). A new method of fabrication to avoid charging effects (manuscript I) and read-out methods for sample evaluation were developed (manuscripts V & VI).

The pMALDI-MS probes can be used to identify peptides and proteins, present in complex sample mixtures, by the prefractionation and/or preconcentration process that can be carried out on their surfaces. The affinity of the peptides and proteins to the surface can be customized by using tailor-made plastic materials according to different chromatographic principles base on

- hydrophobic interactions (manuscripts I, II & VI),
- ionic interactions (manuscript II), or
- affinity interactions (i.e. metal ion affinity, manuscript III).

These probes can be also used as bioreactors, in particular to identify proteins using the peptide mass fingerprint database search (manuscript IV) or to study enzymes activities (manuscript VI). Additionally, we have demonstrated that these polymeric planar devices can also be used for identification of single stranded DNA fragments in order to identify a virus (manuscript V).

4. Summary

In order to elucidate the biochemical processes underlying the survival strategies of living organisms in nature, the ability to perform proteomic analysis quickly and easily is becoming increasingly important.

There are several techniques used to carry out proteomic research. The workflow associated with these techniques depends on the goal of the proteomic study (i.e. proteome characterization, protein identification, protein quantification, function determination, etc.). Nevertheless, all platforms share a common technological base, the need of highly efficient methods of peptide/protein separation (i.e. methods for protein extraction, prefractionation, and/or isolation), in combination with extremely sensitive peptide/protein detectors such as mass spectrometer analyzers for comparative profiling of peptide/protein expressions or peptide/protein identification through database matching.

The two most widely used mass analyzer techniques for proteomic research are electrospray ionization tandem mass spectrometry (ESI-MS/MS), and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS).

A new sample preparation method has been recently used for proteomic research studies. It is called surface-enhanced affinity-captured mass spectrometry (SEAC-MS). It combines MALDI-TOF/MS measurement with peptide and/or protein separation on chromatographic or biochemical surfaces, on which a UV-laser is used to desorb/ionize the peptide and/or protein (with or without the assistance of a matrix).

Depending on the type of modified target surfaces used in SEAC-MS (hydrophobic, anion exchange, cation exchange, metal affinity, antibodies, etc.), the adsorption of a target group of peptides and/or proteins is preferred. By comparing them with the MS data of reference samples, proteins can be identified, and/or the protein expression due to an external controlled stimulus can be quantified. Additionally, using SEAC-MS technology, subsets of a proteome can be selectively profiled, separated and characterized from complex sample mixtures (such as crude plant extracts, bodily fluids, cell lysates, etc.).

In order to satisfy both scientific and economic considerations for proteomic studies, several limitations of MALDI and SEAC-TOF/MS need to be overcome. Proteomic methods must become not only more sensitive, more selective, more reproducible, and more robust; but also less expensive and more user-friendly than they currently are. Disposable plastic MALDI (pMALDI) chips are a cost-effective and time-saving alternative that may replace previously mentioned methods.

This thesis addresses one of the major drawbacks in the use of plastic surfaces for MALDI/SEAC measurements, the charging effect. This effect is attributed to the inability of non-conductive surfaces to remove the photo-electrons produced during irradiation of the MALDI matrix on a non-conductive surface with a UV-laser (this effect is called “the charging effect”). As a result, a localized time-dependent electric field is generated, and it disturbs the electric field which is applied between the target and the extraction grid of the MALDI-TOF/MS instrument. The variation in the *in source* electric field makes the assumption used to simplify equation 1 to equation 2 no longer valid, hence the signals are shifted to apparently higher *m/z* values. The use of “a smart chip design” and/or the use of

electron traps during the MALDI measurements demonstrated that this effect can be compensated for and the deviation from the ideal (equation 2) can be minimized.

To emphasize that pMALDI chips are a viable alternative to the currently commercialized SEAC probes, this thesis focuses on the main application for which SEAC probes are used: "on-probe" prefractionation/preconcentration of analytes from complex sample mixtures. Thus, the experimental conditions (e.g. polymerization time, monomeric ratio, pH and ionic strength for adsorption, etc.) were optimized for various functionalized polymeric surfaces, which share the properties of SEAC probes, such as

- hydrophobic surfaces,
- ionic surfaces,
    - cation exchange,
    - anion exchange, or
- metal ion affinity surfaces.

Additionally, this thesis (manuscript VI) describes how pMALDI chips can be used for protein activity studies, for characterizing protein-surface interactions, and for genomics research (manuscript V).

In conclusion, this thesis describes my contribution to developments in the field of plastic MALDI chips.

5. Zusammenfassung

Um die biochemischen Prozesse innerhalb der Überlebensstrategien von Organismen in der Natur aufzuklären, gewinnt die Fähigkeit, proteomische Analysen schnell und einfach durchzuführen, zunehmend an Bedeutung.

Um Proteomik-Forschung zu betreiben, stehen verschiedene Techniken zur Verfügung. Arbeitsabläufe, die mit diesen Techniken verbunden sind, hängen dabei von der jeweiligen Fragestellung ab (Proteom-Charakterisierung, Proteinidentifikation, Protein-Quantifizierung, Funktionsbestimmung etc.). Trotz dieser Unterschiede gibt es Basis-Techniken, die unabhängig von dem Arbeitsziel benötigt werden. Diese Basis-Techniken betreffen sehr effiziente Methoden zur Peptid/Protein-Auftrennung (z.B. Proteinextraktion, Vortrennung und/oder Isolierung) in Kombination mit sehr empfindlichen Peptid/Protein-Detektoren wie Massenspektrometern, die ein vergleichendes Profiling von Peptid/Protein-Expressionsmustern oder Peptid/Protein-Identifikationen durch Vergleiche mit Datenbanken erlauben.

Die beiden am häufigsten verwendeten Masseanalyse-Techniken in der Proteomik-Forschung sind Electrospray Ionisation Tandem Mass Spectrometry (ESI MS/MS) und Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF-MS).

Eine neue Methode zur Probenvorbereitung, die auf der MALDI-Technik beruht, findet seit kurzer Zeit Anwendung in der Proteomik-Forschung. Diese Technik, Surface-Enhanced Affinity Captured Mass Spectrometry (SEAC-MS) genannt, basiert auf einer Kombination von MALDI-TOF/MS-Messungen und der Trennung von Proteinen und/oder Peptiden auf chromatographischen oder biochemischen Oberflächen. Die Bestrahlung solcher Oberflächen mit einem UV-Laser dient zur Desorption/Ionisation der Peptide und/oder Proteine. Dies kann mit oder ohne Hilfe einer Matrix geschehen.

Abhängig von der Art der modifizierten Target-Oberfläche, die bei der SEAC-MS verwendet wird (hydrophob, Anionen-Austausch, Kationen-Austausch, Metallionenaffinität, Antikörper etc.), wird die Adsorption einer Zielgruppe von Peptiden und/oder Proteinen bevorzugt. Durch den Vergleich mit den MS-Daten von Referenzproben können Proteine identifiziert werden, und/oder die Protein-Expression kann mit Hilfe eines kontrollierten externen Stimulus quantifiziert werden. Zusätzlich besteht durch die SEAC-Technik die Möglichkeit, Teile eines Proteoms selektiv aus einem komplexen Probengemisch (z.B. Pflanzenextrakte, Körperflüssigkeiten, Zelllysate etc.) zu identifizieren, abzutrennen und zu charakterisieren.

Um den wissenschaftlichen und ökonomischen Anforderungen an die Proteomik-Forschung zu genügen, müssen einige Grenzen der MALDI und SEAC-TOF/MS überwunden werden. So müssen die in der Proteomik angewendeten Methoden nicht nur empfindlicher, selektiver, reproduzierbarer und robuster werden, sondern auch kostengünstiger und benutzerfreundlicher, als sie es im Moment sind. Einweg-MALDI-Chips aus Plastik (pMALDI) wären eine kosteneffektive und zeitsparende Alternative, die die oben genannten Methoden ersetzen könnten.

Im Verlauf dieser Arbeit wurde das Augenmerk auf einen der größten Nachteile der Plastikoberflächen für MALDI/SEAC-Messungen – den Effekt der elektrischen Aufladung (charging effect) – gelegt.

Dieser Effekt entsteht dadurch, dass nichtleitende Oberflächen nicht in der Lage sind, die produzierten Photoelektronen abzuleiten. Diese entstehen bei der Bestrahlung der auf eine nichtleitende Oberfläche aufgebrachten MALDI-Matrix mit einem UV-Laser. Auf diese Weise wird ein lokalisiertes zeitabhängiges elektrisches Feld generiert, das das elektrische Feld stört, welches zwischen Target und Extraktionsgitter des MALDI-TOF/MS-Gerät angelegt wird. Durch die Schwankungen im „in source“ elektrischen Feld sind die Annahmen, die zu den Vereinfachungen der Gleichungen 1 und 2 führten, nicht länger gültig. Augenscheinlich führt dies zu einer Verschiebung hin zu höheren m/z-Werten. Ein „smart surface design“ und/oder die Verwendung von Ionenfallen während der MALDI-Messungen zeigen, dass dieser Effekt kompensiert werden kann und die Abweichung vom Idealzustand soweit minimert werden kann, dass sie vernachlässigbar ist.

Um zu zeigen, dass pMALDI Chips brauchbare Alternativen zu den momentan kommerziell verwendeten SEAC-Proben sind, wird der Fokus dieser Arbeit auf das Hauptanwendungsgebiet der SEAC-Proben gelegt. Bei diesem handelt es sich um die „on-probe“ Vortrennung/Vorkonzentrierung von Analyten aus komplexen Probengemischen.
Aus diesem Grund wurden die experimentellen Bedingungen (z.B. Polymerisationszeit, pH-Wert und Ionenstärke für die Adsorption, etc.) für diejenigen unterschiedlichen Polymeroberflächen optimiert, deren Oberflächeneigenschaften mit denen der SEAC-Proben übereinstimmen.

Hierbei handelt es sich um folgende Oberflächen:

- Hydrophobe Oberflächen,
- Ionische Oberflächen,
    - Kationen-Austausch,
    - Anionen-Austausch,
- Metallionenaffinitätsoberflächen.

Zusätzlich beschreibt die vorliegende Arbeit, wie MALDI-chips zur Bestimmung der Protein-Aktivität (z.B. Hemmungsstudien), zur Charakterisierung der Protein-Oberflächen-Interaktionen und zur Genom-Forschung (durch die Bildung einer Mikroarray-Plattform) verwendet werden können.

6. References

(1) Werner, T. *Mass spectrom. Rev.* **2004**, *23*, 25-33
(2) Humphery-Smith, I; Cordwell, S.J.; Blackstock, W.P. *Electrophoresis* **1997**, *18*, 1217-1242.
(3) Anderson, N.G.; Anderson, L. *Clinical Chemistry* **1982**, *28*, 739-748
(4) Wasinger V.C.; Cordwell S.J.; Cerpa-Poljak A.; Yan J.X.; Gooley A.A.; Wilkins M.R.; Duncan M.W.; Harris R.; Williams K.L.; Humphery-Smith I. *Electrophoresis*, **1995**, *16*, 1090-1094
(5) Twyman, R. M. **2004**. *Principles of proteomics*. First edition, W.H. Garland Science/BIOS Scientific Publishers.
(6) Mirsei, H. *Journal of Chromatography B* **2005**, *817*, 23–34
(7) Goldsby, R.A.; Kindt, T.J.; Osborne, B.A., **2003**. *Kuby Immunology*. Fourth edition, W.H. Freeman and company.
(8) Pandey, A.; Mann, M. *Nature* **2000**, *405*, 837-846.
(9) Quadroni, M.; Peter, J. *Electrophoresis* **1999**, *20*, 664-677
(10) Guzman, N. *Anal Bioanal Chem* **2004**, *378*, 37–39
(11) Chiari, M.; Cretich, M.; Damin, F.; Ceriotti, L.; Consonni, R. *Electrophoresis* **2000**, *21* (5), 909-916.
(12) Lilley, K.S.; Razzaq, A.; Dupree, P. *Current Opinion in Chemical Biology* **2002**, *6*(1), 46-50
(13) Görg, A.; Weiss, W.; Dunn, M.J. *Proteomics* **2004**, *4*(12), 3665-3685
(14) O'Farrell, P.H. *Journal of Biological Chemistry* **1975**, *250*, 4007-4021
(15) Righetti, P.G.; Castagna, A.; Herbert, B.; Reymond, F.; Rossier, J.S. *Proteomics* **2003**, *3*, 1397–1407
(16) Lee, Y.-S.; Mrksich, M. *Trends in Biotechnology* **2002**, *20*(12), s14-s18
(17) Halla, D.A; Ptaceka, J.; Snyde, M. *Mechanisms of Ageing and Development* **2007**, *128*(1), 161-167
(18) Aebersold, R.; Mann, M. *Nature* **2003**, *422*, 198-207
(19) Ong, S.-E.; Mann, M. *Nature Chemical Biology* **2005**, *1*, 252-262
(20) Tanaka, K.; Waki, H.; Ido, Y.; Akita, S.; Yoshida, Y.; Yoshida, T. *Rapid Comm. in Mass Spectrom.* **1988**, *2*(20), 151-153
(21) Karas, and Hillenkamp. *Anal. Chem.* **1988**, *60*, 2299-2301
(22) Karas, M.; Bachmann, D.; Hillenkamp, F. *Anal. Chem.* **1985**, *57*, 2935-2939.
(23) Changa, W.C.; Huanga, L.C.L; Wanga, Y.-S.; Penga; W.-P.; Changb, H.C.; Hsuc, N.Y.; Yanga W.B.; Chen, C.H. *Analytica Chimica Acta* **2007**, *582*(1), 1-9.
(24) Nordhoff, E.; Ingendoh, A.; Cramer, R.; Overberg, A.; Stahl, B.; Karas, M.; Hillenkamp, F.; Crain, P.F. *Rapid Commun Mass Spectrom.* **1992**, *6*(12), 771-776.
(25) Stults, J.T. *Current opinion in Structural biology* **1995**, *5*, 691-698
(26) Tang, Y-W; Stratton, C.W. **2006.** *Advanced Techniques in Diagnostic Microbiology*. First Edition, Springer Science Business Media, LLC.
(27) Murgasova, R.; Hercules, D.M. *International Journal of Mass Spectrometry* **2003**, *226*(1), 151-162.
(28) Herod, A.A.; Zhuo, Y.; Kandiyoti, R. *Journal of Biochemical and Biophysical Methods* **2003**, *56*(1-3), 335-361.
(29) Guan, B.; Lu, W.; Fang, J.; Cole, R.B. *J Am Soc Mass Spectrom.* **2007**, *18*(3), 517-24.
(30) Shroff, R.; Muck, A.; Svatos, A. *Rapid Communications in Mass Spectrometry* **in print**

(31) Roe, M. R.; Griffin, T. J. *Proteomics* **2006**, *6*, 4678-4687.
(32) Peterson, D. S.; Rohr, T.; Svec, F.; Frechet, J. M. J. *J. Proteome Res.* **2002**, *1*, 563-568.
(33) Gobom, J.; Nordhoff, E.; Ekman, R.; Roepstorff, P. *Int. J. Mass Spectrom. Ion Processes* **1997**, *169/170*, 153-163.
(34) Park, Z-Y.; Russell, D. H. *Anal. Chem.* **2001**, *73*, 2558-2564.
(35) Hutchens, T.W.; Yip, T.-T. *Rapid Commun in Mass Spectrom.* **1993**, *7*, 576-580.
(36) Goshe, M. B. Brief. Funct. *Genomics Proteomics* **2006**, *4*, 363-376.
(37) Laremore, T. N.; Murugesan, S.; Park, T.-J.; Avci, F. Y.; Zagorevski, D. V.; Linhardt, R. J. *Anal. Chem.* **2006**, *78*(6), 1774-1779.
(38) Mank, M.; Stahl, B.; Boehm, G. *Anal. Chem.* **2004**, *76*(10), 2938-2950.
(39) Armstrong, D. W.; Zhang, L.-K.; He, L.; Gross, M.L. *Anal. Chem.* **2001**, *73*(15), 3679-3686.
(40) Tang, N.; Tornatore, P.; Weinberger, S.R. *Mass spectrometry reviews* **2004**, *23*, 34-44
(41) Nordhoff, E.; Egelhofer, V.; Giavalisco, P.; Eickhoff, H.; Horn, M.; Przewieslik, T.; Theiss, D.; Schneider, U.; Lehrach, H.; Goborn, J. *Electrophoresis* **2001**, *25*, 2844-2855.
(42) Manz, A.; Graber, N.; Widmer, H.M. *Sens. Actuators B* **1990**, *1*, 244-248
(43) DeVoe, D.L.; Lee, C.S. *Electrophoresis* **2006**, *27*, 3559-3568.
(44) Limbach, P.A.; Meng, Z. *Analyst* **2002**, *127*, 693-700.
(45) Laurell, T.; Nilsson, J.; Marko-Varga, G. TrAC *Trends in Analytical Chemistry* **2001**, *20*, 225-231.
(46) Miliotis, T.; Marko-Varga, G.; Nilsson, J.; Laurell, T. J. *Neuroscience Methods* **2001**, *109*, 41-46.
(47) Marko-Varga, G.; Ekström, S.; Helldin, G.; Nilsson, J.; Laurell, T. *Electrophoresis* **2001**, *22*, 3978-3983.
(48) Ekström, S.; Nilsson, J.; Helldin, G.; Laurell, T.; Marko-Varga, G. *Electrophoresis* **2001**, *22*, 3984-3992.
(49) Berhane, B.T.; Limbach, P.A. *Anal. Chem.* **2003**, *75*, 1997-2003.
(50) Dominick, W.D.; Berhane, B.T.; Mecomber, J.S.; Limbach, P.A. *Anal. Bioanal. Chem.* **2003**, *376*, 349-354.
(51) Frankevich, V. F.; Zhang, J.; Friess, S. D.; Dashtiev, M.; Zenobi, R. *Anal. Chem.* **2003**, *75*, 6063–6067.
(52) Strupat, K.; Karas, M.; Hillenkamp, F.; Eckerskorn, C.; Lottspeich, F. *Anal. Chem.* **1994**, *66*, 464-470.
(53) Walker, A.K.; Wu, Y.; Timmons, R.B.; Kinsel, G. *Anal. Chem.* **1999**, *71*, 268-272.
(54) Walker, A.K.; Qiu, H.; Wu, Y.; Timmons, R.B.; Kinsel, G *Anal. Biochem.* **1999**, *271*, 123-130.
(55) Walker, A.K.; Land, C.M.; Kinsel, G.; Nelson, K.D. *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 62-68.
(56) Griesser, H.J.; Kingshott, P.; McArthur, S.L.; McLean, K.M.; Kinsel, G.R.; Timmons, R.B. *Biomaterials* **2004**, *25*, 4861-4875.
(57) Owen, S. J.; Meier, F. S.; Brombacher, S.; Volmer; D. A. *Rapid Comm. Mass Spectrom.* **2003**, *17*, 2439–2449.
(58) Ibáñez, A.; Muck, A.; Svatoš, A. *Journal of Mass Spectrometry* **2007**, *42*, 634–640
(59) Henry, A.C.; Tutt, T.J.; Galloway, M.; Davidson, Y.Y.; McWhorter, C.S.; Soper, S.A.; McCarley, R.L. *Anal. Chem.* **2000**, *72*(21), 5331-5337.
(60) Svatoš, A.; Muck, A., *Pat. App.* **E102004053458, WO2006048306**

(61) Muck, A.; Nesnërová, P.; Pichová, I.; Svatoš, A. *Electrophoresis* **2005**, *26*, 2835-2842.
(62) Muck, A.; Wang, J.; Jacobs, M.; Chen, G.; Chatrathi, M. P.; Jurka, V.; Vyborny, Z.; Schöning, M. J., *Anal. Chem.* **2004**, *76*, 2290–2297.
(63) Karas, M.; Glückmann, M.; Schäfer, J. *J. Mass Spectrom.* **2000**, *35*(1), 1-110.
(64) Frankevich, V.; Zhang, J.; Dashtiev, M.; Zenobi, R. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 2343-2348.
(65) Dashtiev, M; Frankevich, V; Zenobi, R. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 289-291.
(66) Knochenmuss, R. *Anal. Chem.* **2004**, *76*, 3179-3184.
(67) Knochenmuss, R.; Zhigilei, L.V. *J. Phys. Chem. B* **2005**, *109*, 22947-22957.
(68) Setz P.D.; Knochenmuss R. *Journal of physical chemistry. A* **2005**, *109*(18), 4030-4037
(69) William, H.T.; Tai-Tung, Y. *US. Pat.* **US6225047**
(70) William, H.T.; Tai-Tung, Y. *US. Pat.* **US6579719**
(71) William, H.T.; Tai-Tung, Y. *US. Pat.* **US2001014461**
(72) William, H.T.; Tai-Tung, Y. *US. Pat.* **US2003096224**
(73) Din, S.; Lennon, A.M.; Arnott, I.D.R.; Hupp, T.; Satsangi, J. *Nature Clinical Practice Gastroenterology & Hepatology* **2007**, *4*, 372-385
(74) Liang, Y.; Fang, M.; Li, J.; Liu, C.B.; Rudd, J.A.; Kung, H.F.; Yew, D.T. *Exp. Mol. Pathol.* **2006**, *81*(2), 176-80.
(75) Nelson, D.L.; Cox, M.M. **2004** *Lehninger Principles of Biochemistry*, W. H. Freeman; 4 edition.
(76) Brown, P.O.; Botstein, D. *Nat. Genet.* **1999**, *21*, 33-37.
(77) Zhou, X; Wu, L.; Zhou, J. *Langmuir* **2004**, *20*, 8877-8885.
(78) Wong, A.; Krull, U. *Anal. Bioanal. Chem.* **2005**, *383*, 187-200.
(79) Finnskog, D.; Ressine, A.; Laurell, T.; Marko-Varga, G. *J. Proteome Res.* **2004**,*3*, 988-994.
(80) Fodor. S.P.A. *Science* **1999**, *277*, 393-395.
(81) Schena, M.; Shalon, D.; Davis, R.W.; Brown, P.O. *Science* **1995**, *270*, 467-470.
(82) Okamoto, T.; Suzuki, T.; Yamamoto, N. *Nature* **2000**, *18*, 438-441.
(83) M. Schena. **2003.** *Microarray Analysis*. First edition. John Wiley's & Sons.
(84) Mirkin, C.A.; Letsinger, R.L.; Mucic, R.C.; Storhoff, J.J. *Nature* **1996**, *382*, 607-609.
(85) Li, J.; Xue, M.; Lu, Z.; Zhang, Z.; Feng, C.; Chan, M. *IEEE Transactions on Electron Devices* **2003**, *50*, 2165- 2170.
(86) Fritzsche, W.; Taton, T. A. *Nanotechnology* **2003**, *14*, R63-R73
(87) Möller, R.; Powell, R. D.; Hainfeld, J. F. Fritzsche,W. *Nano Letters* **2005**, *5*, 1475-1482.
(88) Willner, I.; Baron, R.; Willner, B. *Adv. Mater.* **2006**, *18*, 1109-1120.
(89) Zhang, C.Y.; Yeh, H.C.; Kuroki, M.T.; Wang, T.H. *Nat. Mater.* **2005**, *4*, 826-831.
(90) Wang, J. *Small* **2005**, *1*, 1036-1043.
(91) Nebling, E.; Grunwald, T.; Albers, J.; Schäfer, P.; Hintsche, R.. *Anal. Chem.* **2004**, *76*, 689-696.
(92) Wang, J. *Nucl. Acids Research* **2000**, *28*, 3011-3016.
(93) Yguerabide, J.; Yguerabide, E. E. *J. Cell. Biochem. Suppl.* **2001**, *37*, 71-81.
(94) Kukanskis, K.; Elkind, J.; Melendez, J.; Murphy, T.; Miller, G.; Garner, H. *Anal. Biochem.* **1999**, *274*, 7-17.

(95) Kepper, P.; Reinhardt, R.; Dahl, A.; Lehrach, H.; Sauer, S. *Clin. Chem.* **2006**, *52*, 1303-1310.
(96) Schuerenberg, M.; Laubbert, C.; Eickhoff, H.; Kalkum, M.; Lehrach, H.; Nordhoff, E. *Anal. Chem.* **2000**, *72*, 3436-3442.
(97) Doherty, E.A.S.; Meagher, R.J.; Albarghouthi, M.N.; Barron, A. *Electrophoresis* **2003**, *24*, 34-54.
(98) Eteshola, E.; Leckband, D. *Sensors and Actuators B* **2001**, *72*, 129-133.
(99) Rollin, D.; Whistler, T.; Vernon, S.D. *Proteome Science* **2007**, *5*, 9
(100) Henderson, N. A.; Steele, R.J.C. *The surgeon* **2005**, *3*(6), 383-390
(101) Sivashankari, S.; Shanmughavel, P. *Bioinformation* **2007**, *1*(9), 376-378.

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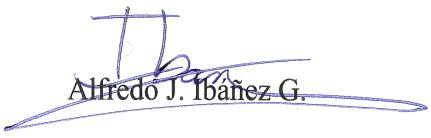
8. Declaration of Independent Assignment

- I declare in accordance with the conferral of the degree of doctor in philosophy from the School of Biology and Pharmacy of Friedrich Schiller University Jena that the submitted thesis was written only with the assistance and literature cited in the text.

- I declare that the people who assisted in the experiments, data analysis, and writing of the manuscript are listed as co-authors of the respective manuscripts. I was not assisted by a consultant for doctorate theses.

- I declare that the content of this thesis has not been previously submitted for the conferral of the degree of doctor in philosophy either to the Friedrich-Schiller-University, Jena, or to any other University.

Jena, 23 of October, 2007



Alfredo J. Ibáñez G.

Alfredo J. Ibanez
Küntzel Str. 6
45147 Essen, Germany
E-mail: a.j.ibanez@gmail.com
Phone: +491727374749

Objective

My professional objective is to develop the field of clinical analysis, in particular the branches of proteomic analysis (e.g. protein identification, quantization, interaction, etc), and clinical analysis (e.g. biosensors, immunoassays, etc) and their applications, to the greatest extent possible to solve the current problems, which affect state-of-the-art instrumentation such as Lab-on-a-chip and microarray devices.
I hope to find an interesting position involving interdisciplinary research projects, and in the future lead a research group in which my team and I share our knowledge, understanding and research experience, pooling our strengths to pursue profitable goals, making an impact in society by developing novel technologies for genomic, proteomic and clinical analysis.

Background

- Analytical skills and knowledge includes: separation techniques (HPLC, LC, GC, CE, and 2D-PAGE), microscopy techniques (optical, electron, and scanning probe microscopy), spectroscopy techniques (UV-, IR-, Raman-, and NMR), electroanalytical methods (amperometry, and voltammetry), and mass spectrometry.
- Data handling and interpretation, writing and reviewing of analytical methodologies, standard operating procedures (SOP's) and scientific articles.
- Development and applications of microfabricated well/liquid planar structures made of polymeric materials that can mimic stationary phases that are commonly used in liquid chromatography.
- Research and development for MALDI-MS-based proteomics and genomic applications.
- Maintenance of MALDI-TOF/MS instruments (e.g. TofSpec 2E, MALDI micro MX).
- Research and development of micro total analysis systems using different electrochemical and optical detectors.
- Design, use and maintenance of in-house built capillary electrophoresis-based Lab-on-a-chip systems and piezoelectric immunosensors for clinical applications.
- Training and supervisory skills

Professional record

Employer:	Scienion AG. Germany
Function:	Industrial internship
From - Until:	April, 2008 – Present

Details:	• Development of polymeric surface-enhanced affinity-capture MALDI-MS targets for proteomic analysis using MALDI-TOF/MS instruments.
Employer:	Walter Perich (Perser S.A.), Peru
Function:	Independent consultant
From - Until:	January, 2005 – Present
Details:	• Provide specialized assessment on new discoveries and potential industrial implications in the area of Silanes • Advise regarding raw material providers, and new markets.
Employer:	Patricia Anaya (LEA & Knit S.A.C.), Peru
Function:	Independent consultant
From - Until:	September, 2004 – Present
Details:	• Provide specialized assessment on new discoveries and potential industrial implications in the area of artificial colorants. • Advise regarding raw material providers, and new markets.
Employer:	Del Oro S.A., Peru
Position:	Internship in the Quality control area.
From - Until:	January, 1996 – March, 1996
Details:	• Quantitative/qualitative chemical analysis • Reading and reviewing of analytical data. Writing reports.

Research Experience

Employer:	Max Planck Institute for Chemical Ecology, Germany
Function:	Graduate Researcher
From - Until:	April, 2005 – April, 2008
Details:	• Use of MALDI-TOF/MS instruments (Waters: TofSpec 2E, MALDI micro MX) and AP/MALDI-LCQ (Thermo Fisher Scientific). • Development (i.e. design and set-up) of in-house built surface-enhanced affinity capture MALDI-MS targets for proteomic analysis using MALDI-TOF/MS instruments. • Development (i.e. design and set-up) of in-house built DNAchip for MALDI-TOF/MS genomic studies. • Development (i.e. design and set-up) of in-house built protein arrays for enzyme characterization studies (e.g. activity, possible inhibitors, etc.), and for phosphoproteome studies (e.g. identification, and characterization of phosphopeptides in a mixture). • Reading and reviewing of analytical proteomic and genomic data. Writing reports and articles.
Employer:	Groningen University Institute for Drug Exploration, The Netherlands

Function:	Graduate Researcher
From - Until:	October, 2003 – March, 2005
Details:	• Fabrication of a miniaturized capillary electrophoresis separation system (i.e. Lab-on-a-chip) coupled to a laser-induced fluorescence detector. • Automatization, using LabView software, of the above system. • Reading and reviewing of analytical data. Writing reports.
Employer:	New Mexico State University, USA
Position:	Teaching Assistant / Graduate Researcher
From - Until:	July, 2000 – July, 2003
Details:	• Development (i.e. design and set-up) of heterogeneous and homogeneous immunoassays using miniaturized capillary electrophoresis separation device (i.e. Lab-on-a-chip) coupled to amperometric and/or conductivity detector. • Development (i.e. design and set-up) of enzymatic assays using miniaturized capillary electrophoresis separation device (i.e. Lab-on-a-chip) coupled to amperometric and/or conductivity detector. • Design & use of in-house built macro- to micro-world interfaces, as well as automatization, using LabView software, of the above mentioned systems. • Responsible for training, supervising and providing analytical technical support to research technicians and students (MSc postgraduate & undergraduates). • Reading and reviewing of analytical data. Writing reports and articles.
Employer:	Pontificia Universidad Catolica del Peru, Peru
Position:	General Chemistry Lecturer / Undergraduate Researcher
From - Until:	March, 2000 – July 2000
Details:	• Development and fabrication of a Piezoelectric Immunosensor for a growth hormone biomarker (detection of 3,3',5-Triiodo-L-thyronine). • Reading and reviewing of analytical data. Writing reports. • Responsible for teaching, supervising and providing support to first, second, and third year university students (undergraduates).
Employer:	Instituto de Corrosión y Proteccion (ICP-PUCP), Peru
Position:	Internship in the Consulting (R&D) area.
From - Until:	January, 1998 – August, 1998
Details:	• Quantitative/qualitative chemical analysis • Reading and reviewing of analytical data. Writing reports.

Education

Dr. rer. Nat.(Ph.D.):	Biology (Biophysics)
University:	Friedrich Schiller University; Max Planck Institute for Chemical Ecology, Jena, Deutschland
Supervisor:	Prof. Dr. Hans-Peter Saluz (FSU); Dr. Aleš Svatoš (MPI-CE)
From - Until	April, 2006 – February, 2008
Graduation Date:	2008 (expected)
M.Sc.:	Chemistry (Analytical)
University:	New Mexico State University, Las Cruces, USA
Supervisor:	Prof. Dr. Joseph Wang
From - Until	July, 2000 – December, 2002
Graduation date:	May, 2003
Licentiate:	Chemistry (Analytical)
University:	Pontifical Catholic University of Peru, Lima, Peru
Supervisor:	Dr. Eric Cosio
From - Until:	January, 2000 – July, 2000
Graduation Date:	July, 2000
B.Sc.:	Chemistry
University:	Pontifical Catholic University of Peru, Lima, Peru
From - Until:	March, 1995 – December, 1999
Graduation Date:	July, 2000

Language proficiency

- Spanish — Native language
- English — Level: Proficient (oral/written)
- German — Level: Beginner (oral/written)

Fellowships and awards

International Max Planck Research School fellowship on "The Exploration of Ecological Interactions with Molecular and Chemical Techniques" (IMPRS-MCE), in Jena, Germany. (April, 2006 – April, 2008)

Personal data

Birth date : 23rd of August 1977
Status : Married
Availability : Worldwide, no restriction
Hobbies : Travel, movies, open-air activities

Profile : An honest, responsible and pro-active person, I am good at organize a team to carry out long-term, complex projects. Eager to share my experience and increase my knowledge, I will bring enthusiasm and creativity to any position. I keep a global view of situations, despite deadlines and pressure.

References : Prof. Dr. Hans-Peter Saluz, Cell and Molecular Biology department head

- Beutenbergstr. 11a, 07745 Jena
  Thüringen, Germany
- Fax: +49 3641656689
- E-mail: hanspeter.saluz@hki-jena.de

Dr. Aleš Svatoš, Mass Spectrometry research group leader

- Hans-Knöll-Str. 8, 07745 Jena
  Thüringen, Germany
- Fax: +49 3641571701
- E-mail: svatos@ice.mpg.de

Patricia Anaya, LEA & Knit S.A.C., CEO

- Bustamante y Ballivian 170, San Isidro
  Lima, Peru
- Fax: +5114400822
- E-mail: anayapatricia@gmail.com

List of Publications & Presentations

Publications:

- A dual fluorescent/MALDI chip platform for the rapid and specific analysis of enzymatic activity and protein profiles
  Halim, V.A.; Muck, A.; Hartl, M.; Ibáñez, A; Giri, A.; Erfuth, F.; Baldwin, I.T.; Svatoš, A.
  Molecular & Cellular Proteomics, **submitted**
- DNA detection using a novel triple read-out optical/AFM/MALDI planar microwell plastic chip.
  Ibáñez, A.; Schüler, T.; Möller, R.; Fritzsche, W.; Saluz, H.-P.; Svatoš, A.
  Analytical Chemistry, **submitted**
- Metal-chelating plastic MALDI (pMALDI) chips for the enhancement of phosphorylated- peptide/protein signals
  Ibáñez, A.; Muck, A.; Svatoš, A.
  Journal of Proteomic Research **2007**, *6*, 3842-3848.
- Dissipation of Charge on MALDI TOF Polymeric Chips using an Electron-acceptor: Analysis of Proteins
  Ibáñez, A.; Muck, A.; Svatoš, A.
  Journal of Mass Spectrometry **2007**, *42*, 634–640.
- Trypsin-linked copolymer MALDI chips for fast protein identification
  Ibáñez, A.; Muck, A.; Halim, V.; Svatoš, A.
  Journal of Proteomic Research **2007**, *6*(3), 1183-1189.
- Atmospheric molding of ionic copolymer MALDI-TOF MS arrays: a new tool for protein identification/profiling
  Muck, A.; Ibáñez, A.; Stauber, E. J.; Mansurova, M.; Svatoš, A.
  Electrophoresis **2006**, *27*, 4952-4959.

- Extending the potential of Polydimethylsiloxane microchips
  Ibáñez, A.
  Revista de Química **2005**, *19*(1), 21-26
- On-Chip integration of enzyme and immunoassays: simultaneous measurements of insulin and glucose.
  Wang, J.; Ibáñez, A.; Chatrathi, M.P.
  Journal of the American Chemical Society **2003**, *125*, 8444-8445.
- Micromachined separation chips with post-column enzymatic reactions of "class" enzymes and end-column electrochemical detection: Assays of amino acids.
  Wang, J.; Chatrathi, M.P.; Ibáñez, A.; Escarpa, A
  Electroanalysis **2002**, *14*, 400-404.
- Microchip-based amperometric immunoassays using redox tracers.
  Wang, J.; Ibáñez, A; Chatrathi, M.P.
  Electrophoresis **2002**, *23*, 3744-3749.
- Electrochemical enzyme immunoassays on microchip platforms.
  Wang, J.; Ibáñez, A; Chatrathi, M.P.; Escarpa, A
  Analytical Chemistry **2001**, *73*, 5323-5327.
- Glucose biochip: dual analyte response in connection to two pre-column enzymatic reactions.
  Wang, J.; Chatrathi, M.P.; Ibáñez, A.
  The Analyst **2001**, *126*, 1203-1206.

Oral presentations:

- Plastic MALDI chips (pMALDI)
  Ibáñez, A.
  2nd Beutenberg-Campus-Workshop **(June 22nd, 2007)**

Poster presentations

- Plastic Development of multiple read-out planar microarray plastic devices for genomic and proteomic applications
  Ibáñez, A.; Muck, A.; Halim, V.; Schüler, T.; Möller, R.; Saluz, H.-P.; Svatoš, A.
  Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy (Pittcon 2008), New Orleans, USA **(March 2nd – 6th, 2008)**
- Development of polymeric planar microarray devices (pMALDI)
  Ibáñez, A.; Muck, A.; Halim, V.; Svatoš, A.
  1st Central and Eastern European Proteomic Conference and 3rd Czech Proteomic Conference, Prague, Czech Republic **(October 29th – 31th, 2007)**
- Plastic MALDI chips (pMALDI): enhancing protein analysis using high-density polymer microarrays in combination with MALDI-TOF/MS
  Ibáñez, A.; Muck, A.; Halim, V.; Svatoš, A.
  55th ASMS Conference on Mass Spectrometry, Indianapolis, USA **(June 2nd – 8th, 2007)**
- Plastic MALDI chips (pMALDI)
  Ibáñez, A.; Muck, A.; Halim, V.; Svatoš, A.

40th Diskussionstagung der Deutsche Gesellschaft für Massenspektrometrie, Bremen, Germany **(March 11th – 14th, 2007)**

- Protein profiling using co-polymer MALDI-TOF/MS chips
  Ibáñez, A.; Halim, V.; Mansurova, M.; Stauber, E.J.; Muck, A.; Svatoš, A.
  International Mass Spectrometry Conference, Prague, Czeck Republick **(August 27th – September 1st, 2006)**
- Electron acceptor-enhanced dissipation of charge on polymeric chips used for MALDI-TOF analysis of proteins
  Ibáñez, A.; Muck, A.; Svatoš, A.
  39th Diskussionstagung der Deutschen Gesellschaft für Massenspektrometrie, Mainz, Germany **(March 8th – 11th, 2006)**

Interviews:

The doctors' view in Peru
Zubritsky, E.
Analytical Chemistry **2004**, *76*, 349A-350A.