Dissertation

"Polymer-basierende Probenträger für die MALDI (matrix-unterstützte Laserdesorption/ionisation) Massenspektrometrie von Biomolekülen und intakten Zellen"

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Univ.-Prof. Mag. Dr. Günter Allmaier
Institut für Chemische Technologien und Analytik/164-IAC

ingereicht an der Technischen Universität Wien

Fakultät für Technische Chemie

von

Mag. rer. nat. Stefan Bugovsky
Matrikelnummer e0002102
Matznergasse 40/16, 1140 Wien

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I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale. We should not allow it to be believed that all scientific progress can be reduced to mechanisms, machines, gearings, even though such machinery also has its beauty. Neither do I believe that the spirit of adventure runs any risk of disappearing in our world. If I see anything vital around me, it is precisely that spirit of adventure, which seems indestructible and is akin to curiosity.

Marie Curie
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1. Zusammenfassung


Neben der Entwicklung und Evaluierung eines solchen MALDI Targets mit entsprechender Kontrolle der Einsetzbarkeit (Erhalt bzw. Verbesserung der MALDI MS Kenndaten (massenspektrometrische Auflösung, Sensitivität, Reproduzierbarkeit)) auf dem Weg zu einem fertigen Großserienprodukt wurde dieses schließlich auch in seiner Gesamtheit charakterisiert, d.h. Stabilität der Kalibration, abdeckbarer m/z-Bereich sowie Detektions- und Quantifizierungsgehalt gegenüber einem traditionellen Vollmetall-Target. Weiters wurden auch andere Qualitätsparameter wie Planarität und Oberflächenbeschaffenheit des neuen MALDI Probenträgers ermittelt.


In allen Anwendungsbereichen konnte sich das polymerbasierende MALDI Target mit nanobeschichteter Metalloberfläche als äquivalent oder sogar überlegen gegenüber dem Standardreferenztarget (Edelstahl-Vollmetalltarget) erweisen.
2. **Abstract**

The aim of this work is the establishing of a polymer-based sample support for MALDI (matrix assisted laser desorption/ionization) mass spectrometry. The up to this date predominant sample supports ("targets") are based on metal as bulk material (e.g. stainless steel or nickel coated aluminum). A polymer-based product promises some useful properties that cannot be realized with a conventional full metal target. Among these are the use as a disposable device, no need for a cleaning step after a series of analyses, consistent surface properties and affordable archiving. The use of a disposable device mitigates sample and matrix carryover from previous measurements. The omittance of a cleaning step saves costs in many forms and helps reduce the amount of solvent waste. Furthermore, the defined surface of a disposable polymer target with every sample preparation facilitates the adherence to existing GLP requirements in labs.

Besides developing and evaluating such a MALDI target during development with according usage tests (retaining or improving of MALDI MS key parameters (mass spectrometric resolution, sensitivity, reproducibility)) on the way to a mass produced product, it was also characterized fully, i.e. by its stability of calibration, achievable m/z-range and limit of detection and limit of quantification in comparison to a traditional full metal-target as a reference. Other quality parameters like planarity and surface properties of the new MALDI sample holder were also studied.

For the documentation of the usability of the new MALDI target in various fields of application Intact Cell Mass Spectrometry was used for differentiation of various bacterial and fungal strains and the results compared to a classic full metal target. Furthermore, the application of this target type for quality control in oligonucleotide synthesis via MALDI mass spectrometry was evaluated. The use of the new polymer based target as an archiving platform for MALDI protein and peptide preparation was studied for a time frame of 6 months and und under different environmental conditions.

The polymer based disposable target with nano-coated metal surface proved to perform equal to or even better than the reference (stainless steel full metal target) in all selected applications.
3. Introduction

Metal sample supports (“targets”) have long been the standard in MALDI mass spectrometry, although there were always research groups that tried to develop polymer substitutes. A full metal target seemingly offers enough benefits over a polymer product, but as is the case in most situations, this depends on the circumstances. The thought that MALDI sample supports made from metal can be used \textit{ad infinitum} is easily understandable, but is a flawed conclusion. Over time, these devices collect a number of scratches, and accumulate local chemical modifications due to previous samples or cleaning procedures or, in the case of metal targets coated by a different metal (e.g. nickel-coated aluminum) can develop bubbles as was evidenced in our lab and others, too. Often operators will also encounter sample material such as polymer samples than simply cannot be completely removed from the surface. While these remnants are often visible with the naked eye, sometimes an improper cleaning procedure can lead to measuring artifacts from previous samples or hamper the actual analysis. Chemical modifications or scratches may lead to matrix/sample preparations that appear different, sometimes to the extent that the sensitivity of the measurement decreases. All of these problems have been encountered in the course of this doctoral thesis and by numerous other laboratories worldwide without any dedicated publications concerning these issues.

Disposable polymer targets on the other hand forego most of these problems due to the fact that they are pristine and defined every time a new measurement is made and discarded after measurement. Alternatively, these types of targets can be stored at proper archiving conditions for the first time to permit re-analyzing samples at a later time or analyzing it for the first time later on due to the fact of the low costs of these targets. Archiving would be prohibitively expensive (approx. 550 € per microtiter plate format target) using regular metal targets.

Developing a polymer equivalent of an established metal product is not without challenges or pitfalls. Among these problems is the correct choice of polymer, the need to ensure planarity of the surface, establishing conductivity of the surface, tailoring the surface to a particular contact angle to avoid spreading of the sample spots (i.e. making the target surface hydrophobic) and of course guaranteeing a suitable shelf-life.
In this thesis, three different types of targets were used for evaluation, specifically a polymer based metal coated target in microscope slide format (“PBT”, Fleximass DS™), a full metal stainless steel target in microscope slide format (“FMT”, Fleximass™) and a nickel coated aluminum target in microtiter plate format (“MTP”, TO-474)

4. Polymers

4.1. Defining properties

A polymer is by definition more than one subunit – or monomer – linked together by a chemical bond. There are an almost endless number of polymer architectures possible, of which a few are listed below:

- Block polymer: Two or more subunits linked together linearly
- Star polymer: Branching out from a center point
- Comb polymer: Linear backbone with branches
- Brush polymer: Same as comb polymer, but with more than one branch extending from the same branch starting point
- Ring polymer: Closed polymer in a ring shape
- Dendrimers: Branched star-like polymer with a sub-unit as the center. Every generation adds another “shell” to the polymer
- AB₂ star, palm-tree ABₙ (where A and B are polymers of differing composition), Coil-cycle-coil, ...

Another defining property of a polymer is its overall chain length. A consequence of increasing chain length is also increasing melting or – if applicable – boiling point and viscosity [1]. For descriptive purposes as to the chain length the degree of polymerization ($DP$) is usually used [2]. It is simply calculated by the following equation:

$$DP_n = \frac{M_{\text{Polymer}}}{M_{\text{Monomer}}}$$

M...molar mass

When encountering copolymers - polymers with more than one type of monomer - further description is needed. As an example alternating copolymers ([AB]ₙ) and periodic copolymers (AₙBₘ) could be named. The indexes $m$ and $n$ represent the number of polymer subunits that are linked together, where $m$ and $n$ may be equal or differ from each other in number.
All these properties are of a descriptive nature containing the structure of a polymeric material, but practically more relevant are for example the tensile strength and Young’s modulus of elasticity. Both describe the mechanical properties of a polymer. The former is used to quantify the stress a material can endure before breaking, while the second describes, as the name implies, the elasticity, i.e. the deformation a material goes through upon linear stretching.

Polymer degradation is another important factor that determines the shelf-life of any product made of such a material. Degradation can happen due to a large number of effects that often appear not solely by themselves but combined. Environmental conditions such as light, heat and chemical reactions lead to a disruption of the polymeric chains, often through hydrolysis. This manifests itself in physical form as brittleness, cracks in the surface of the product, discoloration and opacity. Countering these adverse effects is sometimes possible by mixing in additives, which in turn might change the behavior of the product to some extent. In any case it is important for companies to get an understanding of the shelf life that can be expected from a polymer based product [3].

For some applications a failure after a month can be tolerable, or even be desired (biodegradable packaging, while in other instances a failure is only acceptable after decades (archive-grade optical media such as CDs or DVDs). To arrive at an estimated shelf life, the properties of the object in question have to be compared shortly after production and in certain intervals at typical storage conditions. Often harsher conditions are also used in parallel, to simulate product transport from production to customer.

A product that is being shipped, for example from Austria to Australia in winter would have to pass a number of different climate zones: At first it would be shipped at subzero temperatures to the airport. During flight it would be subjected to even harsher conditions. Arriving in Australia, the shipment would endure relatively high temperatures, possibly up to 50°C during transport. The temperature differences during transport would then amount to 100°C.

The above example illustrates the conditions a polymer product has to endure during transport. These, of course, are only interesting for short periods of time, as it is not expected that the product remains subjected to these conditions for long. For shelf life determination storage at ambient conditions for extended periods of time is needed. Often tracking key properties over this period allows the extrapolation of a theoretical failing point of the
product. Products that have been very thoroughly investigated are digital media like CDs, DVDs and Blu-Ray disks [4].

4.2. Examples for polymers

A number of polymers have been used for developing MALDI MS targets either as a bulk material or a coating.

Polytetrafluoroethylen (Fig. 1), more commonly known by its brand name Teflon™ (DuPont), is a linear polymer with strong hydrophobic properties. It has one of the lowest coefficients of friction and is very heat and chemical resistant and is thus used for example as a non-stick coating for cooking ware or as a lubricant to reduce wear in machinery. All of these properties make this material to a certain extent ideal for targets in MALDI mass spectrometry [5-8].

![Fig.1 Polytetrafluoroethylen](image)

**Polyurethane** (Fig. 2), a group of polymers rather than a single substance, has also been used as a MALDI MS sample support [9, 10]. The polymer was used in the form of a thin membrane instead of a bulk material. Polyurethane is composed of a chain on organic subunits linked together by urethane links. This is done by a condensation step. It is a very diverse material which properties are defined by the other reaction partners.

![Fig.2 Example of polyurethane](image)

**Silicone** [11, 12] (Fig.3), or more correct polisiloxanes, is an organic-inorganic polymer with a silicon-oxygen backbone with organic side groups such as methyl, ethyl or phenyl. The
most often encountered member of this group is polydimethylsiloxane (PDMS), which has also found its way into labs as a fast prototyping material for developing lab-on-a-chip applications [13].

![Polydimethylsiloxane](image)

*Fig.3 Polydimethylsiloxane*

Linear poly(methyl methacrylate) (PMMA) and linear polystyrene have been used as coating material [14] for MALDI targets to act as hydrophobic anchor to which proteolytic peptides would be able to adsorb in order to be able to desalt a sample. Other applications for surface modification include the monitoring of the redox cycle of low-molecular peptides, which was realized with coating the target with polyfluorodioxidole [15], rendering the surface hydrophobic. Also worth mentioning is a diamond-like carbon coating, which aimed to facilitate laser desorption/ionization without a matrix, but also improve results when using MALDI [16]. The concept of a matrix in MALDI will be explained in chapter 5.1.

Other approaches using either a specially coated target or a target completely based on polymer substance can be found in literature [17-20].

### 4.3. Polyolefins/Polypropylene

The basic building block, or monomer, of a polyolefin is as the name implies an olefin, an alkene with the general formula C\textsubscript{n}H\textsubscript{2n}. The defining properties of such a polymer are a very high chemical inertness and decreased structural strength at lower temperatures. Like most other polymers, a polyolefin is an electric isolator.

One member of this group of polymers is polypropylene (PP) (Fig.4). It is used in a wide range of applications where stability, flexibility, chemical inertness and affordability are critical. Among these applications are packaging, containers, lab equipment, car components and more.
While the material is very durable and resistant against chemicals, it is sensitive to degradation by UV light [21]. UV-absorbing additives to counter that effect are available [22] and can be easily incorporated. An agent that can be used is for instance carbon black, which at high enough concentration in the bulk material also introduces a certain conductivity to the material [23, 24], which is very beneficent for MALDI mass spectrometry, i.e. the application of high voltages between the MALDI target and the first lens or grid in the ion source. It is well-known that a conductive polymer-based MALDI target would have a negligible effect on the electromagnetic field lines in the desorption/ionization region of the ion source compared to a target made of an insulator due to the dielectric nature of polypropylene. A dielectric material is an isolator that can be polarized by applying an electric field [25]. This in turn can cause distortions in the electromagnetic field with detrimental effects on the MALDI performance.

4.4. Physical methods for defining selected polymer product’s properties

4.4.1. Contact angle determination

The contact angle is a simple way to measure the hydrophobicity of a surface, or to be more precise, the interaction between a liquid and a solid surface [26]. This value is measured between the surface and the angle of the outer surface of the liquid at the point where they interface. The contact angle ranges from 0° to 180°, where 0° means the highest possible attraction between the two media. In the case of water being the liquid, 0° equals absolute hydrophilicity. From 90° to 180° the surface is considered hydrophobic.

There are three different ways to measure a contact angle, namely the sessile drop method [27], where a droplet is deposited on a surface and the contact angle measured shortly thereafter (Fig.5). Although this is the most practiced method due to its simplicity, but it has a
few shortcomings. During evaporation, which occurs the entire time the liquid is resting on
the surface to be measured, the volume of the droplet decreases, changing the contact angle.
Further effects that can occur are interaction, chemical reactions or physical effects (e.g.
swelling or dissolution of the solid surface, transport of surface active substances from the
solid to the surface).

![Fig.5 Contact angle measurement of acetonitrile on a polymer-based metal-nanocoated MALDI target](image)

The other two methods to determine the contact angle is by constantly adding volume to the
drop and constantly measuring the contact angle on the forefront, the so-called dynamic
contact angle [28]. In contrast to that, the angle can also be measured by sucking the liquid
from the surface and constantly measuring the angle of the receding front. Both of these
techniques are not suitable for rough surfaces [29], because the receding or advancing drop
would be deformed by the ridges of the surface or lose fluid on extreme features (spikes,
microscopic pillar structures,…), distorting the form of the drop.

The contact angle is usually measured by an optical system (a camera) coupled with a
specifically tailored software. The photos (or video in case of the last two presented methods)
are recorded against a light backdrop or a white light source in order to increase the contrast.

Various mathematical models exist to approximately calculate the contact angle. Due to the
different shapes the droplet may have (flat, round, etc.) the calculation model has to be
adapted accordingly. The theoretically most precise method is based on the Young-Laplace
formula discussed earlier. Other techniques are the so called “circle fitting” and “tangential
line” calculations [30].

The shape of the droplet on the surface is described by the Young-Laplace equation [31].

\[
\Delta p = 2 \gamma H = \gamma \left( \frac{1}{R_1} + \frac{1}{R_2} \right)
\]

Young-Laplace equation
In the above equation, $\Delta p$ is the pressure difference of the interface of the binary system, $\gamma$ is the surface tension, $H$ is the mean curvature and $R_1$ and $R_2$ are the radii of the curvature in each of the axes that are parallel to the surface.

### 4.4.2. Morphology imaging by scanning electron microscopy

While conventional light microscopy is sufficient for most surface studies, some minuscule features can only be observed by means of electron microscopy. A particular subdivision of this field is called scanning electron microscopy (SEM) [32, 33]. Here, an electron beam scans over the target area in a raster pattern. The interaction with the atoms at or near the surface (up to 1 nm depth) result in variety of signals, among them secondary electrons, back-scattered electrons, X-rays and cathodoluminescence. Normally, secondary electron detectors are used for detection. They provide a look at the object, while other signals like characteristic X-rays can provide information like the atomic number of the sample.

SEM allows a view at the microstructure of the sample (Fig.6), in the case of this work a look at the surface of the MALDI target. Here, abnormalities due to the production process are easy to spot, which is especially important in the development phase.

![Fig.6 Comparison of 100x magnifications of metal coated polymer target (left) and full metal target (right)](image)

### 4.4.3. Planarity by chromatic-confocal distance measurement

While planarity is not strictly speaking a feature that is important for polymers per se, it is an important feature of a MALDI target so as to negate a difference in m/z of the acquired signals due to longer flight time because of the higher distance from surface to detector or the
other way round [34]. On full metal targets this is facilitated to a limited extent by precise milling or polishing of the surface to a defined height. This approach cannot be taken with polymers. Products that originated from an injection molding process are defined by the mold the polymer is pressed into. After the initial production step, shrinkage may occur which has to be taken account for in the mold, so that the end product reaches its end dimensions correctly [35]. This may result in a skewed product which inevitably leads to problems during MALDI MS measurements. This effect is further enhanced by thin plastic products. Distortions of the material may be counteracted by using a different polymer or different production parameters (e.g. temperature, cooling time.) Some degree of skew or warping will be present in the end product and countermeasures during MALDI MS measurement are to slide the target into an adapter/holder device (see Fig. 7) which acts as a sample stage for the vacuum chamber of the mass spectrometer. Here, the target is pressed from below into the frame above by means of a spring plate (see Fig. 8).

Fig. 7 Target adapter with polymer target inserted; in the top right featured is a full metal target
Planarity can be measured in various ways. Two that were employed in this work are the direct chromatic-confocal distance measurement [36, 37] and the indirect measurement of the m/z value differences (by applying the same calibration) of a MALDI calibrant across the whole target (details see manuscript “Polymer-based metal nano-coated disposable target for matrix-assisted and matrix-free laser desorption/ionization mass spectrometry”, chapter 9.1)

Chromatic-confocal distance measurement uses the dispersion of white light through a lens. This means that the white light is split into all colors due to the different angles the individual wavelengths exit the lens. The beam is reflected by the surface of interest and the beam is fed through the same lens. A spectrometer then determines the dominant wavelength of the reflected light, which can be translated to the distance the object was from the lens.

While not strictly a feature of planarity, surface roughness is also worth elaborating on. The surface roughness of the polymer MALDI target determines crystal formation of the MALDI matrix/sample preparation and affects droplet form, although the main contributor to that is, of course, the surface energy, i.e. the hydrophilicity of the material. The principle by which the roughness of the surface is measured is interferometry [38], which uses the superposition of electromagnetic waves. The topology of the object that is placed under the interferometer produces interference patterns which can be used to calculate height information over an x-y plane with a measurement resolution of up to 10 nm in the z-axis.

4.4.4. Planarity by mass spectrometric data

Differences in m/z value over the whole sample support give only crude information about the planarity of the surface, but it highlights the effects the skew and warp has on the measurement. For this, mass spectra from a known analyte which has been deposited in a
large raster (e.g. 4 by 12 sample spots) are acquired and the shift in m/z value noted. A plot of this data against the x/y-values of the individual measurements location shows the planarity of the target.

5. Matrix-Assisted Laser Desorption/Ionization time-of-flight mass spectrometry (MALDI TOF MS)

Over the years, mass spectrometry has developed a great number of ways to desorb, ionize, separate and detect an analyte. This work will focus on the techniques used in the line of the work of developing the disposable polymer targets. Therefore, the terms “MALDI” and “time of flight” will be explained in detail, while all other methods that were not actively used will at the most be only hinted at.

The principle of MALDI-MS was in fact developed by two groups around the same time (1985-1988). The first approach used ultra-fine cobalt powder suspended in glycerine as liquid matrix [39, 40], while the other used an organic aromatic substance which was cocrystallized with the analyte to form a solid [41, 42]. The ability to choose a wide range of small organic molecules as matrix addressing various analytic needs (m/z range, resolution, sensitivity) made the method developed by Karas and Hillenkamp [35] more versatile, which is why it is the method of choice in use today.

5.1. Concept of the MALDI process

MALDI belongs along with ESI (electrospray ionization) to the so-called soft ionization techniques (allowing separation and detection of polar, thermolabile and high-mass molecules without breaking these apart). Here, the analyte is mixed up to a thousand fold with a substance that is called a matrix, which will be elaborated upon later on. The process – in short – starts with the analyte-matrix mix being irradiated with a high-energy laser beam, which excites the matrix substance. The resulting thermal expansion gives a small plume containing analyte and matrix. Two theories exist which try to explain the subsequent ionization process in case of not preformed ions [43, 44]. The older often encountered theory relies on photoionization [45]. The theory assumes that analyte molecules are incorporated in the matrix crystals without charge. Upon irradiation with an UV laser pulse, the UV
susceptible matrix then becomes photo-ionized. In a second step, the charge of the matrix molecules is then transferred to the analyte in the plume. [46].

The second theory ("lucky survivor model") by Karas et al. [47] assumes that the analytes are incorporated into the matrix as ions even before irradiation. Most of these then later become neutralized within smaller desorbed clusters of analyte and matrix. The remaining ones are the lucky survivors that can be accelerated into the analyzer.

The theory suggests that proteins, e.g., retain their charge state in solution even incorporated in the crystal lattice of the matrix. This rests on the observation that pH indicators retain their colour when incorporated into the matrix, suggesting that they are stabilized as ions (solvated) in the matrix [48]. In acidic matrices, proteins and peptides usually carry one or more positive charges which will be countered by matrix anions or trifluoroacetate, which was used to acidify the sample. Upon desorption the matrix crystal lattice would break into smaller clusters or even single analyte ions. Some of these clusters would carry one or more positive or negative charges, either by deficit or an excess of counterions [49]. Neutrals like neutral matrix and solvent molecules are separated from the analyte ion in the plume. Free counterions associate with the analyte, effectively neutralizing all but a few analyte ions. While this produces mostly single charged molecules, multiple charged analytes are possible. The usual yield of ionization for the analyte ranges between 0.1 to 1%. The elegance of the lucky survivor model is that it explains the formation of both negatively and positively charged analytes (depending on type of analyte, solvent and matrix). It also explains the suppression of one analyte by another, meaning that one species may compete with one another in the cloud and fail to produce charged particles [50].

The MALDI process can also produce a high number of fragments as a result of the excess energy imparted by the laser.

### 5.1. Principle of time-of-flight MS

The ions, charged clusters and neutrals travel in this plume a short distance from the surface until the high voltage electromagnetic field gets hold of the ions and – depending of the polarity of the field and the charge of the ions – accelerates and focuses them by means of ion lenses. Ions are accelerated according to their mass and charge ratio.

\[
v = \sqrt{\frac{2ezU}{m_i}}
\]
The above equation gives the velocity \( v \) that the ion attains from a starting velocity of zero, where \( z \) is the number of charges \( e \) of an electron that the ion possesses, \( U \) is the voltage of the applied field and \( m_i \) is the ion’s mass.

Neutrals do not enter the mass analyzer. Heavier ions are not as fast accelerated as small ones according to their inertia and enter the TOF analyzer later. The second factor that determines the acceleration of charged particles in an electromagnetic field is, of course, the charge the particle carries. The higher the charge number is, the shorter the resulting flight time. The principle employed is what lends the mass analyzer described here its name: A time-of-flight mass analyzer.

Extending the flight time is therefore a logical way to increase resolution. If the length of a linear flight path becomes impractical, the path has to be changed. To achieve this the so called reflector has been devised [51], a system of lenses that allows charged particles to enter but expels them in the opposite direction. The higher the kinetic energy of the ion, the farther it will penetrate into the system. In this way these ions will also spend a longer time in the reflector, effecting a correction of the flight time that greatly enhances the resolving power, but diminishes sensitivity and introduces a mass limit [52]. Due to the longer flight path, particularly smaller m/z values benefit from a reflector TOF. The spread of flight times of one species of ions, i.e. of the same m/z, due to the ionization process will also be greatly reduced enabling higher mass resolution. This is also achieved by focusing all ions into a small region in direction to the detector. A further iteration in the development of this device is the so called curved field reflector that uses a curved field instead of a linear one as the normal reflector does [53].

### 5.2. MALDI Matrix

MALDI matrices are usually crystalline solids of low vapor pressure that have a high absorption for wavelengths of common lasers in the UV or IR range. Some liquid matrices like ionic liquids or nitrobenzyl alcohol are also known to work, though these are more rare. A wider range of substances are available for use with IR-MALDI. Depending of the wavelength, O-H, N-H and C-O bonds become excited, allowing the use of urea, glycerol and malonic acid among others as matrix. In UV-MALDI, matrices carry a chromophore, very
often an aromatic system. Usually, to be more specific, they are small organic molecules with often acid functional groups.

In order to add a charge to the analyte (either positive or negative), the matrix also acts as a protonating or deprotonating agent. Apart from this, the matrix also helps to isolate the analyte molecules so as to avoid larger analyte clusters. The matrix substance is usually more than a 100 times the amount of the analyte present in the matrix-analyte preparation. Choosing the correct amount of energy can be a difficult task as a setting too low would not induce desorption, while a setting to high could potentially damage the analyte. This is especially a concern for higher molecular proteins.

Due to the high amount of matrix substance, MALDI mass spectra usually consist of not only the protonated or deprotonated matrix ion peak, but a variety of matrix cluster ions as well. The good news in this case, however, is that these are well documented for matrices in common use [54]. Therefore, they can be used for calibration purposes.

Examples for MALDI matrices in common use (and used in this work) are as follows:

<table>
<thead>
<tr>
<th>Substance name</th>
<th>Common name or abbreviation</th>
<th>formula</th>
<th>Absorbance maxima [nm]</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-cyano-4-hydroxycinnamic acid</td>
<td>CHCA</td>
<td><img src="image" alt="Image" /></td>
<td>337, 355</td>
<td>Peptides, proteins, lipids</td>
</tr>
<tr>
<td>2,5-dihydroxy benzoic acid</td>
<td>DHB</td>
<td><img src="image" alt="Image" /></td>
<td>266, 337, 355</td>
<td>Peptides, nucleotides, oligonucleotides</td>
</tr>
<tr>
<td>3,5-dimethoxy-4-hydroxycinnamic acid</td>
<td>Sinapinic acid, SA</td>
<td><img src="image" alt="Image" /></td>
<td>266, 337, 355</td>
<td>Peptides, proteins, lipids</td>
</tr>
<tr>
<td>3-hydroxy picolinic acid</td>
<td>HPA</td>
<td><img src="image" alt="Image" /></td>
<td>337, 355</td>
<td>Oligonucleotides</td>
</tr>
<tr>
<td>4-hydroxy-3-methoxycinnamic acid</td>
<td>Ferulic acid</td>
<td><img src="image" alt="Image" /></td>
<td>266, 337, 355</td>
<td>Proteins</td>
</tr>
</tbody>
</table>
5.3. Sample preparation techniques

The way analyte and matrix are mixed, or better, how they both are arranged on the sample holder, is crucial to the MALDI process. While all matrices crystallize in different ways (Fig.9), from small fine crystals to large needles or even flakes that seem to peel of the surface of sample holder, there are a few ways how the analyte-matrix mix can be deposited on the target.

**Fig.9 Matrix preparations on a full metal target**
Various types of sample preparation techniques for MALDI MS have been developed over the years. Not only can the MALDI matrix be adapted to the analyte, but the way analyte and matrix substance are prepared, mixed and put on the target changes the quality on the mass spectrum acquired. Among these are the following:

- **Dried droplet technique**: This technique may be the most commonly used. Small volumes of solutions of matrix substance and analyte are mixed thoroughly and a 0.5 to 2 µL droplet of this mix deposited on the target. After drying the sample spot is ready for loading into the mass spectrometer. Depending on matrix substance used, the droplet may consist of fine crystals evenly dispersed, or in the case of DHB, rough crystals growing from the outer border of the preparation towards the center. This usually results in “sweet spot” searching. These spots are areas where the highest signal intensity can be found.

- **Fast evaporation technique** or **Thin layer technique**: In this method, the matrix substance is dissolved in acetone (containing 1-2% H₂O or 0.1% aqueous TFA). 0.5 µL of this solution pipette onto the target will quickly spread out and evaporate very fast leaving a very thin layer of extremely small matrix crystals. Shortly after, 1 µL of analyte solution is pipetted on the center of this matrix spot. After allowing to dry by itself the sample preparation can be measured. The aim of this technique is to produce an even layer of matrix/analyte co-crystallization without any “sweet spots”, improving reproducibility.

- **Two-layer technique**: The basis of this method is a microcrystalline matrix layer as produced accordingly to the first step of the fast evaporation technique. For the second layer, a saturated matrix solution in a solvent that the matrix is not as soluble in as for the first layer is mixed with the analyte and applied to the first layer. The thin layer should not be completely dissolved. This method is thought to improve measurements of proteins and peptides.

- **Sandwich technique**: The sandwich method is a mix of the previous techniques mentioned above. On a thin layer of matrix crystals and analyte produced according to the fast evaporation technique another droplet of matrix solution (not in fast evaporating solvent as acetone) is placed. This leaves a three-layered sandwich preparation.
5.4. Types of lasers

In comparison to other methods, LDI and MALDI both use relatively simple ion sources, although the desorption/ionization mechanisms themselves are not. A sample preparation, in case of MALDI mixed with a matrix substance, is irradiated by laser light causing desorption and ionization.

The laser light usually illuminates a spot 0.05 to 0.2 mm in diameter. This implicates a number of considerations when preparing the sample for measurement. Often when a sample is being measured, mass spectra are not only obtained from a single spot, but from a number of places. A single laser shot on a particular spot may leave no further analyte to be analyzed due to low concentration or it could be an area with high analyte concentration. While sweet spot searching has its merits, for comparison of data a raster of automated laser shots over a whole area may be beneficial. The data gathered this way is usually averaged, eliminating signals from impurities found in just one laser shot. Knowing the laser beam’s diameter on the target surface thus allows determination of the lowest distance between individual shots of the raster (Fig.10). Imaging mass spectrometry [55], where one shot in a matrix translates to a pixel in a 2D “data image”, does not use averaging of individual profiles, as that would defeat the purpose of the method. Thus, the laser diameter ideally should ideally equal the size of the pixel/laser spot for optimal spatial resolution for this purpose.

![Laser spot diameter and distance between shots](image)

Fig.10. Laser shots of a raster with laser spot diameter equaling the distance between separate shots

The irradiance - or intensity - of the laser is determined by a rotatable absorbance filter that can be finely adjusted from 100% to 1% transmission. High irradiance corresponds to higher signal strengths, but ultimately also to higher fragmentation due to in-source decay [56].
Higher signal intensity is usually also bought along with higher noise and lower resolution. The ability to fine tune the irradiance is thus an important feature.

Other parameters that are used to describe the laser are pulse length and frequency. Pulse length has an impact on how much energy is imparted onto the sample, as the longer the irradiation lasts, the more energy will be transferred.

Two different types of lasers are used in MALI mass spectrometry: ultraviolet (UV) and infrared (IR). The former is by far the most often encountered. The following table illustrates some wavelengths and laser types employed [57]:

<table>
<thead>
<tr>
<th>Category</th>
<th>λ [nm]</th>
<th>Laser type</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV</td>
<td>193</td>
<td>ArF Excimer laser</td>
</tr>
<tr>
<td>UV</td>
<td>248</td>
<td>KrF Excimer laser</td>
</tr>
<tr>
<td>UV</td>
<td>266/355</td>
<td>Nd:YAG laser</td>
</tr>
<tr>
<td>UV</td>
<td>308</td>
<td>XeCl Excimer laser</td>
</tr>
<tr>
<td>UV</td>
<td>337</td>
<td>Nitrogen laser</td>
</tr>
<tr>
<td>UV/UV/IR</td>
<td>266/355/1060</td>
<td>Nd:YAG laser</td>
</tr>
<tr>
<td>IR</td>
<td>2940</td>
<td>Er:YAG laser</td>
</tr>
<tr>
<td>IR</td>
<td>1700 – 2500</td>
<td>Optical parametrix oscillator laser</td>
</tr>
<tr>
<td>IR</td>
<td>10600</td>
<td>CO₂ laser</td>
</tr>
</tbody>
</table>

5.5. Types of detectors

The detector is the part of a mass spectrometer that translates the ion current to a measureable signal. Several different devices have emerged to address the need of ever more sensitive mass spectrometers that can still detect analyte amounts in the femtomol domain and below.

The simplest detector is the Faraday cup, where charges from the ions are simply registered by electron transfer thus generating a current. Today’s detectors are secondary electron multipliers (SEM) that rely on the emission of secondary electrons upon impact of energetic ions. SEMs are generally used for techniques such as MALDI and ESI MS, but not for FT-ICR and Orbitrap instruments. Those are based on a completely different detection principle, namely image current detection. Different detector types based on the SEM principle have been developed:
• **Discrete Dynode Electron Multipliers** [58]

This detector type consists of separate (or discrete, hence the name) electrodes that arranged in a zig-zag array produce an avalanche like effect, with ever more electrons being emitted by each stage upon electron impact. 12-18 dynode stages (at 100 V) generate enough current to be registered.

• **Channel Electron Multipliers** [59]

In contrast to the Discrete Dynode Electron Multiplier discussed above, the Channel Electron Multiplier consists of a continuous tube but works on all other aspects along the same principle. A curved design of the tube has proven to deliver better results compared to a linear design, particularly due to shorter paths for ion acceleration which leads to decreased noise due to ion-feedback.

• **Microchannel Plates** [60]

Microchannel Plates can be seen as the next evolutionary step after Channel Electron Multipliers. They consist of millions of linear channel electron multipliers in an array on a plate. The stacking of such plates results in an even higher gain, although spatial resolution is reduced. This is a result of electrons from the first plate spreading over more than one channel on the following plate and so forth.

• **Post-Acceleration and Conversion Dynode** [61]

While the previous methods tend to discriminate slower ions, which translates to higher mass ions, post-acceleration detectors foregoes that problem by accelerating the ions shortly before they hit the detector with a voltage of 10-30 kV. Conversion dynodes on the other hand, while also being placed before the SEM, work by the same principle as a SEM, attracting ions with a voltage of about 15 kV, and generating electrons which are then fed into the detector. The added benefit of such a construction is the avoidance of neutrals during detection.

• **Focal Plane Detectors** [62]

Focal plane detectors are employed in junction with magnetic sector instruments. It allows the detection of several m/z values (2-5%) around the center mass at a time. The device uses two multi-channel plates in a chevron arrangement. As a result peak
broadening occurs, which significantly lowers the resolution. The theoretical (maximum) resolution is limited by the number of channels on the multi-channel plate.

5.6. MALDI target for sample carrying

Surprisingly few publications can be found about MALDI sample supports in particular, especially from the beginning of the method. Modifications made on or with a target structurally or chemically are well documented, while publications about unmodified stainless steel targets could not be discovered, which are often quoted as being “standard” in MALDI MS [63, 64]. Thus, while no literature as to what makes a MALDI sample support suitable for measurement exists, the following list shall give an overview of properties deemed necessary after working on this subject for the duration of this thesis:

- **Chemical inertness to the analytes**
  
  Usually chemical reactions between analyte and target surface are undesirable to acquire a complete mass spectrum of a sample, but sometimes interaction can be desirable to purify or concentrate the analyte by interaction of one or more components with the modified surface [5, 65, 66]

- **Planarity**
  
  The planarity of a target ensures that all sample measurements happen at the same distance from the detector, thus ensuring the same flight length and therefore an identical m/z value for the same analytes. To eliminate errors stemming from irregularities in the planarity, it is important to cluster sample measurements around a calibration measurement.

- **Suitable hydrophobicity**
  
  As discussed in 2.4.1., good sample preparation depends heavily on the contact angle of the sample-matrix preparation on the target, i.e. if the drop covers a small or a large area (spreading). This can also be partially controlled by changing the solvent system the preparation is dissolved in.
• Smooth surface
While it was seen that measurements were possible on a visually rough surface (data not shown here), those mass spectra exhibited very low signal-to-noise ratio. It was found, however, that small imperfections on the surface facilitated a good crystallization of the sample/matrix preparation. A good example of a very smooth surface without these imperfections due to the omission of carbon black in the bulk material can be seen in Figure 8 below.

• Conductivity
Whether conductivity of the sample support is essential is debatable. It is thought that conductive materials affect the field lines in the ion source during measurement less than non-conductive ones, allowing higher resolution and/or sensitivity. This has been witnessed with polypropylene targets without carbon black filling (data not shown). Mass spectra were acquired, but their quality very poor. This, however, could also be attributed in part to improper crystallization of the sample/matrix preparation due to an excessively smooth surface.
An important feature of the MALDI target is its surface. Shown in Figure 11 are the same preparations of CHCA in acetonitrile/water (1:1, w/w) on different targets resulting in very different crystallization patterns.

*Fig. 11 CHCA matrix preparations in acetonitrile/H₂O (1:1, v/v) on various target surfaces*
6. Selected classes of analytes investigated

6.1. Proteins

Proteins are a large group of biopolymers (Fig. 12). They consist of amino acid subunits liked together by a peptide bond. Their so called primary structure, a proverbial “chain of pearls”, is simply the sequence in which they are bound together. This dictates the shape the protein takes in the 3D realm. Parts of the sequence align to each other in z-patterns called beta-sheets, while other parts might take the form of a spiral or a “barrel” due to their hydrophobic or hydrophilic side groups. This is called the secondary structure. Tertiary and quaternary structures are essentially based on the interactions between the structural elements introduced by secondary structure. A consequence of this intricate design is that heat, a change in pH or concentration can lead to changes in this three dimensional structure, possibly leading to a loss of biological activity.

![Crystal structure of uncleaved ovalbumin at 1.95 A resolution.][67]

Proteins have a large mass range, presenting a great challenge to mass spectrometry. They range from the smallest known protein, Trp-cage (2168 D) [68] to Titin (3.6 MD) [69].
reality, proteins with masses of up to approximately 1 MD are achievable, meaning that the majority of proteins will be detectable with MALDI.

A good matrix for proteins are CHCA for small proteins and sinapinic acid and in some cases 2,5-dihydroxybenzoic acid for larger proteins. Proteins also aggregate together to form multimers, as can be seen in Fig.13, a positive ion mass spectrum of Ovalbumin. Less stable proteins tend to fragment either during the MALDI process or in flight (post source decay), introducing another challenge.

![Mass spectrum of Ovalbumin](image)

*Fig. 13 Positive ion mass spectrum of ovalbumin featuring its molecular ion and (from left to right) its dimer, trimer and tetramer.*

### 6.2. Peptides

Peptides are small proteins or fragments of these (Fig. 14) and may be linear or cyclic. Like regular proteins, they are composed of a defined sequence of amino acids linked together by a peptide bond. The difference between proteins and peptides is simply a matter of length. While no exact definition exists, molecules with a length larger than 100 amino acids are generally considered proteins. The biological function of peptides varies greatly are too diverse to describe. They can be divided in a variety of classes according to their origin, among them artificial peptide fragments, ribosomal and non-ribosomal peptides and milk peptides.
Due to their polar nature and small size they are usually relatively easy to analyze by MALDI MS. While the same matrix used for proteins may yield adequate results, $\alpha$-Cyano-4-hydroxycinnamic acid has proven to be the matrix of choice in most situations. Oligonucleotides are best measured in reflectron mode to achieve a better resolution because of their small size. Peptides range between about 500 to below 10,000 Daltons.

The mass spectrum shown in Fig. 15 illustrates a typical example of a peptide mix used for calibration purposes.
The determination of peptides is perhaps most used for the identification of proteins. Protein samples separated by 2D-gel electrophoresis are extracted out of the gel by cutting and dissolution of the gel. The proteins are then identified in mass spectrometry by cutting them into peptides via digestion with e.g. trypsin. The resulting mass spectrum is then used to compile a list of representative peaks (without known contaminants such as keratin, alkali adducts and trypsin remnants) that can be correlated with database entries. This method is called peptide mass fingerprinting (PMF) [70-72]. The challenge in PMF is preventing and eliminating contaminations such as keratin (hair). An example of a peptide mass fingerprint can be found in the manuscript “Polymer-based metal nano-coated disposable target for matrix-assisted and matrix-free laser desorption/ionization mass spectrometry” in chapter 9.1.

6.3. Oligonucleotides

Oligonucleotides are short nucleic acid polymers (Fig.16), whereby short translates to a length of up to 50 monomers joined together. A strict definition of which length can still be called an oligomer does not exist. They play an integral role in biochemistry and molecular biology, and today even in diagnostic medicine.

A very important use of oligonucleotides is the determination of gene functions. Here, oligonucleotides with complementary sequences to a specific mRNA are used, so called antisense-oligonucleotides that inhibit a specific gene, leading to a loss-of-function phenotype. This way, a biological function can be associated with a gene.

A newer and very promising method that can be used to induce a loss of function is to exploit a phenomenon called RNA interference, RNAi for short. Small double stranded RNA molecules, it has been found, can be used to target specific mRNA strands, calling upon cell inherent enzymatic machinery to degrade them [73].

Another method that employs oligonucleotides is the polymerase chain reaction (PCR), where they are used as primers. Elevated temperature is used to separate DNA strands, short specifically to the target DNA tailored oligonucleotide sequences are aligned to their counterpart, allowing in a third step the polymerase to write the complementary DNA. [74]

Synthetic oligonucleotides often contain contaminations, synthesis byproducts or even changes in their sequence. These are typically eliminated by size exclusion chromatography. Quality control however is very often done with mass spectrometry [75]. A change in the
sequence would then be easy to check for. Electrophoretic methods, which are also still used in this field, usually suffer from long measurement time and bad resolution compared to mass spectrometry. In the case of DNA, the smallest difference in mass to be expected when checking for synthesis errors is 7 Da (difference between A and T). RNA, however, can differ in as little as 1 Da (difference between U and C). This means that a good resolution is vital for analysis, especially for longer chains.

Although nucleic acids belong to the bioanalytes with the highest polarity, which usually helps when acquiring mass spectra, they are rather difficult to measure because of their polyanionic nature. They form rather easily bonds with alkali ions such as Na\(^+\) or K\(^+\) and their phosphodiester groups, often leading to complex mass spectra as well as difficult to measure sample/matrix preparations. Additionally, beak broadening may occur, leading to loss of sensitivity. Efforts were made, however, to reduce the alkali levels usually present due to the synthesis. [76]

In theory, the mass spectrum may include anything from the molecular ion as well as single charged multimers and monomers and multiple charged multimers, although at lower intensities [77, 78].

Typical matrix substances suitable for oligonucleotides include 3-hydroxypicolinic acid and 2,4,6-trihydroxypicolinic acid [79-81]. Both matrices perform well for UV lasers, producing intact molecular ions. In the beginning of the analysis of nucleic acids with MALDI MS, only oligonucleotides with small masses could be measured. 3- hydroxypicolinic acid is favorable to use, as it gives comparable results for both positive and negative ion detection modes.

Further information regarding the measurement of oligonucleotides can be found in the manuscript “\textit{MALDI measurements of oligonucleotides on disposable polymer targets for routine quality control}” in chapter 9.4.
In contrast to DNA synthesis found in nature, oligonucleotide synthesis in labs is done in a 3’ to 5’ direction. The phosphoramidite method [82, 83] starts with a solid support on the 3’ end (Fig.17). To this starting point phosphoramidite subunits are added, which are derived from 2’-deoxybucleosides or ribonucleosides, as naturally occurring nucleotides are too chemically unreactive to produce high enough yields. Protecting all functional groups of the building blocks not pertinent to the linkage process avoids undesired side reactions. After the desired oligonucleotide is generated, these protecting groups have to be removed. After the synthesis, the product is purified by preparative HPLC. All these steps are fully automated and highly
reproducible. In general, sequences up to 200 subunits are technically possible to synthesize. Longer oligonucleotides exhibit a higher rate of synthesis errors. In practice, sequences between 15 and 25 units are most often produced.

![Phosphoramidite method for oligonucleotide synthesis](image)

**Fig.17** Phosphoramidite method for oligonucleotide synthesis

The synthetic cycle begins with the removal of the DMT group from the 5’ end by the addition of an acid (2% trichloroacetic acid or 3% dichloroacetic acid). The second step sees the activation of nucleoside phosphoramidite (0.02 to 0.2 M solution) with e.g. an acidic azole catalyst. The activated compound is then added in a 1.5 to 20 molar excess to the starting nucleotide bound to the solid support or (if further in the synthesis) the oligonucleotide precursor. Any unbound reagents or side products are subsequently washed away. Any unreacted 5’-OH groups are then capped with a mixture of acetic anhydride and 1-methylimidazole to ensure that oligonucleotides with missing base in sequence do not occur. After capping iodine, water and a weak base are added to oxidize the unstable phosphite triester linkage that binds the newest subunit to the product. Thus, a tetra-coordinated phosphate triester is formed that is more stable under synthesis conditions and is a precursor of the phosphate diester of the naturally occurring product.
6.4. Microorganisms – Intact Cell Mass Spectrometry (ICMS)

Identification and differentiation of microorganisms is important in many fields. The first that come to mind being hospitals, where samples have to be checked for pathogens (hospital-acquired infections from *Staphylococcus aureus*, *Candida albicans* and *Pseudomonas aeruginosa* among others), bioterrorism threats (*Bacillus anthracis*, *Clostridium botulinum*, *Yersinia pestis* ...) and food testing (pathogenic fungi). While methods for the determination of microorganism have been known for decades, they generally relied upon the cultivation of samples on diverse media, incorporation of dyes, immunoreaction to certain antibiotics and others. Newer methods rely on PCR analysis which allow for faster response time [84]. Mass spectrometry, however, allows even faster analysis times.

The idea behind ICMS is the desorption/ionization of surface bound proteins of a microorganism by laser [85-87]. The resulting mass spectrum is unique not even to the species, but also to the strain [86-88]. The cell wall of the microorganism is here of particular interest, as it contains all the important features (surface bound proteins) needed for the cell to interact with the environment. While it is also surely possible to differentiate between microorganisms based on their cell extracts [89], ICMS has to deal with fewer proteins making interpretation easier. An additional benefit, of course, is the ability to skip extraction steps that would be needed for the analysis of the extract.

The ability to differentiate between strains is of particular interest in medical care and the food industry, as one strain of an organism might be pathogenic, while another might be benevolent. Intact Cell MS enables a fast response for that. The lower limit of detection was 1000 cells [90]. It is even possible to “filter” gram-positive and gram-negative cells by choice of matrix. CHCA for example is specific for gram-negative organisms.

The mass spectra acquired from the microorganisms are unique enough to even make a distinction between strains of microorganisms and perhaps even between vegetative states of e.g. fungi. From these mass spectra a certain number of “significant” peaks, i.e. peaks that are distinct to a particular microorganism are compiled to a “master spectrum”, which is added to a database. Newly acquired mass spectra can then be compared to existing entries in this database. Mathematical models are then used to give an indication of how close a match the mass spectrum is to the data of a particular microorganism. It allows the identification of microorganisms present in the sample and sometimes also differentiation between strains. This, of course, is an over-simplistic explanation of the whole concept [91]. An example for
such a search algorithm linked with a database is SARAMIS™, which was developed by AnagnosTec [92].
7. References


8. Aims of the thesis

This work was conducted with the following objectives:

1) Evaluation of the mass spectrometric performance of the individual iterations of the polymer-based and metal nano-coated MALDI MS targets produced by Sony DADC Austria AG. The following aspects were used to evaluate the targets:
   a) Optical and microscopic inspection of the target in terms of cracks or artifacts from the injection molding or sputtering process related to the coating
   b) Evaluation of a defined sample/matrix preparation (CHCA) spotted onto target and comparison with classical metal target
   c) Evaluation of mass spectra in terms of mass spectrometric performance of a defined peptide mixture based on signal strength, resolution, noise level, possible additional peaks

2) Definition of the final MALDI MS target product in terms of achievable limit of detection and quantification for peptides and proteins, m/z range, mass spectrometric resolution, but also planarity and contact angle of the generated target surface

3) Long time archiving of peptide and protein samples spotted on polymer-based and metal nano-coated MALDI MS targets under different storage temperatures to determine the best archival parameters and realistic maximum storage times.

4) Evaluating real of polymer-based and metal nano-coated MALDI MS targets for various fields of real-world applications including
   a) Intact Cell Mass spectrometry studying a range of different fungi and bacteria
   b) Quality control of oligonucleotide synthesis products
   c) Metabolomics: Steviol glycosides and tyrocidines
9. Manuscripts and application notes

9.1. Polymer-based metal nano-coated disposable target for matrix-assisted and matrix-free laser desorption/ionization mass spectrometry

9.2. Intact cell/intact spore mass spectrometry (IC/ISMS) on polymer-based, nano-coated disposable targets

9.3. Long time storage (archiving) of peptide, protein and tryptic digest samples on disposable nano-coated polymer targets for MALDI MS/MALDI measurements of oligonucleotides on disposable polymer targets for routine quality control

9.4. Quality control of oligonucleotide synthesis by means of matrix-assisted laser desorption/ionization linear time-of-flight mass spectrometry on a nanocoated disposable target

9.5. Application Note: Analysis of a Tryptic Protein Digest by MALDI-RTOF Mass Spectrometry using Microscope Slide-format FlexiMass™-DS target

9.6. Application Note: Metabolomics: MALDI RTOF MS of Steviol Glycosides and LDI/MALDI RTOF MS of Tyrocidines using Microscope Slide-format FlexiMass™-DS targets

9.7. Application Note: Analysis of Small and Large Proteins with MALDI-TOF Mass Spectrometry using Microscope Slide-format FlexiMass™-DS targets

Polymer-based metal nano-coated disposable target for matrix-assisted and matrix-free laser desorption/ionization mass spectrometry

Stefan Bugovsky a, Wolfgang Winkler a, Werner Balika b, Manfred Koranda b, Günter Allmaier a,⇑

a Institute of Chemical Technologies and Analytics, Vienna University of Technology, Vienna, Austria
b Sony DADC BioScience Austria, Anif, Austria

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A B S T R A C T

The ideal MALDI/LDI mass spectrometry sample target for an axial TOF instrument possesses a variety of properties. Primarily, it should be chemically inert to the sample, i.e. analyte, matrix and solvents, highly planar across the whole target, without any previous chemical contact and provide a uniform surface to facilitate reproducible measurements without artifacts from previous sample or matrix compounds. This can be hard to achieve with a metal target, which has to be extensively cleaned every time after use. Any cleaning step may leave residues behind, may change the surface properties due to the type of cleaning method used or even cause microscopic scratches over time hence altering matrix crystallization behavior. Alternatively, use of disposable targets avoids these problems. As each possesses the same surface they therefore have the potential to replace the conventional full metal targets so commonly employed. Furthermore, low cost single-use targets with high planarity promise an easier compliance with GLP guidelines as they alleviate the problem of low reproducibility due to inconsistent sample/matrix crystallization and changes to the target surface properties. In our tests, polymeric metal nano-coated targets were compared to a stainless steel reference. The polymeric metal nano-coated targets exhibited all the performance characteristics for a MALDI MS sample support, and even surpassed the – in our lab commonly used – reference in some aspects like limit of detection. The target exhibits all necessary features such as electrical conductivity, vacuum, laser and solvent compatibility.

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1. Introduction

Conventional MALDI MS targets consisting of plain metal (e.g. stainless steel) often require intensive cleaning involving copious amounts of solvents, additives and detergents, which adds to cost and creates an increased environmental footprint. The type of cleaning required depends on the analyte and target surface. For example, microorganisms need to be rendered inert after measurement via sterilization. Strongly adhering lipids, polymers or photo-polymerized UV MALDI MS matrices may need to be removed from the sample support’s surface by force (abrasion). Furthermore the costs of microtiter plate-sized targets are considerable and make the use of a large number of targets expensive and longer storage of samples hard to justify. The surface properties and chemistry are also subject to change over time (e.g. rendering them more hydrophilic or hydrophobic) and even morphological damage of the surface can easily happen which was observed in our lab with nickel-coated aluminum targets.

In fields of work where a defined surface is not only required by the sample preparation method but also important for validation such as in the pharmaceutical industry or forensic sciences, it is necessary to ensure the exact same conditions for every measurement performed. For avoiding external influences disposable targets are an easy way of guaranteeing standardization, e.g. GLP requirements. Also in areas where high risk analytes such as human-pathogenic microorganisms or toxins are analyzed routinely, it is necessary to safely dispose the analyzed samples (because very often considerable parts of the samples are not completely consumed by the measurement). In such situations conventional full metal targets carrying the unused sample residues must be subjected to rigorous cleaning, such as sterilization. Obviously, in these instances inexpensive disposable sample holders are a great benefit.

In multi-user environments it is also convenient to have access to a repository of targets in contrast to having only a few expensive targets available that need to be communally shared. This is particularly true for the high-cost metal MTP (microtiter plate) format targets.

A number of polymer-based targets made it to the market and were evaluated [1–3]. Among these were the AnchorChip [4–6]
(Bruker Daltonics) disposable targets with pre-spotted MALDI matrix with a varying number of spots for sample and calibrant, and the Mass-Spec-Focus Chip by Qiagen that features a focusing effect especially for phosphopeptides [7] and proteins [8]. This is achieved by creating an ultrahydrophobic boundary outside of the more hydrophilic sample well. Both offer specific functionality that comes with inherent limitations. The prespotted PAC AnchorChip is only available for Bruker mass spectrometers and is not suitable for samples which require different matrices and non-standard solvents. The Mass-Spec-Focus Chip is on the other hand limited by the low binding capacity and useable solvent systems [8]. Recently, Bruker brought the MALDI Biotarget 48 to market, a disposable MALDI target specifically designed for microorganism identification on their mass spectrometers.

We compare a disposable polymer target (DPT) consisting of a polypropylene support with carbon black (to facilitate optimal conductivity) coated with a stainless steel layer in the nanometer range with a standard stainless steel full metal target (FMT). The thin metal layer is a product of a sputtering process to emulate the surface usually encountered on current commercial available metal-based targets. The morphology [9] and surface composition [10,11] of a MALDI MS target greatly affects parameters such as sensitivity [1,12] and even selectivity [13]. The use of a patterned nano-structured gold film has even been demonstrated [11] to facilitate LDI measurements. Chemistry and morphological structure of the surface have a direct effect on the crystals (shape, crystal size/form/number) formed in the MALDI MS sample preparation [14–16], adding further complexity to the already difficult task of selecting the best matrix and solvent system for a given analyte. Even when using the same material composition, the way a target surface was crafted (e.g. polished by hand or machine, electro-polished, brushed) also has a direct influence on its microstructure. While most metal targets are polished to guarantee a certain area planarity and hydrophobicity, this creates microscopic polishing marks. The polymer-based target employed here is surface-treated with a sputter-coating process, resulting in a very smooth and uniform surface as will be shown later. The advantage here is that any small imperfections in the polymer surface are essentially leveled out due to the sputter deposition.

2. Experimental section

2.1. Targets

Disposable polymer targets [DPT; FlexiMass-DS targets, TO-430, Shimadzu Kratos Analytical] were designed and manufactured by Sony DADC Austria. The targets have the following dimensions, 76.2 mm × 26 mm × 1.6 mm (±0.1 mm, flatness and parallelism significantly less) and consist of carbon black-filled polypropylene with a stainless steel coating on the up-facing side of about 300 nm. The format used for the targets is based on the microscope slide-sized FlexiMass MALDI targets (Shimadzu Kratos Analytical). Four times 12 sample spot areas (2.8 mm ID) were marked allowing the preparation of 48 samples (four columns with 12 rows, designated A–L on the long side and 1–4 on the short; see Fig. 1(a)) and furthermore three calibration spot areas (2.3 mm ID) located in the top, center and bottom area of the target between the two central sample spot columns (see Fig. 1(a)). The targets were produced by an injection molding process followed by DC-pulsed magnetron sputtering of stainless steel onto the DPT’s sample-carrying side.

As a reference target for performance comparison a full metal target (FMT; FlexiMass-targets TO-483800, Shimadzu Kratos Analytical) which consists completely of stainless steel and exhibits a machine-polished surface was used.

An MTP-sized target holder (Precision adapter, TO-488, Shimadzu Kratos Analytical) was used for carrying the two different kinds of target (DPT and FMT) on the sample stage in the ion source. In this target holder up to 4 targets can be mounted and introduced simultaneously into the vacuum system of the ion source. The targets are pressed from the bottom into a frame (see Fig. 1(a), left target) by means of a spring plate to obtain optimal planarity across the whole target for optimal desorption/ionization.

2.2. Chemicals

Tyrosine solution (kindly provided by the Vienne Chamber of Pharmacy; 1 µg/mL in ACN (acetonitrile);H₂O (1:1, v/v)), angiotensin II (Sigma-Aldrich, A8846; 10 pmol/µL in H₂O), α-cyano-4-hydroxycinnamic acid (CHCA; Agilent Technologies, G2037A) matrix solution (6.2 mg/mL in methanol (MeOH):ACN:H₂O (36:56:8, v/v/v)), sinapinic acid (5A, Sigma-Aldrich, S8313) matrix solution (10 mg/mL in ACN/0.1% aqueous trifluoroacetic acid (TFA) (1:1, v/v)), 2,5-dihydroxybenzoic acid (DHBr; Fluka, 85707) matrix solution (20 mg/mL in ethanol:H₂O (1:1, v/v)), Pullulan 5600 Da (Polymer Standards Service; 2.02 mg/mL in H₂O with 0.5% NaNO₃), IgM from human serum (Sigma-Aldrich, 18260, reagent grade; 0.8 mg/mL in 0.05 M Tris-HCl, 0.2 M NaCl, 15 mM Na₂CO₃, pH 8.0), and γ-globulin (Sigma-Aldrich, 49030; 2 mg/mL in 0.01% aqueous TFA) were used without further purification. The peptide calibration mix consisted of angiotensin I (Sigma-Aldrich, A3178; [MH]⁺ m/z = 1296.69, monoisotopic value), Glu1-fibrinopeptide B (Sigma-Aldrich, F3261; [MH]⁺ m/z = 1570.68, N-acetyl renin substrate (Sigma-Aldrich, R5380; [MH]⁺ m/z = 1800.94), ACTH 1-17 (Sigma-Aldrich, A2407; [MH]⁺ m/z = 2093.09) and ACTH 18-39 (Sigma-Aldrich, A0673; [MH]⁺ m/z = 2465.20) and contained these peptides at a concentration of 100 fmol/µL in ACN; 0.1% aqueous TFA (1:1, v/v) except for ACTH 7-38 (Sigma-Aldrich, A1527; [MH]⁺ m/z = 3657.93) which was present in the mix at 150 fmol/µL ACN (Merck, 100003), ethanol (Merck, 100983, absolute), TFA (Riedel-de-Haën, 61030) and methanol (Merck, 106007) were of analytical grade. Water was obtained from a reverse osmosis facility and further purified with Simplicity UV (Millipore). This water quality was used in all applications.

2.3. Mass spectrometer

Positive ion MALDI and LDI mass spectra were obtained by means of a time-of-flight instrument (Axima CFRplus, Shimadzu Kratos Analytical) in the linear or reflectron mode. In case of the MALDI analysis of the glycoprotein IgM in the linear mode the mass spectrometer was employed in conjunction with an ultra-high mass detector (HM1 High-Mass System, CovalX) instead of the standard detector [17,18]. The instrument was used with a nitrogen laser (337 nm) at a pulse rate of 20 Hz and at an average analyzer pressure of 1.6 × 10⁻³ mbar.

2.4. Determination of mass spectrometric resolution, limit of detection (LOD), limit of quantification (LOQ) and linearity

Angiotensin II solutions (from 0.2 to 2 fmol/µL) were mixed in an Eppendorf vial with an equal amount of the described CHCA matrix solution. A volume of 1 µL of this mixture was pipetted on the DPT and the FMT and allowed to dry at room temperature (RT), resulting in absolute amounts of peptide from 0.1 to 1 fmol on target (preparation area of approx. 4.5 mm²). The data obtained for these amounts were used to determine the LOD with an appropriate S/N ratio. Positive ion MALDI mass spectra of these spots were recorded in reflectron mode (484 unselected laser shots in a 700 µm × 700 µm raster) in the m/z range of 500–3000. All ions
below m/z 500 were removed before entering the analyzer ("blanked out"). Pulsed extraction was set to m/z 1400 for optimal resolution. No smoothing or baseline subtraction was applied to the generated and shown MALDI mass spectra.

For the exact determination of the mass spectrometric resolution in the reflectron mode a 10 fmol/μL angiotensin II solution was used (mixed in equal amounts with CHCA matrix solution), resulting in 5 fmol absolute amount of peptide on target.
The linear range for quantification was determined by preparing angiotensin II dilution series ranging from 0.4 fmol/μL to 200 fmol/μL and mixed with CHCA matrix solution in equal volumes. A volume of 0.5 μL of the sample mixture was then spotted onto the targets to achieve 0.1, 0.5, 1.5, 10 and 50 fmol of analyte on target. Positive ion mass spectra of these spots were recorded in reflectron mode (484 unselected laser shots in a 700 μm × 700 μm raster) in the m/z range of 500–3000. All ions below m/z 500 were removed before entering the analyzer. Laser irradiance was set to achieve maximum signal intensity while staying below signal saturation (2000 mV). Pulsed extraction was set to m/z 1400 for optimal resolution. No smoothing or baseline subtraction was applied to the generated and shown MALDI mass spectra. For all concentration this setting was used and these experiments were conducted with DPT as well as FMT.

The noise for the determination of the LOQ was determined by averaging all signals in the m/z range of 1010–1080 excluding the angiotensin II signal from three different measurements of the 0.5 fmol analyte amount on both target types. Ten times the noise was entered into the regression line equations and the LOQ was calculated.

2.5. Calibration stability across the target (m/z drift over the whole target)

The above described peptide calibration mix was combined in an Eppendorf vial with an equal amount of CHCA matrix solution. A volume of 0.5 μL of this mixture was deposited on all 48 sample positions and three calibration positions of the MALDI targets (DPT and FMT) resulting in 25 fmol of each peptide per position after drying at RT. MALDI mass spectra were recorded according to the instrumental parameters defined for the determination of the LOD. Pulsed extraction was changed to m/z 2400 to improve the coverage of the m/z range of all standard peptides. Calibration was performed on sample spot A1 and then used for all succeeding measurements on all other 47 prepared sample spots and 3 calibration positions. The m/z value of the monoisotopic peak of ACTH 18–39 was used as reference to determine the change of its m/z value in relation to the x-y-position on target. No smoothing or baseline subtraction was applied to the generated mass spectra.

2.6. LDI reflectron TOF MS mode

A volume of 1 μL of the tyrocidine solution was deposited directly onto the target and allowed to dry at RT. Positive ion mass spectra of these spots were recorded in reflectron mode (484 unselected laser shots in a 700 μm × 700 μm raster) in the m/z range of 500–3000 while ions below m/z 500 were eliminated. Pulsed extraction was set to m/z 1500 for optimal mass resolution. No smoothing or baseline subtraction was applied to the mass spectrum.

2.7. Determination of the upper m/z limit in MALDI linear TOF MS mode [19,20]

IgM was used without any prior cleaning step and mixed in an Eppendorf vial with an equal volume of SA matrix solution.
A volume of 1 µL of the mixture was spotted on the DPT and allowed to dry at RT. MALDI mass spectra were recorded in positive ion mode (484 unselected laser shots on a sweet spot) in the m/z range of 120,000–1,000,000 with elimination of all ions below m/z 100,000. No pulsed extraction was used. Mass spectra were smoothed by means of the company-supplied Savitsky-Golay algorithm [21] (filter width set to 46). Baseline subtraction was also performed (filter width 500 channels).

IgG was used as starting point to evaluate the m/z limit and applied again without purification and mixed in equal volumes with SA matrix solution. A volume of 1 µL of this mixture was spotted onto the DPT and FMT and dried at RT. Mass spectra were recorded in positive ion mode (100 unselected laser shots on a sweet spot) in the m/z range of 40,000–200,000 with elimination of ions below m/z 35,000. No pulsed extraction was used. Mass spectra were smoothed again by means of the Savitsky-Golay algorithm [21] (filter width set to 46) but without baseline subtraction.

2.8. Determination of polymer molecular mass distribution in MALDI linear TOF MS mode

From the large group of natural occurring oligo- and polysaccharides the SEC reference sample pullulan was selected [22,23]. A 5 mg/mL solution of pullulan 5600 Da was prepared and mixed in equal parts with the described DHB matrix solution. A volume of 1 µL of the mixture was then deposited on the DPT and FMT. Positive ion mass spectra of these sample spots were recorded in the linear mode based on 484 unselected laser shots in the rastering mode (750 µm × 750 µm) in the m/z range of 500–20,000 with elimination of ions below m/z 500. Pulsed extraction was not employed. MALDI mass spectra were smoothed by means of the Savitsky-Golay algorithm [21] (filter width set to 40). Baseline subtraction was also performed (filter width of 2000 channels).

2.9. Scanning electron microscopy

SEM images of the target surfaces of DPT and FMT were taken using a Hitachi S-4000 (Hitachi) with 15 kV accelerating energy, extraction voltage set to 5 kV and electron current to 8 µA (250× and 500× magnification).

2.10. Light microscopy

Photos at 20× and 80× magnification were taken with the SMZ 800 (Nikon) microscope in combination with the camera control unit DS-L1 (Nikon).

2.11. Contact angle measurements

Measurements were performed using the Easy Drop DSA 20 (Kruess) with an angle resolution of ±0.1°. A 2 µL drop of deionized water (predistilled water purified by the ion exchange system Arium UV (Sartorius) and filtered with a 0.3 µm filter) was deposited on the target by means of a needle (static sessile drop method). All 48 sample positions on the target were tested with water to test for the spot hydropobicity distribution across the whole target surface. Also, the following solvents and mixtures were tested for their contact angle on the DPT and FMT: ACN, methanol, ACN:0.1% aqueous TFA (1:1. v/v) and the solvent system employed for the CHCA matrix solution as described above (MeOH:ACN:H2O (36:56:8, v/v/v)). Contact angles were calculated with the tangent approximation method provided by the instrument manufacturer.

2.12. Surface roughness

DPT and FMT were scanned with MicroSpy Topo, a confocal microscope that allows the measurement of surface roughness based on interferometry (Fries Research & Technology). Three sample spots on one target were used for measurements on the FMT, whereas roughness on the DPT was determined on 10 sample spots distributed over 3 different targets. Applied lateral resolving power of the device was 2.46 µm and vertical resolution 10 nm.

2.13. Surface topology

DPT and FMT were investigated with the MicroProf 200 (Fries Research & Technology) device with a chromatic white light sensor (CWL 3 mm). The device determines surface planarity using the principle of chromatic aberration. By shining white light on a surface, the wavelength of the scattered light allows the calculation of the height of the surface. A measurement area of 250 mm × 200 mm with a measurement resolution in the z-axis of ±10 nm was used. The scans were performed on the two target types with and without being fixed in the sample target holder.

3. Results and discussion

Before detailed mass spectrometric data and some selected applications will be presented the quality of the DPT will be shown in terms of the parameters surface roughness and planarity, maximum valley/peak depths and hydrophobicity/contact angle.

3.1. Surface roughness and planarity

The surface roughness was measured on the smooth sample spot areas. For the FMT, the arithmetic average of roughness, R_a, was determined to be 0.041 ± 0.013 µm, whereas DPT exhibits a value of 0.045 ± 0.001 µm. That means that both targets are almost identical when it comes to roughness. The standard deviation is higher for the FMT surface.

The maximum peak height (R_p) and maximum valley depth (R_g) give an indication of the topography of the targets. For the FMT the mean values are R_p = 1.076 ± 0.434 µm and R_g = 1.481 ± 0.775 µm. Measurements for DPT reveal a surface without such extremes: R_p = 0.361 ± 0.024 µm and R_g = 0.306 ± 0.069 µm.

Comparing these numbers allows the conclusion that the DPT possesses an almost identical roughness compared to the FMT, but at the same time with fewer topographic extremes, eventually a result of its manufacturing process involving molding and subsequent sputter-coating of the polymer surface (i.e. a kind of leveling out valleys). This combination of production processes generates a very homogenous target surface as can be seen in the SEM images of Fig. 1(b).

The holder used to fix the targets (up to four) as a means to transport the microscope slide formatted DPT into the ion source of the MALDI MS instrument is also important in guaranteeing a planar surface across the whole target whilst performing measurements. The achieved planarity is made visible by a heat map shown in Fig. 1(c). Here three DPTs were put into the holder (left, Fig. 1(c)), which presses the targets towards the frame and significantly improves the flatness compared to the DPT without frame support (center of Fig. 1(c)). Fig. 1(d) displays a topographical cross-section of the three DPT and one FMT. The FMT, which appears much more planar in the x-axis, sits in a much lower position in the adapter. The adapter was specifically designed for the DPT and also accepts FMT but is not flush with the adapter surface when using these targets. This does not impact MS performance. The noticeably higher
noise which can be seen on the DPT in the cross-section is due to the larger part of the target being intentionally rough so that the sample spots stand out. The roughness measurement at the beginning of 3.1 was done at the smooth target positions.

A high planarity of the DPT in the holder as well as high smoothness is important in terms of calibration stability and effective sample preparation (i.e. good crystallization of the matrix/analyte mix).

3.2. Contact angle measurements

The contact angle is a good indicator for the interaction between a fluid and the surface it is in contact with. The closer the angle is to 0° the more similar are their respective surface energies. In contrast, a high value (above 90°) represents a difference in properties between the solid surface and the liquid. For plain water, a hydrophilic surface yields a contact angle between 0 and 90° whereas values above 90° would indicate a surface with hydrophobic properties. The contact angle is therefore a good indication of the wettability of a surface, an important feature for MALDI MS targets. A MALDI MS matrix/analyte solution that forms a droplet with only a small interface area will very likely produce a preparation different to one that spreads over a larger area. Both phenomena – focusing of a preparation (generating a small spot) and spreading over a larger diameter – have their applications. In the first case (focusing) a concentration effect as described for the AnchorChip [5] targets or Mass-Spec Focus Chips [7] will be generated. The second case, for instance, is achieved with acetone as a solvent, resulting in a thin film (for acetone soluble matrices) and counteracts to some degree effects of high salt concentrations. Three sample preparations on DPT are shown in Fig. 1(e). SA and CHCA form large spots and crystallizations very comparable to those seen on FMT. DHB on the other hand shrinks to a small spot which is beneficial. In some instances the operator may be able to adapt the solvent system in which the selected matrix substance is dissolved and/or change the matrix concentration to meet the analytical needs.

For deionized water, the contact angle was found to be 106.2° ± 0.2° on DPT, i.e. a hydrophobic surface (82.2° ± 4.5° in case of the applied FMT, i.e. a slightly hydrophilic surface characteristic). Methanol gave 28.6° ± 1.3° on DPT (29.6° ± 6.7° on FMT) and ACN 43.4° ± 2.2° on DPT (29.7° ± 6.5° on FMT). More complex solvent systems like ACN:aqueous 0.1% TFA (1:1, v/v), often used in MALDI MS resulted in 61.2° ± 8.0° on DPT (47.9° ± 2.8° on FMT). Methanol: ACN: H₂O (36:56:8, v/v/v) gave a contact angle of 55.4° ± 3.1° on DPT and 43.6° ± 0.5° on FMT. In conclusion the contact angle measurements for the DPT indicate a higher hydrophobic character (compared to FMT). Fig. 2(a) visualizes the homogeneity of the contact angle of water over a whole DPT. The contact angle ranges from 106.5° to 108° and this difference is negligible in most scenarios, but can become an issue for some specific preparations as was encountered when performing, for instance, intact mass spectrometry of bacteria.

3.3. LOD, LOQ, linearity of quantification, mass spectrometric resolution and drift in m/z value determination

The limit of detection for a small peptide (in this instance angiotensin II), using a 3:1 signal-to-noise (S/N) ratio and the monoisotopic protonated molecule, was determined on both DPT and the conventional FMT at 500 attomole on target. The S/N ratio for that amount of peptide was for the DPT calculated to be 12:1. The FMT reached a ratio of 8:1 for this amount of peptide under the same acquisition conditions. The LOD for the DPT was therefore calculated to be around 125 attomole on target. The FMT exhibited a slightly higher LOD. Fig. 2(b) illustrates the two mass spectra acquired with 500 attomole on both target types. The mass spectrum derived from DPT exhibits also the isotopic pattern expected from the theoretical calculation due to the better S/N ratio.

To determine the linearity of the response a dilution series of angiotensin II between 100 attomole and 50 fmol on target was plotted against the signal intensity and is shown in Fig. 2(c). A linear regression was calculated for the two types of targets, DPT and FMT. Here the polymer target (y = 29.49, x ± 5.068, R² = 0.9996) exhibits a linearity across approx. three orders of magnitude as well as a better correlation coefficient than the metal target (y = 24.21, x ± 20.74, R² = 0.9956). While the correlation coefficient suggests a very high linearity for both targets, the zoomed in portion of Fig. 2(c) reveals that especially for the lowest concentrations, the data points of the DPT fit the regression line much better.

Various definitions of the exact value of the LOQ exist [24–26], the one employed here is the value of ten times the mean blank signal (noise in the region of interest), which in this case was derived from three measurements in the range between m/z 1010 and 1080 (excluding the angiotensin II signal). The above line equation (determination of linearity) were used to calculate the concentrations equaling the LOQ (DPT: 325 attomole, FMT: 700 attomole). The results from the DPT are therefore not only in good accordance with results achieved with MTP but also surpass these.

In terms of mass spectrometric resolution, DPT appears to be slightly better compared to the conventional target (FMT) used as a control (see Fig. 2(d)). The former generated a mass spectrometric resolution (FWHM) of 9790 ± 510 (n = 7), whereas FMT, the reference, gave 9200 ± 360 (n = 7). The results corroborate with earlier findings derived from a prototype target [27].

It is also of interest to study the m/z value drift of a molecular ion of interest that occurs when one target is calibrated by a calibrant and based on this calibration is then applied to other measurements on different sample spots on the same target. Although only small differences in mass would be expected, it is prudent to use the provided calibration spots nearby the sample preparation spots when measurements are performed. Fig. 2(e) compares the m/z drift of ACTH 18-39 between DPT and the conventional FMT. The calibration was performed at spot A1, with succeeding measurements using this calibration/reference point. The selected analyte was in this case the ACTH fragment because it was part of our peptide calibration mix. It is interesting to see that the DPT batches used appear to result in a consistent calibration error of ±1.0–1.2 Da, corresponding to a difference in the planar area of 0.1–0.2 Da, i.e. 40–81 ppm after A1 and A2 (mass spectra were recorded in the direction A1 to L1 and then again starting from A2 to L2 and so on). The upper left edge of DPT in this particular case was not optimally pressed against the lid of the target holder. The FMT on the other hand exhibits a clear dividing line between rows F and G showing a small error of 0–0.4 Da (A to F) and a large error of 0.6–1.2 Da (G to K) which might be a result of non-perfect target production. This illustrates the necessity of multiple calibration points in the case of FMT and that one calibrant sample spot is not enough. In the case of DPT it is sufficient to use the three calibrant sample spots provided in the center of the 48 sample spots solving the problem of the above mentioned non-optimal pressing of the DPT against the target holder.

3.4. Applications

3.4.1. LDI reflectron MS of a small molecule mixture

LDI measurements are beneficial in avoiding interference from MALDI matrix substances and of the low mass contaminants in small molecule analysis [28]. Fig. 3(a) shows LDI mass spectra of tyrosidine, a natural occurring mixture consisting of at least 10 cyclic peptides [29,30]. The mass spectrum acquired using the DPT exhibited much higher signals (49 mV) while exhibiting less
noise compared to the traditional FMT (5.2 mV). On the FMT the following molecular species were found: tyrocidine A [MH]+ (FMT: m/z 1270.67 (7.9 ppm), DPT: m/z 1270.69 (23.6 ppm), theoretical: m/z 1270.66), tyrocidine A1 [MH]+ (FMT: m/z 1284.67 (0 ppm), DPT: m/z 1284.68 (7.9 ppm), theoretical: m/z 1284.67), tyrocidine B [MH]+ (FMT: m/z 1309.70 (22.9 ppm), DPT: m/z 1309.70 (22.9 ppm), theoretical: m/z 1309.67), tyrocidine B1 [MH]+ (FMT: m/z 1323.76 (60.4 ppm), DPT: m/z 1323.78 (75.5 ppm), theoretical: m/z 1323.68), [Trp3,Trp5] tyrocidine A [MH]+ (FMT: m/z 1332.83 (112.6 ppm), DPT: m/z 1332.81 (97.5 ppm), theoretical: m/z 1332.68), tyrocidine C [MH]+ (FMT: m/z 1348.93 (185.3 ppm), DPT: m/z 1348.94 (192.7 ppm), theoretical: m/z 1348.68), tyrocidine C1 [MH]+ (FMT: m/z 1363.05 (264.2 ppm), DPT: m/z 1363.07 (278.9 ppm), theoretical: m/z

Fig. 2. (a) Heat map of contact angles of plain water droplets measured across a whole DPT. (b) Molecular ion region of the MALDI mass spectrum of 500 attomole of deposited angiotensin II on FMT (top) and DPT (bottom). (c) Molecular ion signal of angiotensin II plotted against sample amount deposited between 0.1 fmol and 50 fmol. Inset shows range 0.1–5 fmol. (d) Molecular ion region of MALDI mass spectrum of angiotensin I for mass spectrometric resolution (FWHM) determination of the monoisotopic ion (5 fmol absolute amount) on FMT (top) and DPT (bottom). (e) Heat map of drift of m/z value for ACTH 18-39 ([MH]+ starting spot A1 m/z 2465.20) over a complete DPT (left) and FMT (right) surface. Calibration was done on spot A1 and the m/z difference was compared to the theoretical value for every other spot.
1362.69) and tyrocidine D [MH]+ (FMT: m/z 1372.19 (364.5 ppm), DPT: m/z 1372.11 (306.2 ppm), theoretical: m/z 1371.69), whereas on DPT all these were found with the addition of [Trp3] tyrocidine A [MH]+ (DPT: m/z 1293.68 (7.7 ppm), theoretical: m/z 1293.67). All theoretical m/z values are based on published structures [29].

An LDI mass spectrum of the blank DPT was acquired under the same MS acquisition conditions (Fig. 3(a), bottom) to show that the DPT target does not generate any additional peaks under the experiment’s conditions. LDI measurements generally require more fluence compared to MALDI. If the irradiation fluence is set too high damage to the metal film is possible. Only noise in the range up to 0.4 mV signal intensity was measured, which is about 100 times lower than the data from the tyrocidine sample acquired under the same conditions.

While DPT can be affected by very high laser fluence (the laser beam could penetrate the nanolayer), the laser fluence required for LDI measurements does not cause damage to the DPT. Normal samples in MALDI MS were usually measured with a relative laser intensity of 55–60 au (au, arbitrary units; the maximum selectable setting is 180 au). LDI mass spectra were usually recorded at around 70. Surface damage to the DPT occurred above approx. 120 au in the LDI mode.
Fig. 3. (a) Molecular ion region of the positive ion LDI mass spectra of tyrocidine deposited on a cleaned FMT and a new, unused DPT (m/z 1240–1500). The inset depicts the noise of a blank DPT acquisition (m/z 1300–1380); (b) Positive ion MALDI mass spectrum of γ-globulin (IgG) prepared on a DPT with the MALDI matrix SA; (c) Positive ion MALDI mass spectrum of IgM prepared on a DPT with the MALDI matrix SA; (d) MALDI mass spectrum of Pullulan 5600 Da, a size exclusion chromatography standard, prepared on an FMT (top) and DPT (bottom) with the MALDI matrix DHB.
Fig. 3 (continued)
3.4.2. MALDI linear TOF MS of an ultra-high molecular mass glycoprotein

The MALDI mass spectra of γ-globulin (IgG) on both DPT and FMT are quite similar and of almost the same absolute signal intensity (Fig. 3(b)), except the noise level is slightly better in the latter.

Polyclonal IgM exhibits a molecular weight close to 1 MDa [19,20] one of the largest glycoproteins still accessible with MALDI linear TOF MS. Fig. 3(c) shows the positive ion MALDI mass spectrum using a DPT and employing the ultra-high mass detector (425 fmol on target, 484 single shots). Singly, doubly and triply charged IgM molecules are visible (m/z 942,000, 471,000 and 314,000, respectively); a quadruply charged IgM is suspected, but not annotated). Based on these three ion species an average molecular mass of 942,000 Da was calculated, which is in line with the published data [19,20]. Other data clearly show that the same performance characteristics in terms of m/z range could be obtained with FMT (data not shown, but similar to data already published [19]).

3.4.3. MALDI linear TOF MS of a natural carbohydrate polymer mixture

Polymers of natural and synthetic nature are a substance group that usually requires very rigorous target cleaning after measurement, as most polymers tend to stick well to the target surface. For this reason, polymer measurement is a prime application area for DPT. In this instance, a 5600 Da pullulan reference (with an average degree of polymerization of 11.1) used in size exclusion chromatography [31] was chosen for DPT evaluation. In Fig. 3(d) two mass spectra of this pullulan sample acquired on both FMT and DPT are compared, exhibiting only sodiated molecules. A mass difference of around 486.4 from 3 glucose units was found throughout a polymer distribution ranging from m/z 2472.11 to 8309.20 in the case of the FMT. On the DPT, it was possible to see distinct peaks at higher m/z values as well. For FMT, an M_w value of 4610 and an M_z value of 4900 was calculated from the mass spectrum. In the case of DPT, an M_w of 5750 and an M_z of 5180 was found. The M_w and M_z determined from the measurement on the DPT are much more in accordance with the data provided by the vendor which was determined by size exclusion chromatography (M_w = 5900 and M_z = 5400). As can be seen, the m/z range which exhibits peaks with good S/N ratio is higher on DPT compared to FMT. Additionally, both spectra exhibit a second polymer distribution towards the lower m/z range showing mass differences in accordance with heoxse subunits (Δm/z = 162.1) These can be seen more clearly on the DPT and can be attributed either to in-source fragmentation or a sample impurity.

4. Conclusions

To determine mass spectrometric performance of a new MALDI MS target for axial MALDI and LDI TOF MS it is important to evaluate the achievable mass spectrometric resolution, m/z range, limit of detection and of course the surface itself, as this has a significant effect on all measurements performed. In this regard, the here presented disposable polymeric and nano-coated MALDI target (DPT) proves itself to be on par, and in several aspects, even better than the conventional full metal target (FMT) reference. The investigated targets exhibited a high flatness that is important for targets used in axial TOF mass spectrometers. Electrical conductivity, which is essential to avoid charging-induced peak shifting, vacuum and laser compatibility (for typical laser fluences applied in MALDI and LDI), no polymer-derived background ions (due to the metal layer nano-coating) and chemical compatibility to typical MALDI/ LDI MS sample/matrix solvents are given. DPT achieved higher analyte ion signals for LDI measurements, often better signal-to-noise ratios, a slightly higher resolution and better linearity for very low concentrations near the LOQ. Aside from this, real world measurements like polymer and protein analytics show that this new target is suitable for a broad range of applications.

One benefit of using disposable targets lies in the fact that for every sample a clean and fresh target surface can be used, thus avoiding possible sample memory. No required cleaning eliminates solvent/detergent and disposal costs, an important factor as they normally have to be used in large volumes. Assuming that the FMT is used 25 times, i.e. cleaned 25 times, the saving per sample spot is approx. 0.44 € with the one-way DPT vs. FMT. Furthermore, this means no changes in target surface morphology or chemistry can occur from contact with reagents or cleaning agents, ensuring compliance with cleaning validation requirements. An additional feature of this target type is the ability to perform LDI measurements on small analytes with good S/N ratio. In tests in our lab the DPT was used to archive peptide and protein samples directly on the target for re-measuring purposes [32]. To allow easier identification of targets laser-engraving of a bar code is possible. Recently, the DPT has also shown its usefulness for intact cell mass measurements like polymer and protein analytics show that this new target is suitable for a broad range of applications.

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Conflict of interest

SB, WW and GA have no financial or personal conflict of interest. WB and MK are full-time employees of Sony DADC Austria.

References


Intact cell/intact spore mass spectrometry (IC/ISMS) on polymer-based, nano-coated disposable targets

Stefan Bugovsky a, Wolfgang Winkler a, Werner Balika b, Manfred Koranda b, Günter Allmaier a,∗

a Institute of Chemical Technologies and Analytics, Vienna University of Technology, Getreidemarkt 9/164, Vienna A-1060, Austria
b Sony DADC, BioSciences Business Unit, Anif, Austria

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A B S T R A C T

Identification and differentiation of microorganisms has and still is a long arduous task, involving culturing of the organism in question on different growth media. This procedure, which is still commonly applied, is an established method, but takes a lot of time, up to several days or even longer. It has thus been a great achievement when other analytical tools like matrix-assisted laser desorption/ionization (MALDI) mass spectrometry were introduced for faster analysis based on the surface protein pattern. Differentiation and identification of human pathogens as well as plant/animal pathogens is of increasing importance in medical care (e.g. infection, sepsis, and antibiotics resistance), biotechnology, food sciences and detection of biological warfare agents. A distinction between microorganisms on the species and strain level was made by comparing peptide/protein profiles to patterns already stored in databases. These profiles and patterns were obtained from the surface of vegetative forms of microorganisms or even their spores by MALDI MS. Thus, an unknown sample can be compared against a database of known pathogens or microorganisms of interest.

To benefit from newly available, metal-based disposable microscope-slide format MALDI targets that promise a clean and even surface at a fraction of the cost from full metal targets or MTP (microtiter plate) format targets, IC/ISMS analysis was performed on these and the data evaluated. Various types of bacteria as well as fungal spores were identified unambiguously on this disposable new type of metal nano-coated targets. The method even allowed differentiation between strains of the same species. The results were compared with those gained from using full metal standard targets and found to be equal or even better in several aspects, making the use of disposable MALDI targets a viable option for use in IC/ISMS, especially e.g. for large sample throughput and highly pathogenic species.

1. Introduction

Sample supports (“targets”) for MALDI MS have a great number of features that are tailored to the need of the application, these being ideally a perfect planarity, no damages on the surface, being easy to clean after analysis and facilitating an ideal crystallization of the sample/matrix mix. While metals (e.g. stainless steel or nickel-coated aluminum) have long been the main material used for constructing these devices, there are a number of considerations that have led researchers to explore other materials such as silicon or polymers. Polymers have the benefit of being very cost efficient, leading to new ways on using targets. Targets with hard to remove samples (e.g. synthetic polymer samples) do not have to be cleaned after measurements they can be simply discarded after use, something especially polymer analytics can very much benefit from. In some areas, a defined surface such as this of a newly produced polymer MALDI target is demanded by law or lab policy (GLP) to guarantee unambiguous results free of measurement artifacts (e.g. in the pharmaceutical or human diagnostic industry).

Here we compare the application of disposable polymer nano-coated targets (DPT) for Intact Cell/Intact Spore Mass Spectrometry (IC/ISMS) of fungal and bacterial samples from various sources. IC/ISMS is an invaluable tool for fast typing of a large range of unknown microorganisms [1–7], requiring only a small sample amount [8]. While other methods to identify microorganisms exist such as infrared spectroscopic methods [9] or molecular biology techniques based on the 16S-rRNA method [10] they either are very time consuming or require specially trained personnel for
interception of the data. IC/IMS on the other hand is a technique that can be performed in minutes and also be automated.

Healthcare institutions can analyze patients’ samples, determine if a pathogenic strain is present and treat accordingly, often within just hours or even minutes after sample acquisition. In the food industry, it is also necessary to control e.g. crops for fungus like *Fusarium* sp. as some strains exhibit toxicity towards humans and livestock [11] and disturb the seed development leading to losses in for example wheat [12]. Even if most peaks’ identities are unknown, the m/z patterns they produce during mass spectrometric analysis allow differentiation between species and strains. With the help of elaborate algorithms and a database system these mass spectra can also be compared to already known mass spectra of microorganisms [13–15]. To establish effective databases for identification/differentiation, highly reproducible measurements and sample preparation protocols are necessary. These prerequisite cannot be met if MALDI target surface properties change due to target cleaning procedures, autoclaving or carryover from previously deposited samples.

As was shown in earlier reports mass spectra acquired on disposable polymer targets can exhibit a higher sensitivity [16,17] as well as better resolution compared to full-metal target, something IC/IMS can greatly benefit from. In high throughput scenarios where a large number of samples have to be processed, time per measurement should be kept low. An important influence on that is the crystallization of the sample/matrix mix, which in turn is highly influenced by the surface (material, structure) of the target. Small groves in the top layer of the target can provide the matrix/sample-solution a starting point for crystallization which, if unevenly distributed, can result in an uneven crystallization. A very smooth surface can on the other hand also result in unusable preparation. The surface of the polymeric targets appear to facilitate a very uniform and reproducible crystallization, as will be shown later herein.

2. Materials and methods

2.1. MALDI targets

Disposable polymer nano-coated target (FlexiMass-DS targets, TO-430, Shimadzu Kratos Analytical) was made by Sony DADC Austria. The device (Fig. 1) has the following dimensions, 76.2 × 26 × 1.6 mm and consists of black carbon filled polypropylene with a stainless steel nano-coating on upwards facing side of less than 400 nm. The format used for the targets is based on microscope slides. 48 sample spot areas with 2.8 mm diameter were marked allowing the preparation of 48 samples (four columns with 12 rows, designated A-L on the long side and 1–4 on the short, and furthermore three calibration spot areas located in the top, center and bottom area of the target between the two central sample spot columns — column 2 and 3). The targets were produced by an injection molding process followed by DC pulsed magnetron sputtering of stainless steel onto the DPT’s top layer.

As reference target (Fig. 1) for comparison in performance a full metal target (FMT, FlexiMass-targets TO-483R00, Shimadzu Kratos Analytical) which consists completely of stainless steel and exhibited a machine-polished surface. *Fusarium* samples were instead of the before mentioned FlexiMass target prepared on 384 well microtiter plate (MTP) format nickel-plated aluminum-based target (TO-474, Shimadzu Kratos Analytical). MTP did not require a separate target holder for introducing it into the ion source.

A MTP sized target holder (Precision adapter, Shimadzu Kratos Analytical) was used for carrying the two different kinds of target (DPT and FMT) for the introduction of the samples into the MALDI ion source (Fig. 1). In the applied target holder up to 4 targets can be fixed and introduced simultaneously into the vacuum system of the ion source. The targets are pressed from the bottom into the frame of the holder by means of a spring plate to obtain optimal planarity across the whole target for optimal desorption/ionization conditions.

2.2. Chemicals

Sinapic acid matrix solution (SA, Sigma–Aldrich, product number 58313; 10 mg/mL sinapic acid in ACN (acetonitrile)/0.1% aqueous trifluoroacetic acid (TFA) (1:1, v/v)), ferulic acid matrix solution (FA, Fluka, product number 46280); 10 mg/mL in acetonitrile/0.1% aqueous trifluoroacetic acid (7:3, v/v), α-cyano-4-hydroxycinnamic acid matrix solution (CHCA, Sigma–Aldrich, product number C-2020; saturated solution in water ACN: ethanol (1:1:1,v/v/v) containing 3% TFA), 2,5-dihydroxybenzoic acid (DHB, Fluka, product number 85707; 75 mg/mL in water: ACN: ethanol (1:1:1,v/v/v) containing 3% TFA). The following solvents were used: ACN (Merck, product number 100003, analytical grade), ethanol (Merck, product number 100983, absolute analytical grade), trifluoroacetic acid (Riedel-deHaën, product number 61030), formic acid (Sigma–Aldrich, product number 06440) and water (from reverse osmosis facility and further purified with Simplicity apparatus (Millipore), 18.2 MQ cm resistivity at 25 °C). This water quality was used in all applications.

2.3. Microorganisms


2.4. Mass spectrometry

Two different mass spectrometers were used to acquire the mass spectra of the selected samples. *Aspergillus* and *Fusarium* samples: Positive ion MALDI mass spectra were obtained by means of a MALDI time-of-flight (TOF)
instrument (Axima CFRPlus, Shimadzu Kratos Analytical) in the linear mode. The device uses a nitrogen laser (337 nm) at a pulse rate of 20 Hz at an average pressure of 1.6 × 10⁻¹⁰ mbar. Mass spectra were averages of 900 single, unselected laser profiles (one shot per profile) in a 1000 × 1000 μm raster. All measurements were blanked (“removal of low mass ions”) up to m/z 1000. Mass spectra for samples were acquired in the m/z range 1000–30,000 and for Fusarium samples from m/z 1000 to 15,000. Pulsed extraction was set to m/z 5000 to cover the wide selected m/z range.

All other bacteria and Candida samples: Positive ion MALDI mass spectra were recorded by means of the MALDI TOF mass spectrometer Axima Confidence (Shimadzu Kratos Analytical) in the linear mode. The device uses again a nitrogen laser but at a pulse rate of 50 Hz. Mass spectra consisted of 34 averaged profiles of 5 shots each (170 shots in a circular raster). All measurements were blanked until m/z 1800. Mass spectra were acquired from m/z 1800 to 20,000 and pulsed extraction was set to m/z 8330.

2.5. Sample preparation

**A. clavatus and A. fischeri.** 15 μL of the suspension (spores and hyphae in water) were washed to eliminate contaminants derived from cultivation three times with water using Nanosep MWCO 10 kDa centrifugal devices (Pall) at 16,100 g for 10 min and resuspended in 3 μL water. 3 μL of the purified suspension were spotted onto DPT and FMT for comparison and allowed to dry at room temperature (RT). Then 1 μL of SA matrix solution was added to the preparation and again allowed to dry before measurement at RT.

**F. graminearum strains.** [18] Fusarium conidia spores suspensions contained glycerol (for long term storage). 86 μL of F. graminearum CPK 2983 and 22 μL F. graminearum CPK 2986 suspensions were washed three times (to remove glycerol and contaminants) with 100 μL ACN: 0.5% aqueous formic acid (7: 3, v/v) using Nanosep MWCO 10 kDa centrifugal devices at 16,100 g for 10 min and resuspended in 5 μL water. For MS sample preparation, a specifically designed 2-layer volume technique was used [19]. Briefly, FA matrix solution was premixed in equal parts with the purified conidia spore suspension and vortexed. 1 μL of this mixture was spotted on the different types of targets (DPT and MTP) and allowed to dry at RT. Additionally, further 0.5 μL of the matrix solution were deposited on top of the dried sample matrix preparation and also allowed to dry again at RT.

**Bacteria and C. albicans.** [20] A microorganism colony picked from the agar plate was suspended in 60 μL 25% aqueous formic acid and subjected to 10 s ultrasound treatment. This suspension was split into two aliquots and then mixed with 60 μL CHCA matrix solution and the second aliquot with 60 μL DHB matrix solution, respectively. 1 μL of the CHCA/sample mixture and 0.55 μL of the DHB/sample were then deposited on both target types (DPT and FMT) and allowed to dry at RT.

**Light microscopy image.** Images at 20 × and 80 × magnification were taken with SMZ 800 (Nikon) microscope in junction with camera control unit DS-L1 (Nikon).

3. Results

3.1. Spores and hyphae of A. clavatus and A. fischeri

A comparison of the mass spectra of the two different species of Aspergillus, (namely A. fischeri and A. clavatus) clearly shows that a distinction between the samples is possible based on their peptide/protein pattern (data not shown). Not all peaks were annotated due to the sheer number of signals present, but the 15 most prominent and representative (discerned from a comparison of 10 measurements of different samples within one Aspergillus species; peaks present in 9 out of 10 measurements were deemed representative, the 15 most intense of these peaks were chosen) were highlighted in the mass spectra shown (Fig. 2). The mass spectra clearly share some peaks, although some peaks can only be found in one mass spectrum or the other. The peaks chosen to be representative for A. fischeri are as follows: m/z 3502, 3926, 4042, 5164, 5294, 5364, 5555, 5953, 6628, 7009, 7557, 7674, 11,711 and 14,013. Representative peaks for A. clavatus were found at m/z 2405, 2592, 4047, 4834, 6798, 7340, 7405, 7469, 7516, 7630, 7734, 8504, 11,783, 14,147 and 15,793. These peaks are present on both DPT and FMT.

Mass spectra (Fig. 2) acquired from different targets (DPT and FMT) exhibit a very similar number of peaks in the mass range below m/z 10,000, while usage of DPT generated a greater number of peaks above m/z 10,000 albeit with increased noise level (particularly for A. fischeri). This observation could be used to identify a far greater number of key signals thus giving a higher degree of confidence when determining species or strains.

Preparations of both species were also studied under the microscope (Fig. 3) at 20 × and 80 × magnifications. While the crystallization was quite normal for SA for A. clavatus on both FMT and DPT, the photographs clearly show abnormal results for A. fischeri on both target types, i.e. no discernible crystallization and a ring of clear substance around the preparation. This was due to the gelatinous biopolymer, in which the A. fischeri hyphae and spores were embedded and which could not be removed by normal washing procedures. Other than that, there was no discernible difference in preparation on FMT and DPT upon comparison within a strain.

3.2. Spores of F. graminearum CPK 2983 and CPK 2986

Both F. graminearum strains were selected to highlight the ability of IC/ISMS to even allow differentiation between spores of two fungal strains. As above, only the most abundant peaks (25 peaks in this case) were highlighted and annotated to illustrate similarities and differences between the mass spectra. For F. graminearum CPK 2986 these were determined to be m/z 2506, 2590, 2635, 2686, 2969, 3119, 3158, 3200, 3432, 3523, 3566, 3585, 3622, 3635, 3714, 3763, 3894, 3979, 4225, 4233, 4385, 4518, 4682, 4811, 4988 and 5006. For F. graminearum CPK 2986 these were determined to be m/z 2506, 2590, 2635, 2686, 3179, 3432, 3566, 3582, 3622, 3635, 3714, 3763, 3894, 3979, 4174, 4225, 4253, 4385, 4518, 4682, 4811, 4988 and 5006. As can be seen in the m/z values listed above and the mass spectra (see Supplement Fig. 1s), both strains possess common as well as different signals. Peaks that only occur in strain CPK 2983 are m/z 3179 and 4174, whereas CPK 2986 exhibited 4 peaks (m/z 2969, 3119, 3158, 3523 and 6093) which were only found with this strain.

The acquired mass spectra on both DPT and MTP exhibited a similar number of peaks and exhibited all of the signals listed above, although the level of noise was found to be slightly higher on the DPT, which may or may not be simply a variation in sample preparation. Fungal samples were found to be more difficult in obtaining suitable mass spectra compared to bacteria.

A side by side comparison of the preparations achieved on polymer and metal targets shows noticeable differences. While the crystallization of CHCA (Fig. 5(a)) yielded slightly smaller and more uniform crystals on the polymer target, it exhibited a rather distinct appearance on DPT, while the crystallization on MTP is typical of that encountered on metal targets in that it exhibited a dense crystalline ring while the preparation in the middle was sparse.

3.3. Bacteria and C. albicans

A side by side comparison of the IC/ISMS preparations achieved on polymer and metal targets shows noticeable differences. While the crystallization of CHCA (Fig. 5(a)) yielded slightly smaller and more uniform crystals on the polymer target, it exhibited a rather distinct...
variations for DHBFig. 5 (b). This matrix usually forms large crystals growing from the rim of the preparation in the direction of the center. This, however, was only observed on the DPT target; crystallization on the FMT consisted of small, but denser crystals along the rim of the preparation. The reason for this is that in the presence of the microorganisms the crystallization is disturbed, leading to smaller crystals.

In contrast to the fungal samples tested above, the acquired mass spectra of the bacterial and C. albicans samples were not compared manually but with the SARAMIS software (Shimadzu Kratos Analytical) and database, which contains a large number of fingerprints of bacteria and fungi allowing automatic identification [20]. The quality of this match is given in percent, where 100% indicates a perfect match. The overall score (sum of all scores) of all microorganisms tested was slightly higher when using DPTs. The bacterial samples were all identifiable with the SARAMIS software and database with 99.9% certainty on DPT, while four measurements out of the 30 samples (10 species of bacteria, three measurements per species) gave scores below 99.9% (but above 94% in

Fig. 2. Positive ion mass spectra of A. fischeri with sinapic acid as matrix substance on FMT (top) and DPT (bottom).

Fig. 3. Microscope images of Aspergillus clavatus (a) and Aspergillus fischeri (b) samples on FMT (left) and DPT (right) before and after the addition of sinapic acid matrix solution at 20× and 80× magnifications.
all cases; with CHCA as matrix) on FMT. Usage of DHB instead of CHCA yielded two measurements out of the 30 samples with scores of 94.0 and 94.1, with the remaining 28 having a score of 99.9 on the DPT (FMT: five below a score of 99.9, one of these even as low as 79.8). An overview of the positive ion mass spectra of the 11 bacteria tested as well as the C. albicans sample is illustrated in Fig. 2S of the supplement. E. coli was selected for a direct comparison of matrices CHCA and DHB on DPT and FMT (Fig. 6). 15 of about 200 peaks recognized by SARAMIS algorithm were chosen to highlight the differences/similarities of these mass spectra: m/z 3851, 4362, 4775, 5096, 5380, 6255, 6411, 7268, 7705, 7865, 8326, 9537, 10,140, 10,300 and 11,728. CHCA provides a marginally better signal-to-noise ratio compared to DHB as a matrix. The recognition score was also slightly better using CHCA (99.9% average instead of 99.6% when using DHB, both on DPT).

The time per measurement, which was necessary to obtain a mass spectrum with 170 laser shots, was also reduced from 73 s (FMT) to 38 s (DPT) (for CHCA as matrix; no improvement was seen for DHB as matrix), as it took less time to acquire the needed number of acceptable single shots (DHB: 1000 shots, CHCA: 500 shots), which were then compiled to the final mass spectrum.

### 4. Discussion

In regard to differentiation between two strains of DPT was equivalent to the MTP. While a minor difference in signal-to-noise ratio of the acquired mass spectra was observable in the mass spectra, these should be disregarded. Spores tended to provide mass spectrometric data with a high level of noise and fewer signals (dozens compared to 100–200 found in bacteria). It is suspected that bacteria provide a higher number of signals as their cell wall may more easily disintegrate in the laser beam thereby gaining access also to the proteins found not only on the cell surface but also in the cytoplasm and cytosol.

Results from the measurements of the bacterial microorganism samples were slightly superior on DPT as indicated by the overall identification score gained from SARAMIS software. While the scores were still all high enough to allow a definite identification bacterial species, there might be times when that small difference might have an influence in the correct determination of a pathogen. A further factor that makes the DPT superior to the FMT is the difference in the time per measurement needed to acquire a useful mass spectrum which is clearly influenced by the co-crystallization of the sample/matrix-mix but also by the morphology of the preparation, i.e. size and distribution of crystals as was seen above in the case of E. coli with DHB. A reduction of this factor has a direct influence on the number of samples that can be analyzed for a given time frame, which makes usage of polymer based targets very economic in high-throughput situations (e.g. hospitals or medical diagnostic centers).

CHCA was shown to be the matrix of choice for IC/ISMS of bacteria on any target, as mass spectra acquired using it exhibited a
slightly better signal-to-noise ratio, had a higher score of identification on the biological samples validated in this test and produced these results in a shorter amount of time. Mass spectra acquired with DHB as matrix yielded a slightly lower identification score whilst taking longer for a suitable mass spectrum to be acquired.

5. Conclusion

The DPT is as good or in some cases even better than the two conventional target types (FMT and MTP format targets) used in this study in terms of number of peaks detected and signal-to-noise ratio of these peaks. Sample/matrix preparations exhibit a slightly different appearance (sample pattern) on DPT, allowing also the long term storage of prepared samples, as was seen in preliminary tests. Furthermore, the introduction of affordable DPTs avoids the problem of target cleaning, which can be especially problematic in laboratories where GLP or GMP require arduous and expensive cleaning procedures, e.g. for highly infectious or pathogenic samples [21].

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mcp.2013.11.001.

Fig. 6. Positive ion mass spectra of E. coli on both FMT and DPT with MALDI matrices DHB (upper two mass spectra) and CHCA (lower two mass spectra). 15 peaks were annotated for better comparison.

References

[17] STEUBIGER G, BOLACEM O, REHULLA P, BICKER W, BINDER BR, BOCHLOV V. Analysis of oxidized phospholipids by MALDI mass spectrometry using 6-aza-2-


Long time storage (archiving) of peptide, protein and tryptic digest samples on disposable nano-coated polymer targets for MALDI MS

Stefan Bugovsky\textsuperscript{a}, Wolfgang Winkler\textsuperscript{a}, Werner Balika\textsuperscript{b}, Günter Allmaier\textsuperscript{a,\ast}

\textsuperscript{a}Institute of Chemical Technologies and Analytics, Vienna University of Technology, Vienna, Austria
\textsuperscript{b}Sony DADC Austria, Anif, Austria

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Archiving of biological specimens is important due to the importance of reanalysis of already prepared MALDI MS samples or simultaneous analysis of a sample series. Proteins/peptides and digests were prepared for measurement on a polymer-based metal nano-coated MALDI target and subjected to various storage conditions (−80 °C to RT) in a vacuum-sealed pouch and at atmosphere for 6 months. The MS data gathered from these preparations illustrate trends in the aging of different samples and to find optimal storage conditions (−20 or −80 °C in low oxygen environment). The disposable/low cost target proved to be a suitable platform for storage and MALDI MS.

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1. Introduction

Long time storage of biological samples is a very difficult but nonetheless essential aspect of bioanalytical processes\cite{1,2}. While some analytes (e.g., polymers) are very resilient to degradation (oxidation\cite{3}, dehydration, . . . ) and can be stored at ambient conditions for a long time, other substances, especially biomolecules, are of a completely different nature as they react strongly to changes of pH, composition of the solvent, radiation and temperature, altering the primary (e.g., hydrolytic cleavages, deamination of Asn), secondary (irreversible damage) and/or tertiary structure (reversible damage)\cite{4} thus possibly losing the activity of a given protein due to changes to the active site\cite{5}. In any case, degradation is always a factor when dealing with biopolymers such as proteins\cite{6,7} and peptides, which is why great care has to be taken when considering storage conditions for such biological samples\cite{8–10}.

Especially biomolecules\cite{11,12} like peptides and even proteins furthermore tend to be adsorbed to the inner walls of the sample containers, lowering the concentration of the analytes even more along with changing the composition of the sample. Solutions to this problem exist in the form of low binding surfaces meant to keep the sample materials from adhering to the surface. This, however, becomes irrelevant when the analyte sample is stored with, e.g., MALDI MS matrix and possibly additives directly on the MALDI MS target. The main reason to store partly consumed MALDI MS preparations is the possibility to perform reanalysis without any new critical preparations for example to perform MS/MS in the high energy CID mode or post source decay fragmentation. Furthermore, the subsequent direct analysis of samples collected at different time points from, for example, 2D gel electrophoresis done at different times, i.e., collect all samples, prepare samples on MALDI targets, store these targets and then perform all MALDI MS analysis at once. The emergence of new and cheap materials\cite{13,14} for MALDI MS targets allows the use of these as disposable targets, which avoids the problem of carryover from previously measured samples and is also a very inexpensive way to archive large sample batches. Possible scenarios where such a need might arise include large-scale proteomics projects based on 2D gel electrophoresis, tissue samples in medical diagnostics or environmental samples. Controversial or data-analyzed samples can be re-analyzed (due to the fact that MALDI MS usually is not consuming the complete sample preparation) at a later time if the need arises, for example determination of a post translational modification by MS/MS or gain of new knowledge about the biological experiment. Here, the specimen would ideally be in a state very close to the first measurement.

The storage conditions chosen for long term archiving include worst (maximal change) and best case scenarios (minimal or no change at all) as baselines against which to compare the effectiveness
of the storage protocol. The worst case scenario here resembles storage of samples under normal ambient conditions (access to atmosphere (oxygen, humidity) and room temperature (RT)), whereas the best case scenario would be –80°C without contact to the normal atmosphere.

2. Experimental

2.1. Chemicals

α-Cyano-4-hydroxycinnamic acid (CHCA) matrix solution (Agilent Technologies, G2037A, solution of 6.2 mg CHCA/mL in methanol: acetonitrile (ACN): H2O (36:56:8, v/v/v)), 2.5-dihydroxybenzoic acid (DHB, Fluka, 85707; 20 mg/mL in ethanol: H2O (1:1, v/v)), ferredoxin (Sigma–Aldrich, F2513, from Spirulina, 1 mg/mL (95.24 nmol/mL) in H2O), bovine serum albumin (Sigma–Aldrich, A2153, BSA1 mg/mL in H2O), γ-globulins (Sigma–Aldrich, 49030, 2 mg/mL in 0.01% aqueous TFA), fetuin (Sigma–Aldrich, from fetal calf serum), ovalbumin (Sigma–Aldrich, from chicken egg white), peptide calibration mix (angiotensin I (Sigma–Aldrich, A3178; [MH]⁺ m/z 1296.69), [Glu]₂-fibrinopeptide B (Sigma–Aldrich, F3261; [MH]⁺ m/z 1570.68), N-acetyl renin substrate (Sigma–Aldrich, R5380; [MH]⁺ m/z 1800.94), ACTH 1-17 (Sigma–Aldrich, A2407; [MH]⁺ m/z 2093.09) and ACTH 18-39 (Sigma–Aldrich, A0673; [MH]⁺ m/z 2465.20) were all processed at 100 fmol/µL in ACN:0.1% aqueous TFA (1:1, v/v), whereas ACTH 7-38 ([MH]⁺ m/z 3657.93) was present in the matrix at 150 fmol/µL, ACN (Merck, 100003, p.a., analytical grade), ethanol (Merck, 100983, absolute, p.a.), trifluoroacetic acid (TFA, Riedel-de-Haën, 61030), denaturing buffer (6 M guanidinium hydrochloride (Sigma–Aldrich, G4505), 0.5 M Tris–HCl, 2 mM EDTA, pH 8.0), DL-dithiothreitol (Fluka, 43819), iodoacetamide (Sigma–Aldrich, I6125), water (from reverse osmosis water quality was used in all applications.)

2.2. MALDI MS targets

Disposable polymer target (FlexiMass–DS, TO–430, Shimadzu Biotech–Kratos Analytical) were manufactured by Sony DADC Austria. The device consists of carbon black particle filled polypropylene with a stainless steel nano-coating on upwards facing side of about 300 nm. The format used for the targets is based on the microscope slide format with following dimensions: 76.2 × 26 × 1.6 mm. A microtiter plate sized target holder (Precision adapter, Shimadzu Kratos Analytical) was used for carrying the target on the sample stage, which is introduced into the MALDI MS ion source. Up to four targets can be fixed onto the target holder and introduced afterwards simultaneously into the vacuum system of the MS.

2.3. MALDI mass spectrometry

Positive ion MALDI mass spectra were obtained by means of an Axima CFRPlus instrument (Shimadzu Kratos Analytical) in the linear (proteins) or reflectron (peptides and PMF) mode. The device uses a nitrogen laser (337 nm) at a pulse rate of 20 Hz at an average ion source pressure of 1.6 × 10⁻⁷ mbar. All data was recorded automatically to eliminate operator influence, i.e., all sample positions were programmed into the device’s acquisition software which then measured all of 48 sample preparations per target automated. The positioning of the target stage was recalibrated before the first data acquisition was performed on every measurement day. The digest calibration was performed via the peptide calibration mix. For the known stock analytes (peptide calibration mix and proteins) their molar mass according to literature was used for calibration.

2.4. Automated MS measurement of proteins

Proteins were measured in positive linear mode in the m/z range from 2,000 to 70,000 for ovalbumin, 2,000 to 50,000 for ferredoxin, and 2,000 to 200,000 for γ-globulin, respectively, with laser irradiance set to 120 a.u. (arbitrary unit, with a maximum of 180). Recorded mass spectra consisted of the average of 673 single profiles recorded in a circular raster with 1.5 mm diameter and a spacing of 55 µm between laser spots. Pulsed extraction was set to m/z 44,000 for ovalbumin, 11,000 for ferredoxin and 180,000 for γ-globulin. Savitsky–Golay algorithm [15] was applied for smoothing of the acquired mass spectra (20 channels). No baseline subtraction was performed. The best mass spectrum (in terms of highest signal intensity and most symmetric peak shape) out of three measurements of different preparations was used for interpretation.

2.5. Automated MS measurements of protein digests and peptide calibration mix

Mass spectra of digests were performed in positive ion reflectron mode between m/z 300 and 5,000 with laser irradiance set to 55 a.u. Acquired mass spectra were comprised of an average of 571 single profiles recorded in a circular raster with a diameter of 1.5 mm and a spacing of 60 µm between laser spots. Pulsed extraction was set to m/z 2400. Neither smoothing nor baseline subtraction were applied. The best mass spectrum (in terms of highest signal intensity) out of three measurements of different preparations was used for interpretation.

2.6. Peptide mass fingerprinting

For peptide mass fingerprinting (PMF) all peaks were annotated and picked manually (all peaks between m/z 600 and 2800 with a signal-noise-ratio of at least 10:1; mass spectrometric settings were according to the described measurements for protein digests). From the peak list eliminated were all known contaminations [16] derived from the MALDI MS matrix, alkali adducts, water cleavages, keratin and autotryptic digestion. The filtered list was entered into a Mascot (Matrix Science) database query [17] (version 2.3.02, database SwissProt, enzyme trypsin, up to 1 missed cleavage, fixed modification: carbamidomethyl, peptide tolerance: ±0.5 Da, m/z values [MH]+, monoisotopic, taxonomy: mammalia). Detailed score values, sequence coverages, number of peaks searched and number of peaks of the highest scoring result were listed in supplement.

2.7. Protein digestion procedure [18]

About 1 nmol protein to be digested (BSA and fetuin, separate) was dissolved in 75 µL denaturing buffer. The solution was then incubated at 37°C for 30 min in a thermostirrer and 50 times the molar amount DTT added. Again, incubation was carried out for 1 h at 37°C. The reduced protein was then cooled in ice and iodoacetamide in excess of the –SH groups present added. The mixture was then incubated for 1 h at RT in the dark. The sample was then cleaned up with a MicroSpin G-25 column (GE Healthcare), diluted in equal volumes with 100 mM ammonium hydrocarbonate solution and 500 ng trypsin (Roche) added. The digestion was performed overnight at 37°C and the digested protein solution then dried by means of the vacuum concentration centrifuge. The resulting dried sample was then redissolved in
25 µL ACN/0.1% TFA solution (1:1, v/v) prior to MALDI MS sample deposition.

2.8. Preparation and storage of samples

1 mg/mL solutions of ferredoxin and ovalbumin and a 2 mg/mL solution of the γ-globulins as well as 1:10 (v/v) and 1:100 (v/v) dilutions with water thereof were mixed in same volumes with DHB matrix solution. 1 µL of these mixes were then spotted on 31 targets (5 storage conditions for 6 measurement days plus one for the reference on day zero) three times per target and allowed to dry at RT. In terms of absolute amount of protein on target, the undiluted solution comes to 47.2 pmol ferredoxin, 11.7 pmol ovalbumin and 6.9 pmol γ-globulin, with a tenth of that for the 1:10 (v/v) dilution, resulting in 4.7 pmol ferredoxin, 1.17 pmol ovalbumin and 690 fmol γ-globulin on target per preparation.

100 fmol/µL and 20 fmol/µL peptide calibration mixes and 1:10 (v/v) dilutions (in ACN: 0.1% aqueous TFA (1:1, v/v)) of the protein digests were mixed in equal volumes with CHCA matrix solution. 0.5 µL of these mixes were then pipetted on the 31 targets from above three times per target and allowed to dry at RT.

One target box was vacuum sealed with a Max-DD (Boss) device. Targets were placed in microscope slide boxes which in turn were placed in water and air tight pouches (Fig. 1). These were positioned enveloping the nitrogen nozzle to ensure a complete as possible flush of oxygen. The device was then evacuated for 30 s and flushed with nitrogen (99.999% AirLiquefir) for 2 s (pressure was regulated to approximately 4 bar on the gas cylinder). Sealing was applied for 1.6 s to ensure a thorough weld.

BSA and fetuin digests were used undiluted (40 pmol/µL theoretical amount without considering any loss during digestion process and 100% digestion efficiency) as well as diluted by the factor of 10 and mixed in equal volumes with CHCA solution. 0.5 µL of these mixes were then pipetted onto the 31 targets three times per target and allowed to dry at RT.

24 of the targets were sealed in water- and air-tight pouches under vacuum and flushed with nitrogen. For each storage temperature (−80 °C, −20 °C, +4 °C and RT) six targets were selected and stored. Additionally 6 targets were stored without a pouch at RT with access to the lab atmosphere as a reference (considered as worst case). The remaining 31st target was used as a reference for day zero and measured immediately. Targets – one per storage condition – were taken out of storage in a roughly monthly timeframe (26, 61, 87, 117, 153 and 181 days) for six months.

3. Results and discussion

3.1. Peptide mixture

The peptides in the peptide calibration mix were chosen for their known stability in storage and good mass spectrometric response—they do not contain methionine or any disulphide-bond and are not susceptible to easy hydrolysis. Our investigations found that this is only valid in part. The 100 fmol/µL peptide mix yielding 25 fmol absolute amount on target exhibited virtually no signs of degradation at −20 and −80 °C. Samples stored on a target that was archived at +4 °C showed small signs of water loss from [Glu1]-ferroin peptide B that aggravated at samples stored at RT, with no difference if the target was in a sealed pouch or not. In these two instances, this phenomenon was so pronounced that the intensity of the [MH–H2O]+ ion was even higher than the intensity of the [M+H]+ peak after 153 days of storage. The loss of water originates from the cyclization of the N-terminal glutamic acid in [Glu1]-ferroin peptide B [19]. Apart from that, no relevant peak deterioration was found for the 100 fmol/µL mix. The 20 fmol/µL peptide mix (resulting in 5 fmol peptide mix on target) exhibited a steady but slow increase in noise level as can be seen in Fig. 2. Interestingly, here the water loss described earlier was only found at samples stored at RT. No valid data could be acquired from measurements performed on the targets stored at −20 °C (sealed) and RT (open) on the 117th day, as all three preparations (per storage condition) proved unusable (possibly non-optimal crystallization). A trend, however, can still be seen even without these two mass spectra. The two targets stored at RT (sealed and open) also displayed steadily rising noise level, which in these cases also go hand-in-hand with loss of peptide intensity, leading to the loss of signal from peptide ACTH 1–17 at m/z 2093.09 after 117 days.

Concluding this observation, it is clear that some peptides are more prone to degradation than others, as shown for [Glu1]-ferroin peptide B. This loss is heavily influenced by temperature. This effect was observed at high as well as at low peptide concentrations. Therefore it is recommendable—and as has been shown to be possible—to store peptide samples on target at temperatures below −20 °C and sealed in a pouch to avoid condensation, oxidation, loss of water and deamination. For these conditions, no change was observed in the peptide pattern in the six months timeframe of the experiment.

3.2. Proteins

Results for proteins were surprising as the data revealed that the analytes were very stable until the end of the testing even when not stored refrigerated. The undiluted ovalbumin solution yielded very well discernible peaks with little difference to the mass spectra acquired on day zero for all storage conditions, even RT. The diluted solution, on the other hand, displayed peaks that were still recognizable, but on the brink of disappearing, i.e., at the limit of detection without search for a sweet spot. The strongest signals here, however, were found on the targets stored at −80 °C. Ferroin samples did not show any degradation trend at all and thus did not prove to be a good indicator for stability tests.

Ferroins are iron and sulfur containing proteins, thus it was assumed to observe different aging products compared to the glycoprotein ovalbumin. This was not observed. Neither additional peaks nor significantly increased noise was found (Fig. 3). Oxidation products might be found in the high m/z tailing of the ferroin molecular ion peak. The protein proved to be stable for the whole timeframe of the experiment and at all temperatures samples were stored at. Both undiluted and 1:10 diluted protein sample gave very similar results. Literature search indicate that

![Fig. 1. MALDI MS targets stored in the container in a sealed, nitrogen-purged pouch (left) and in an open container (right) at RT.](image-url)
ferredoxin is rather stable in the presence of even small amounts of salt [20], which might have happened in our preparations. Significant differences in sample degradation, however, were found for the γ-globulin. Fig. 4 shows γ-globulin peaks at m/z 148,194 (±175) as well as a doubly charged ion at m/z 74,385 (±86). In addition to these, cleavage products of the γ-globulin were observed (m/z 136,402 and 124,488) that increase in intensity with time and temperature whilst a diminishing γ-globulin signal can be observed. These signals are already barely visible from day 0, but are noticeable on the targets after 26 days, after which two cleavages can be observed. This trend is even more pronounced on targets stored at RT, where two other – very weak – signals were found, at m/z 112,884 and 100,995. The results correlate with those found in literature [21] and – among other effects – can be attributed for example to partial hydrolysis.

These trends verify observations made for the other proteins as well as the peptide calibration mix. The MALDI mass spectra from γ-globulin samples stored at −20 °C and −80 °C resemble each other closely (undiluted and 1:10 dilution). The proteins tested for archiving showed a range from completely stable for the allotted time (six months) to showing heavy degradation within the first month (γ-globulin).
3.3. Protein digests (PMF)

The protein digests of BSA and fetuin proved to be good indicators for storage stability testing as these are complex samples where degradation may hinder identification of the digested protein. The 1:10 dilutions of the two digests were used for further investigation. The MASCOT score of the PMFs acquired from digested proteins was used as an indicator of the quality of storage conditions.

![Molecular ion region of positive ion mass spectra](image1)

**Fig. 3.** Molecular ion region of positive ion mass spectra of 1:10 (v/v) diluted solution of ferredoxin deposited on target stored at \(-20^\circ C\) (sealed pouch) and at RT (open system) acquired after 0, 26, 61, 87, 117, 153 and 181 days. \(m/z\) range shown is 9000–12,500.

![Positive ion MALDI mass spectra](image2)

**Fig. 4.** Positive ion MALDI mass spectra of 1:10 (v/v) diluted solution of \(\gamma\)-globulin deposited on target stored at \(-80^\circ C\) (sealed pouch) and RT (open system) were acquired after 0, 26, 61, 87, 117, 153 and 181 days. \(m/z\) range shown is 60,000–165,000.
the archiving process, as smaller peaks vanished into the noise after months of storage, altering the score. The MASCOT score value takes into consideration the number of peaks that were entered and the number of peaks that were matched to peptides from the theoretical digest. Disappearing contaminations can, thus, for example increase the score. Vanishing meaningful peptides on the other hand will decrease the overall score. Results above a score of 60 are considered significant.

The BSA digest at the start of the experiment encompassed 24 peaks at an S/N ratio of 10 to 1, of which 15 were matches and covered 22% of the sequence. This produced a score of 123. The score of the samples stored at different conditions were plotted over the course of time, which can be seen in Fig. 5(a).

As with all MALDI mass spectrometry data, mass spectra do vary according to the distribution of the sample in the preparation among other things, which is why a quantitative approach to this technique is challenging with a reasonable expense. Based on that statement, signals on the threshold of inclusion in the peak list due to their weak intensity may appear in one mass spectrum of the same storage condition and concentration whilst missing in another. Of the three preparations per storage condition and time point, the most intense (in terms of absolute mV value) mass spectrum was selected for this task, hoping to include the highest number of analyte peaks, which is why the scores visualized in Fig. 5(a) and (b) do contain fluctuations. Thus, the general trend should be considered for any assertion instead any single point of data. The variance in the data is illustrated in Fig. 5(c) for the storage of fetuin digest at −80 °C. The diagram contains the average MASCOT score values and the standard deviation of three samples per month. It should be noted that average of month 1 and 2 only contain two scores each instead of three, because one preparation in each of these months failed to yield usable data. While under normal circumstances an average of multiple measurements should be used, in this instance only the mass spectra of the highest quality (e.g., those exhibiting the most information) should be used after archiving. This is the reason why in (a) and (b) only the most intense mass spectra were used.

The targets stored at −80 °C shows an interesting development over the months. Although the scores from +4 °C and −20 °C are almost on par with the −80 °C stored samples for the first two months, the coldest storage condition proves to be best archiving protein digests. The RT stored which was in a sealed pouch appeared to have reached a stable condition after 3 months and while ranging after that time in scores between 60 and 80, an identification of the protein could still be made. This, however, does not hold true for the samples which had contact with the atmosphere, which repeatedly dropped below a score of 60 after 4 months.

In the mass spectra of the fetuin digest 12 peptides were identified by MASCOT. Peaks from known contaminants [16] (matrix clusters, autotryptic peptides, alkali adducts, . . . ) were eliminated from the peak list prior to submitting to MASCOT search. The 12 identified peaks cover 43% of fetuin’s sequence, resulting in a score of 97. Over the course of time (Fig. 5(b)), a steady decrease of the score value is found for the open storage at RT, dropping to score of 65 after 4 months and 68 after 6 months. No usable data was found for these storage conditions after 5 months. Other storage conditions, including RT in a sealed pouch (score value 96), yielded comparable results after 6 months. −80 °C samples delivered a score value of 89, −20 °C a score value of 94 and samples stored at +4 °C even a score value of 99. It has to be concluded that up and downs during duration of the experiment were evident. Especially the +4 °C stored target showed a downward trend until the last month, when the score value improved from 78 (5th month) to 99. This observation can be explained by how the MASCOT search algorithm calculates the identification score value [17]. While the number of matched peaks increases the score value, the number of unmatched peaks affects the score negatively. This means that if unmatched peaks are declining over time, the score value will increase.

4. Conclusions

The results that this investigation generated were quite surprising. It was not expected to still be able to see the peaks associated with the tested analytes (proteins, peptides and protein digests) after six months incorporated into the MALDI MS matrix at
RT. While a decrease in signal intensity is clearly visible, the peaks were very well distinguishable from the background of the MS measurement. The best results were clearly seen in case of storage at the lowest temperatures. We could show clearly that storage of important samples related to protein investigations and to proteomic studies on the polymer-based nano-coated MALDI MS target on a long term basis is feasible at –20 °C and –80 °C, if the targets are sealed in a pouch to protect them from humidity and oxidation. In all cases, though, the samples stored at subzero temperatures fared quite better than their counterparts stored at above zero temperatures. Short time storage in the tested MALDI without optimization of the delay time for a particular long time storage of important proteomic and protein samples. The MALDI MS target makes the device a very convenient platform for low cost (compared to full metal sample targets) of this disposable purposes and on MALDI MS devices from different vendors. The applied in this investigation proved to be usable for archiving from the storage system without impacting other stored samples (particular in its microscopic format allowing the easy removal preparations, especially on large scale testing as was done here.

Finally, the polymer-based nanocoated MALDI MS target (particularly in its microscopic format allowing the easy removal from the storage system without impacting other stored samples) applied in this investigation proved to be usable for archiving purposes and on MALDI MS devices from different vendors. The low cost (compared to full metal sample targets) of this disposable MALDI MS target makes the device a very convenient platform for long time storage of important proteomic and protein samples. The reanalysis of unused MALDI MS preparations will allow the optimization of the delay time for a particular m/z range, the MS/ MS or post source decay fragment analysis of sequence tags (i.e., improved confidence in protein identification) or post translational modifications. Additionally the MALDI MS sample preparations can be performed immediately after the sample generation and later after sample storage the analyses can be performed in one step.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.euprot.2015.07.013.

References

Dear Editor,

Quality control of oligonucleotide synthesis by means of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry on a nanocoated disposable target

Since the first total chemical synthesis of the gene for a protein,[1] oligonucleotide synthesis has become essential[2] for generating primers for PCR (polymerase chain reaction), real-time PCR, DNA microarrays, Southern blots and fluorescent in situ hybridization. It is also employed for the production of artificial genes[3] and, finding its way into the global drug pipeline, of antisense oligonucleotides, miRNA inhibitors or siRNAs.[4] Correctly synthesized oligonucleotides are therefore a prerequisite for any of the above-mentioned techniques. Incorrect oligonucleotide sequences[5] will probably not ligate with the target sequence and, in the worst case, could bind to a wrong counterpart, giving incorrect feedback when used as a reagent. It is thus imperative to verify the product of the synthesis, i.e. final product quality control (QC), and to perform in-process QC. There are two important points that can be addressed relating to the final product: (1) product identity and (2) purity. The best way to do this would be by sequencing, breaking apart the oligonucleotide subunit-by-subunit to verify the correct envisioned sequence. This, of course, is very time-consuming and thus not a realistic approach for high-throughput analysis. The fastest method available here is mass spectrometry (particularly MALDI (matrix-assisted laser desorption/ionization) and ESI (electrospray ionization)), which allows the verification of the calculated molecular mass with the measured m/z value (i.e. product identity), with the additional option to perform tandem mass spectrometry (MS/MS)[6] and final product QC (presence of impurities related to side products) for oligonucleotides.[8] This study focuses on the measurement of oligonucleotides with MALDI-MS in a QC setting.[9,10] Sample supports (‘targets’) for MALDI were until recently mostly full metal targets (FMTs, e.g. stainless steel or nickel-coated aluminum with different surface morphologies). After measurements these have to be cleaned to remove the already measured analytes as well as the MALDI matrix and degradation products. This is – depending on the sample and matrix – done with organic solvents, acids or simply water, and this can over time change the surface morphology of the MALDI targets causing differences in the crystallization behavior of the sample preparation. It can – among other effects (cross-contamination from previous samples, change in surface hydrophobicity/hydrophilicity, etc.) – lead to differences in the quality of the mass spectra. A way to avoid this situation is to use disposable high-quality (in terms of, e.g., target planarity and background ions) targets, although until recently this was not feasible due to high costs. Various research groups have experimented with non-metal materials as a substitute for full metal targets.[11–18] There are now a number of commercially available polymer-based MALDI targets, partly with surface modification (disposable polymer target, DPT)[11,18] that are relatively inexpensive and therefore suitable for single use but not adapted or evaluated for the MALDI-MS analysis of oligonucleotides. Furthermore, with these targets, sample carryover from previously measured difficult-to-remove analytes (e.g. polymers) on target can be avoided, which is a great benefit for QC under GLP/GMP (good laboratory practice/good manufacturing practice) regulations. Usually, these regulations demand defined conditions for every measurement taken, and this can be easily provided with an unused disposable target. Unambiguous results free of measurement artifacts would thus be provided.

The DPT used in this study is a polymer nanocoated target (FlexiMass-DS TO-430; Shimadzu/Kratos Analytical, Manchester, UK). The microscope slide sized device consists of carbon black filled polypropylene with an inox steel (high carbon and chromium content) nanocoating on the top (i.e. the side used for sample and matrix application) and features 48 sample spot areas in 4 columns by 12 rows, and 3 calibration spots in the center. The targets were produced by an injection molding process followed by DC-pulsed magnetron sputtering from inox steel to form a nanolayer onto the upwards-facing side.

To validate the usability of the DPT, mass spectra for oligonucleotides were acquired by MALDI linear TOFMS on a DPT and a standard FMT (FlexiMass-target TO-483R00; Shimadzu/Kratos Analytical) used for comparison. In contrast to the polymer-based target this standard FMT consists completely of stainless steel and features a machine-polished surface. Both target types were introduced into the vacuum chamber, i.e. into the ion source of the MALDI mass spectrometer, using a microtiter plate-sized target holder (Precision adapter, Shimadzu/Kratos Analytical) used for the simultaneous introduction of up to four targets. The targets were fixed in the holder by means of a spring plate pressing the sample targets upwards to the upper frame of the holder. In this way all samples are brought into the right plane (focus of the laser beam), and optimal planarity across the whole target for optimal desorption/ionization conditions is ensured.

For the first experiments at the beginning of this study a 10mer oligonucleotide (5’-GAAATTCGAAT-3’ ([MH]+ m/z 3052.1 (av, average molecular mass)), a 15mer (5’-GAAATTCGAATTCCA-3’ ([MH]+ m/z 4601.1 (av)), and a 20mer (5’-GAAATTCGAATTCAATTCGA-3’, ([MH]+ m/z 6141.1 (av)) were applied (all obtained from Eurofins MWG Operon, Ebersberg, Germany). The modified oligonucleotides used for this study were all synthesized and purified by BVC Biotech Service (Vienna, Austria). A 25mer with the sequence 5’-ACG TAC GTA CGT ACG TAC GTA CGT A-3’ ([MH]+ m/z 7667.0 (av)) was used as the starting sequence, from which four deviating compounds were prepared: 5’-ACG TAC GTA CGT CCG TAC GTA CGT A-3’ ([MH]+ m/z 7643.0 (av)) exhibits an exchange of an A with a C, 5’-ACG TAC GTA CGT CCG TAC GTA CGT A-3’ ([MH]+ m/z 7335.8 (av) displays a deletion, 5’-ACG TAC GTA CGT ACG TAC GTA CGT A-3’ ([MH]+ m/z 8103.4 (av) features a biotin label on


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the 5' end, and 5'-ACG TAC GTA CGT ACG TAC GTA CGT A-3' ([MH]+ m/z 8373.9 (av)) features a cholesterol label attached on the 3' end. All the VBC Biotech Services samples were purified by HPLC (high-pressure liquid chromatography) and diluted to 100 pmol/μL in water before measurement. The MALDI matrix used was 3-hydroxypicolinic acid (HPA, Sigma-Aldrich, St. Louis, MO, USA; 25 mg/mL HPA in acetonitrile/0.1% aqueous trifluoroacetic acid (TFA) (7:3, v/v)). The following solvents were used: acetonitrile (Merck, Darmstadt, Germany; p.a.), ethanol (Merck, absolute p.a.), trifluoroacetic acid (Riedel-de-Haën, Seelze, Germany; ≥99% (alkalimetric)), and water (provided by the reverse osmosis facility of the Vienna University of Technology, Vienna, Austria, and further purified with a Simplicity UV apparatus (Millipore, Billerica, MA, USA), 18.2 MΩ cm at 25°C). Positive ion MALDI mass spectra were obtained by means of an Axima CFRplus time-of-flight (TOF) instrument (Shimadzu/Kratos Analytical) operated in the linear mode. This uses a nitrogen laser (337nm) at a pulse rate of 20Hz with an average ion source pressure of 1.6 × 10⁻⁷ mbar. All measurements were blanked (‘removal of low-mass ions’) up to m/z 2000. Mass spectra were acquired up to m/z 13000. The mass spectra were averaged of 120 single, unselected laser profiles (one shot per profile). Prior to the evaluation experiments various MALDI matrices were tested, particularly for the analysis of the labeled oligonucleotide, and the commonly used HPA[15,21,22] was found to be the most suitable. The sample preparation was as follows: 1 μL of the individual oligonucleotide solutions was diluted with 5 μL acetonitrile/water with 0.1% TFA (7:3, v/v). To this, 5 μL HPA matrix solution was added and 1 μL of this mixture was deposited on the two types of targets and allowed to dry at room temperature. The target with the dried sample preparation was then introduced into the vacuum chamber of the ion source. Insulin (Sigma-Aldrich) and ubiquitin (from bovine red blood cells; Fluka, Buchs, Switzerland) were used for calibration, deposited on the centered reference spots of each target.

Images of typical preparations are shown in Fig. 1. The images at 20× and 80× magnification were taken with a SMZ 800 (Nikon, Tokyo, Japan) microscope in conjunction with a camera control unit DS-L1 (Nikon). As can be seen, the sample preparation in the dried-droplet mode on the FMT generated a wide-diameter ring with a thin rim of crystals (within the 2.8-mm ring). This is completely different on the DPT where, due to a higher contact angle of the solution on the surface, the crystal/sample preparation area is much smaller. Thus, the analyte is more concentrated on a smaller area, which makes the analysis more straightforward. On the DPT one has to perform sweet spot searching. In addition, the morphology of the sample/matrix-crystal layer is quite different on the two targets. The HPA on the FMT has morphology similar to that of 2,5-dihydroxybenzoic acid, i.e. crystals growing from the rim towards the center, albeit very short ones. The DPT in contrast exhibits feathery and thin-looking sample morphology. At lower sample concentrations (not the focus of this investigation and therefore not shown) good signal-to-noise (S/N) ratios were achieved.

In the first step a 10mer oligonucleotide (sequence see above, [MH]+ m/z 3052.1), a 15mer (sequence see above, [MH]+ m/z 4601.1) and a 20mer (sequence see above, [MH]+ m/z 6141.1) were evaluated. Figure 2(A) shows as...
an example the positive ion MALDI mass spectra of the 15mer oligonucleotide prepared identically on the DPT and the FMT. In addition to the dominating protonated molecule, various alkali adducts were detected at low abundance. A comparison between the mass spectra obtained from DPT and FMT introduced via the same target holder (i.e. under identical ion source conditions) proved that the inox steel nanocoated, polymer-based target (DPT) exhibited a much better S/N ratio and absolute signal intensity which is helpful in detecting impurities (m/z theoretical 4601.1; m/z found 4601.2 for the DPT with 417 mV protonated molecule abundance and m/z 4601.3 for the FMT with 71 mV protonated molecule abundance) and performing quality checks in a shorter time.

The positive ion MALDI mass spectra of a number of modified oligonucleotides were recorded using the DPT and FMT. The mass spectra (Fig. 2(B)) of the biotinylated 25mer using both targets are shown as an example. Again both mass spectra exhibit as the dominating ion species the protonated molecule of the target structure. A comparison between the

Figure 2. (A) MALDI mass spectra of a 15mer oligonucleotide prepared on DPT (top) and FMT (bottom). (B) MALDI mass spectra showing the m/z range of interest from 6800 to 8600 of a modified 25mer oligonucleotide carrying a biotin label prepared on DPT (left) and standard FMT (right).
two different types of applied targets shows a better S/N ratio on the DPT than on the FMT and the accuracy is within the expected boundaries (m/z theoretical 8104.4 and m/z found 8104.3 (for both DPT and FMT)). The most probable reason for the much better S/N ratio achieved on the DPT as well as the better absolute signal abundance is the higher concentration of the analyte on a smaller area due to different liquid droplet behavior on the DPT target surface. This allows end product QC to be performed unambiguously in an automatic fashion as well as easy in-process QC in a multi-user environment. Furthermore, in the case of the DPT an abundant fragment ion at m/z 7988.8 was detected representing the neutral loss of deoxyribose (to a certain extent this was also found on the FMTs).

Overall, the DPT applied to oligonucleotides and particular modified forms with molecular mass below 10kDa delivers better S/N ratios and a higher absolute signal intensity. Due to the improved crystallization behavior (more homogenous and focused) of the MALDI matrix/oligonucleotide sample a concentration effect occurred for the HPA matrix. This in turn increases the measurement speed due to the reduction of the sweet spot search time or the possibility of automatic rastering, which is a necessity for automation under HTP conditions. The low costs and disposability of the DPTs are of great value in guaranteeing the same conditions for every measurement, eliminating the cleaning step and physical changes of a target surface during the repeated cleaning procedures. The issue of cross-contamination is completely eliminated which is important under GLP/GMP conditions.

Acknowledgement

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Stefan Bugovsky1, Werner Balika2, Manfred Koranda2, Siegfried Schnabl3 and Günter Allmaier1,*

1Institute of Chemical Technologies and Analytics, Vienna University of Technology, Vienna A-1060, Austria
2Sony DADC Austria Bioscience, Anif A-5081, Austria
3VBC-Biotech Service, Vienna A-1110, Austria

*Correspondence to: G. Allmaier, Institute of Chemical Technologies and Analytics, Vienna University of Technology, Getreidemarkt 9/164, Vienna A-1060, Austria.
E-mail: guenter.allmaier@tuwien.ac.at

REFERENCES

9.5. Application Note: Analysis of a Tryptic Protein Digest by MALDI-RTOF Mass Spectrometry using Microscope Slide-format FlexiMass™-DS target

Introduction

Digestion of proteins (e.g. *in-gel*, *in-solution* or *on-target*) is a method commonly used in combination with MALDI-MS to identify or get indication of the presence of proteins. Furthermore peptide mass fingerprinting (PMF) is applied in the production and QC of recombinant proteins for various applications. In most cases, trypsin is used as protease to cleave proteins at specific positions (Lys and Arg) into small peptides[2, 3]. Mass spectrometric analysis of such often complex mixtures generate a data set that with the aid of a protein sequence database, can be matched to *in silico* digested proteins to allow the identification. Here we show the *in-solution* digestion of ovalbumin, which is the main protein of egg white and a known allergen, and the MALDI mass spectrometric analysis of its derived cleavage peptides. To compare the performance of the new FlexiMass™-DS targets, MALDI mass spectra of the ovalbumin digest prepared onto FlexiMass™-DS and microtiter plate format metallic targets (TO-474) were acquired.

FlexiMass™-DS targets have been developed to be used as disposable targets, eliminating the problem of cross contamination from previous samples, to ease the handling of infectious or pathogens (e.g. microorganisms) materials and to avoid any cleaning procedure. This last aspect allows a significant reduction in solvent consumption, lowering cost as well as ecological footprint.

Methods

*Tryptic Digest* [4]:

About 1 nmol of Ovalbumin (chicken, Grade VI, Sigma) was dissolved in 75 µl denaturing buffer (6M guanidinium hydrochloride, 0.5M Tris-HCl, 2mM EDTA, pH 8.0) and incubated at 37°C for 30 min. For reduction, a 50-fold molar excess of dithiothreitol (DTT) was added to the solution and again incubated for 1 hour at 37°C. The sample was then allowed to cool down to room temperature (RT). For alkylation, iodoacetamide in excess of the -SH groups in the mixture was added and incubated in the dark at RT for 1 hour. To remove excess of DTT, iodoacetamide and guanidinium hydrochloride, the sample was cleaned up using a MicroSpin G-25 column (GE Healthcare). The cleaned sample solution was then diluted with an equal volume 100 mM ammonium hydrogen carbonate solution (pH 8.5) and 500 ng trypsin (recombinant, proteomics grade, Roche Diagnostics) was added. The digestion was performed at 37°C overnight.

The sample was then dried via a vacuum concentrator centrifuge (Univapo 150H, UniEquip), 30 µl formic acid were added and again centrifuged till dryness. This was then redissolved in 250 µl acetonitrile/0.1% trifluoroacetic acid (TFA) (1:9, v/v) and used for MALDI MS measurements.

Molarities of tryptic peptides stated below do not take in account incomplete digestion or loss during cleanup and should be considered as such.

*MALDI MS sample preparation:*

An aliquot of the protein digest solution was mixed in an Eppendorf tube with an equal volume of α-
cyano-4-hydroxycinnamic acid (CHCA) matrix solution (6.2 mg/ml in methanol:acetonitrile:H₂O, (9:14:2, v/v/v)) and 1 µl of this mixture was spotted on the FlexiMass™-DS and on the standard microtiter plate format TO-474 target.

**MALDI mass spectrometry:**
Measurements were performed using an AXIMA CFRplus instrument (Shimadzu Biotech Kratos Analytical) in positive reflector time-of-flight-mode with blanking up to m/z 500. Mass spectra were the result of 121 averaged unselected laser shots. Pulsed extraction was set to m/z 1400 an intermediate value covering a wide range of peptide masses. Mass spectra shown are unsmoothed.

**Mascot® database search:**
The m/z values were obtained manually by selection of monoisotopic peaks with a minimum signal-to-noise ratio of at least 3:1 and were searched against the SwissProt database (Release 57.10 from 4 Nov, 2009) using Mascot® Peptide Mass Fingerprint (www.matrixscience.com). All m/z values known to result from the MALDI MS matrix or alkali adducts were omitted. The search parameters were defined as follows: Enzyme: trypsin, taxonomy: vertebrate, peptide tolerance: ±0.5 Da, fixed modification: carbamidomethylation, variable modification: formylation, missed cleaveages: 1.

**Results**
The MALDI mass spectra of the ovalbumin digest shown in Figure 1 were acquired on both, the standard microtiter plate-format TO-474 (bottom) and FlexiMass™-DS (top), target under identical sample preparation conditions. Peaks at m/z values corresponding to a known (e.g. calculated) peptide of the Ovalbumin digest were marked with an asterisk. Here, an amount of about 20 pmol per peptide was deposited on the targets. The 12 peaks, corresponding to tryptic peptides from ovalbumin, identified on the standard target were also detected on the polymer target FlexiMass™-DS. The absolute intensity of the ions of interest was practically identical. The mass spectrometric resolution (FWHM) achieved on the FlexiMass™-DS targets compared to the conventional target (see insets in Figure 1) showed a very similar result (10690 vs. 10120 for the ovalbumin peptide T144 – T159 at m/z 1859.07; average of ten measurements on ten separate preparations). The higher noise in the mass spectrum derived from the preparation on the FlexiMass™-DS can be attributed to the fact that laser irradiance was set to the same value for both targets, i.e. no optimization for the different targets was done.
Figure 1. Positive ion MALDI RTOF mass spectra of the digested protein sample on two different types of targets (standard target TO-474, bottom and FlexiMass™-DS target, top) (unsmoothed; inset: molecular ion region of peptide T144–159 at m/z 1859.07 (calculated monoisotopic value)

The m/z values picked from the MALDI mass spectra from both target types were submitted to Mascot PMF search resulting in a MOWSE score of 128 (FlexiMass™-DS) and 137 (TO-474) for Ovalbumin from Gallus gallus (Accession number P01012) with a sequence coverage of 53%, which is shown in Table 1, for both targets. Matched parts of the sequence are marked in bold red.

Table 1. 53% Sequence coverage (marked red) of chicken Ovalbumin derived from the in-solution tryptic digest

<table>
<thead>
<tr>
<th></th>
<th>FlexiMass™-DS</th>
<th>TO-474</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MGSIGAASME FCDFVKELK VHHANENIFY CPIAIMSALA MVYLGAKDST</td>
<td></td>
</tr>
<tr>
<td>51</td>
<td>RTQINKVRF DKLPGFGDSI EAQCGTSVNV HSSLRDILNQ ITKPDNYSF</td>
<td></td>
</tr>
<tr>
<td>101</td>
<td>SLASRLYAEV RYPILPEYLV CVKELYRGLG EPINFQTAAD QARELINSWV</td>
<td></td>
</tr>
<tr>
<td>151</td>
<td>ESQNGIIRN VLQPSDVDSQ TAMVLNAAV FKGLWEKAKF DEDTQAMFF</td>
<td></td>
</tr>
<tr>
<td>201</td>
<td>VTEQESKPFQ MMQIGLFRV ASMASEKMKI LELPFASGTM SMLVLLPDEV</td>
<td></td>
</tr>
<tr>
<td>251</td>
<td>SGLEQLESII NFEKLTEWTS SNNMEERKIK VYLPKMKMEE KYNLTSVLM</td>
<td></td>
</tr>
<tr>
<td>301</td>
<td>MGITDFSSS ANLGGISSAE SLKISQAVHA AHAINEAGR EVVGSAEAGV</td>
<td></td>
</tr>
<tr>
<td>351</td>
<td>DAAASVEEFR ADHPFLFCIK HIAATNLFV GRCVSP</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. 53% Sequence coverage (marked red) of chicken Ovalbumin derived from the in-solution tryptic digest

To allow a comparison of sensitivity of the conventional with the disposable FlexiMass™-DS target, the digest was diluted and amounts ranging from 20 pmol to 20 fmol per peptide were deposited on the targets. The side-by-side comparison of the mass spectra can be seen in Figure 2. Peaks matching a tryptic ovalbumin peptide are marked with an asterisk.
As can be seen in Table 2, although MOWSE scores were nearly identical for higher amounts of digest analyzed, lower concentrations of the Ovalbumin digest resulted in a better MOWSE score applying the FlexiMass™-DS target. Only peaks with a signal-to-noise ratio higher than 3:1 were considered for the calculations.

<table>
<thead>
<tr>
<th></th>
<th>TO-474</th>
<th>FlexiMass™-DS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score</td>
<td>Sequence Coverage</td>
<td>Peaks selected</td>
</tr>
<tr>
<td>20 pmol</td>
<td>137</td>
<td>53%</td>
</tr>
<tr>
<td>2 pmol</td>
<td>121</td>
<td>46%</td>
</tr>
<tr>
<td>200 fmol</td>
<td>94</td>
<td>34%</td>
</tr>
<tr>
<td>20 fmol</td>
<td>58</td>
<td>18%</td>
</tr>
</tbody>
</table>

Table 2. Results from MASCOT search at different Ovalbumin digest amounts deposited

**Conclusion**

- MALDI mass spectrometric analyses of a proteolytic digest of Ovalbumin for protein identification perform equally well on FlexiMass™-DS compared to standard microtiter-format metallic targets (e.g. TO-474). Identification of proteins by PMF could be achieved for amounts down to 20 fmol on the target.
- FlexiMass™-DS targets deliver much higher MOWSE score at 20 fmol level.
- The MALDI mass spectrometric performance (resolutions and S/N ratios) achievable using FlexiMass™-DS and metal based targets is equal.

**References**

1. Rehulka, P., J. Salplachta, and J. Chmelik, *Improvement of quality of peptide mass spectra in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and post-source*


Introduction

The metabolome is by definition the complete set of small molecules that actively take part in the process of metabolism in living organisms or are a result thereof. Thus, metabolomics helps to understand the inner workings of an organism and allows recognizing deviations as a result of diseases or genetic variations.

Figure 1. Stevia rebaudiana and Bacillus brevi

Steviol glycosides are naturally occurring sweeteners that can be found in different parts of the stevia plant (Stevia rebaudiana, Figure 1, left). They are especially attractive for the food industry, as they do not induce a glycemic response, making these substances suitable for diabetics. Tests indicate these compounds are up to 300x sweeter than conventional sugar [1]. Steviol glycoside extracts contain four main substances, stevioside, rebaudioside A, rebaudioside C and dulcoside A, of which the former two are the most important ones for use as sweeteners (stevioside glycoside structure see Figure 2, left). Late 2008 the Food and Drug Administration (FDA) accepted steviol glycosides as be “generally recognized as safe” [2].

Figure 2. Structure of stevioside (left) and tyrocidine A (right)
Tyrocidine [3] encompasses a group of cyclic decapeptides (structure example see Figure 2, right) produced by the bacterium Bacillus brevis (Figure 1, right) and was the first commercially available antibiotic [4]. Nowadays it is still used in lozenges against sore throat.

In metabolomics studies related to plants and microorganisms forming important therapeutics the fast and reliable detection and identification is of great importance. Here, MALDI-RTOF mass spectrometry using disposable polymer FlexiMass™-DS targets and 384 well plate TO-474 for comparison were used for the analysis of these compound classes. We analyzed stevioside glycoside and rebaudioside A as well as the tyrocidine complex. Furthermore the suitability of FlexiMass™-DS targets for matrix-free analysis, i.e. LDI mass spectrometry, of complex tyrocidine mixtures as present in the drug formulation was evaluated.

The polymer-based FlexiMass™-DS targets are specifically designed to be ready for use without pre-cleaning and to avoid cross-contamination from previous samples and to reduce the need of solvents, cutting down handling cost and waste produced.

**Methods**

**Samples:**

Rebaudiosid A (Wako Chemicals) and steviosid glycoside(Sigma-Aldrich): 0.1 mg/ml in methanol was used as stock solution and diluted by factor 1:1000 (v/v) with methanol for the final analysis.

Tyrocidine (kindly provided by the Viennese Chamber of Pharmacy): 1 µg/ml tyrocidine was dissolved in acetonitrile:water (1:1, v/v)

**Sample preparation for rebaudioside A and stevioside (dried droplet technique):**

The analyte solution was mixed 1:1 (v/v) with matrix solution (15 mg/ml 2’,4’,6’-Trihydroxyacetophenone in methanol which was saturated by NaCl). 1 µl of this mixture was deposited on the targets. This translates to approximately 6 pmol of glycoside on the target.

**Sample preparation for MALDI MS of tyrocidine sample [5] (dried droplet technique):**

0.7 µl of the analyte solution (1 µg/ml tyrocidine sample in acetonitrile:water (=1:1, v/v)) was mixed with 0.7 µl α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution (6.2 mg/ml in methanol:acetonitrile:H2O (9:14:2, v/v/v), Agilent Technologies) directly on the target and allowed to dry under room temperature (RT).

**Sample preparation for LDI MS of tyrocidine sample:**

1 µl of the analyte solution was deposited directly onto the target and allowed to dry under RT.

**Mass spectrometry:**

Measurements were performed using an AXIMA CFRplus instrument (Shimadzu Biotech Kratos Analytical) in positive reflector time-of-flight (RTOF) mode with pulsed extraction set to m/z 1000 for the steviol glycosides and m/z 1500 for tyrocidine sample. Mass spectra were an averaged result of 121 unselected single laser shots.
Results

The mass spectra of both glycosides (Figure 3) are very comparable and underline the equal applicability of the target for MALDI MS measurements of secondary plant metabolites. Resolution (FWHM) in the mass spectra derived from both type of targets was nearly identical, being 6676 for the TO-474 target and 6317 for the polymer-based FlexiMass™-DS target. These values represent the average of 5 measurements on 5 separate preparations of the main stevioside glycoside peak at m/z 826.95.

The tyrocidine sample, which is a mixture of at least 10 components [5], was used here to illustrate the ability to perform (matrix-free) LDI MS measurements on FlexiMass™-DS.

LDI MS worked very well (see Figure 4) without matrix and also the need to spike with alkali metals, resulting in a mass spectrum containing all of the peaks found in the MALDI MS measurement on the polymer target. The LDI measurement on the 384 well target, which acted as a reference, missed the Tyrocidine C1 MH⁺ peak at m/z 1363.76. Otherwise the number of peaks, height of the peaks in relation to each other and signal-to-noise ratio showed negligible differences, indicating the new FlexiMass™-DS to perform at least as well as conventional metal targets.
Despite the lower intensity of the peaks in the mass spectrum from the standard target TO-474 the peak pattern is distinctively more complex. This is due to the fact that these mass spectra not only contain signals related to the singly protonated species, but also sodium and potassium adducts of the major compounds. The absence of alkali adducts in mass spectra acquired from samples prepared on the FlexiMass™-DS targets clearly show the higher purity of the target surface and the advantage of a single-use target.

**Conclusion**

- The FlexiMass™-DS polymer targets used here yielded comparable results to established metal targets.
- The disposable FlexiMass™-DS targets avoid any carryover from previous sample preparations or interference caused by suboptimal cleaning procedures.
- The MALDI spectra achieved yielded very comparable results in terms of reproducibility and signal-to-noise-ratio, which appeared to be better on the polymer targets for equally intensive laser-settings.
- The results achievable with LDI MS on FlexiMass™-DS was superior to data derived from conventional TO-474, because the alkali contamination in the latter case resulted in a more complex pattern.

**References**

2. Notice to the U.S. Food and Drug Administration (FDA) that the use of Rebiana (Rebaudioside A) derived from Stevia rebaudiana, as a Food Ingredient is Generally Recognized as Safe (GRAS).  2009  http://www.accessdata.fda.gov/scripts/fcn/gras_notices/grn000282.pdf.


Photo of Stevia rebaudiana is taken from Wikimedia Commons and licensed under the Creative Commons License Attribution Share Alike 2.0. Image provided by Ethel Aardvark.
9.7. Application Note: Analysis of Small and Large Proteins with MALDI-TOF Mass Spectrometry using Microscope Slide-format FlexiMass™-DS targets

Introduction

Detection of intact (glyco)proteins is an important aspect in bioanalytical application as it allows to gain a fast overview of the complexity (heterogeneity) of a sample at hand. Particularly in routine applications MALDI-TOF mass spectrometry in the linear mode is a fast and accurate method to determine the molecular mass of a given (glyco)protein. This is also used in profiling of complex samples as recombinant therapeutic proteins or where particular markers are sought to distinguish between these, e.g. healthy and pathological tissue [1].

Here, the performance of the new disposable FlexiMass™-DS in comparison to the conventional 384-well microtiter plate format metallic TO-474 target for the analysis of different protein samples is shown. The chosen samples were the Ovalbumin, a glycoprotein with MW (molecular weight) of 44 kDa, Bovine Serum Albumin (BSA, MW 66 kDa) and a sample of γ-Globulin from bovine serum (again glycoproteins). γ-Globulins [2] are a fraction of serum proteins consisting mainly of immunoglobulins A, D, G, E and M. Of these, Immunoglobulin G (IgG, MW 150 kDa) is by far the most abundant.

![Figure 1. Molecular structure of Immunoglobulin G](image)

FlexiMass™-DS targets have been developed to be used as disposable targets, eliminating the problem of cross contamination from previous samples, to ease the handling of infectious or pathogens materials and to avoid any cleaning procedure. This last aspect allows a significant reduction in solvent consumption, lowering cost as well as ecological footprint.

Methods

Samples

Ovalbumin (grade VI, Sigma): 2 nmol/µl Ovalbumin in 0.1% aqueous trifluoroacetic acid (TFA)

Bovine Serum Albumin (Sigma): A 50 pmol/µl stock solution in 20 mM amminoacetate buffer (pH 7.4) was used, which was diluted to 1 pmol/µl with water

γ-Globulins (Sigma): 2 mg/ml γ-Globulins in 0.01% aqueous TFA
Sample preparation

All samples were prepared by applying the dried droplet technique: Sample and matrix solution (sinapinic acid 10 mg/ml in acetonitrile: 0.1% TFA = 1:1, v/v) was pre-mixed 1:1 (v/v) in an Eppendorf tube and 1 µl of this mixture was spotted on the FlexiMass™-DS and standard microtiter plate format TO-474 target.

Mass spectrometry

Measurements were performed using an AXIMA CFRplus instrument (Shimadzu Biotech, Kratos Analytical) in positive linear TOF (time-of-flight) mode with pulsed extraction set to a value of the approximate molecular weight of the protein. Mass spectra were an averaged result of 100 unselected single laser shots. All mass spectra shown are smoothed by applying the Savitsky-Golay algorithm (20 channels width).

Results

Sample preparation often affects the mass spectra acquired with MALDI mass spectrometry, particularly an adequate crystallization is required. The sinapinic acid employed as matrix in these analyses produced a satisfactory preparation on FlexiMass™-DS (see Figure 2) very similar to results obtained on metal surfaces.

Figure 2. Image at two different magnifications of a preparation of Ovalbumin with sinapinic acid on FlexiMass™-DS target

In Figure 3, the mass spectra of the Ovalbumin sample. The high amount of protein used allows the detection of protein multimers (up to the pentamer at m/z 221000, artefacts of the MALDI MS) besides the singly and doubly charged monomeric species. The mass spectra acquired from both targets are very similar in terms of intensity, S/N ratio, peak width and peak intensity ratios. The observed difference between observed MW and MW calculated from Ovalbumin sequence (42.8 kDa vs. 44.3 kDa) is in good accordance to the MW of the glycosylation (1562 Da, (GlcNAc)_2(Hex)_4) as found in literature [3].
Bovine Serum Albumin (Figure 4) yielded identical results both on FlexiMass™-DS and the normal metal target in terms of intensity. Signal-to-noise ratio on the other hand was slightly better on the new target, exceeding 200 to 1 for 500 fmol on target.
In Figure 5, mass spectra of the γ-globulin sample are shown. The M+ marked peak correlates as expected with the known approximate molecular mass of immunoglobulin G, the main component of γ-globulins. Signal intensity and resolution on FlexiMass™-DS and conventional TO-474 were identical.

![Figure 5. MALDI TOF massspectra of γ-globulin sample (top: FlexiMass™-DS, bottom: TO-474; approx. 340 pmol on target)](image)

The three analyzed (glyco)proteins yielded nearly identical results on both types of targets tested in terms of resolution and sensitivity, allowing the conclusion that the new FlexiMass™-DS is comparable in terms of plain MS performance with conventional metal targets for analyzing small to large (glyco)proteins.

Conclusion

- MALDI mass spectrometric analyses of small and large (glyco)proteins perform equally well on FlexiMass™-DS compared to standard microtiter-format metallic targets (e.g. TO-474).
- Preparation with sinapinic acid resulted in crystallization comparable to metal-based targets
- FlexiMass™-DS and TO-474 both yielded satisfying results for (glyco)protein down to 500 fmol

References


Figure 1 is taken from Anthony Nicholls, Kim Sharp and Barry Honig, PROTEINS, Structure, Function and Genetics, Vol. 11, No. 4, 1991, pg. 281ff and licensed under the Creative Commons License Attribution Share Alike 2.0

Introduction

Fungi belonging to the genus *Fusarium* are well-known and frequently encountered contaminants of cereal plant products. The infection [1] of these plants with fungi results in a production of mycotoxins that can cause serious health problems in livestock and humans. This raises the need for a rapid and easy identification method that can distinguish between the various species.

Intact cell mass spectrometry (ICMS) is an important tool for the fast discrimination and identification of microorganisms [2,3]. Here, it is not necessary to apply any prior extraction steps, as the microorganism is used as a whole, using the different proteins bound to the surface for differentiation. MALDI-MS as a so-called “soft ionization” technique is particularly suited for this task as it allows the desorption/ionization of proteins and peptides from the surface of microorganisms as molecular ions without fragmentation. Furthermore, the inherent insensitivity of this method to salts enables a fast analysis without the need of an arduous cleaning beforehand.

![Image of macroconidia of *Fusarium graminearum*](image)

**Figure 1. Image of macroconidia of *Fusarium graminearum***

MALDI MS (ICMS) is shown within this application note with conidia spores of two strains of *Fusarium* on disposable polymer targets for MALDI MS using a 2-layer volume preparation technique [4] on FlexiMass™-DS targets and an established 384 well metal target in microtiterplate format (TO-474). FlexiMass™-DS targets were specifically designed to be disposable, eliminating the need to clean the targets after use. This is reflected in a lower use of solvents needed, which lowers cost and ecological footprint. Furthermore, no decontamination steps are needed if infectious material or pathological microorganisms were analyzed due to the fact that is a one-way device. Disposable targets additionally eliminate the danger of carryover from previous analyzed microbiological samples.
Methods

Samples:
*Fusarium graminearum* CPK 2983 (isolated from corn) and *Fusarium graminearum* CPK 2986 (isolated from soil) were taken from the strain collection of the Institute of Chemical Engineering (Vienna University of Technology, Vienna, Austria).

Pre-measurement purification [4]:

*Fusaria* conidia spores were stored in glycerol, making a cleaning step necessary [5]. For this, 86 µl of *Fusarium graminearum* CPK 2983 and 22 µl *Fusarium graminearum* CPK 2986 were separately washed three times with 100 µl acetonitrile: 0.5% aqueous formic acid (7: 3, v/v) using Nanosep™ MWCO 10kDa centrifugal devices (Pall) at 16 100 g for 10 min and resuspended in 5 µl water.

Sample preparation:

For preparation, a specifically designed 2-layer volume technique was used [4]. 10 mg ferulic acid (Fluka) dissolved in 1 ml acetonitrile: 0.1% aqueous trifluoroacetic acid (TFA) (7:3, v/v) was used as matrix solution and premixed in equal parts with the purified conidia spore suspension (3 million spores / µl ) and vortexed well. 1 µl of this mixture was spotted on the target and allowed to dry. Additionally, further 0.5 µl of the matrix solution were depositeded on top of the dried sample/matrix preparation.

Mass spectrometry:

Measurements were performed using an AXIMA CFRplus instrument (Shimadzu Biotech, Kratos Analytical) in positive ion linear mode with pulsed extraction set to m/z 5000. Mass spectra were the result of 2500 unselected single laser shots and blanking up to m/z 1000. For acquisition automated measurement was employed with rastering. External 3-point calibration was made with peptide ACTH 7-38 and the single and double protonated cytochrome C. All mass spectra shown are smoothed by applying the Savitsky-Golay algorithm (20 channels width) and baseline subtracted (120 channels width).

Peaks of different mass spectra varying in m/z within a margin of 1 + ([m/z]/3000) were considered identical.

Results

Achieving good results in ICMS very much depends on the quality of the MALDI MS sample preparation, as spores can be evenly distributed and yield good results, but also be clustered in a single area. Figure 2 shows two typical preparations achieved on the new FlexiMass™-DS target and the conventional metallic TO-474 target. The obvious difference in the microscope images is the much denser crystal layer on the disposable target. Using an automatic rastering during measuring requires the preparation to be as uniform as possible, which is clearly the case on FlexiMass™-DS.
Figure 2. Images at two magnifications of preparation of conidia spores from *Fusarium graminearum* with ferulic acid on FlexiMass™-DS (upper row) and TO-474 (lower row).

Figure 3 compares the mass spectra of *Fusarium graminearum* strains CPK 2983 and 2986 on conventional TO-474 metal target to results achieved using the new disposable FlexiMass™-DS target. The m/z range used for comparison was chosen between 2450 and 6550, as most interesting m/z range. No peaks were found above m/z 6550. The relation of the peaks on both targets to each other in terms of intensity varies, which is to be expected in such a complex microbiological sample. The signal-to-noise ratios in the mass spectrum derived from *F. graminearum* CPK 2986 sample observed on FlexiMass™-DS was significantly higher.
Figure 3. MALDI mass spectra of *Fusarium graminearum* CPK 2983 (left column) and CPK 2986 (right column) obtained from preparations on the two different types of targets (standard target TO-474, bottom and FlexiMass™-DS target, top)

To allow a differentiation between the two strains m/z values of selected peaks were counted, noted and compared against each other. In the selected m/z range from 2450 to 6550 25 peaks above a signal-to-noise ratio of 3:1 were counted (excluding peaks originating from alkali adducts; see Table 1) of which 16 occurred in both strains. The remaining nine peaks only occurred in either one of the two samples and allowed a clear differentiation of both fungi strains. Two of these were unique to *F. graminearum* CPK 2983, whereas seven appeared exclusive in to strain CPK 2986.

While strain CPK 2983 compared well on both targets, missing only one peak at m/z 2651 on FlexiMass™-DS, CPK 2986 revealed a few more differences: peaks at m/z 2675, 3870 and 3955 were found only on TO-474, whereas one peak at m/z 4967 was found only on FlexiMass™-DS. These peaks, however, were detected with a very low signal-to-noise ratio (just above the set level), indicating that the missing peaks might be lost in the noise.
Table 1. Comparison of the presence of selected peaks of *Fusarium graminearum* CPK 2983 and 2986 on TO-474 and FlexiMass™-DS. Coloured field indicates presence of given peak.

**Conclusion**

- Preparations of conidia spores using ferulic acid on the new FlexiMass™-DS were superior to those on conventional metal-based TO-474 target as they exhibited a denser and more even crystallization allowing a better shot-to-shot reproducibility employing an automated rastering during data acquisition.

- Due to the use of the one-way FlexiMass™-DS targets the problem of microbiological cross-contamination in ICMS can be avoided which is of particular importance if human pathogen microorganisms are analyzed.

- Differentiation of conidia spores of *Fusarium graminearum* CPK 2983 and 2986 was possible on the new FlexiMass™-DS target as described by applying conventional TO-474.
References


Figure 1 is taken from Wikimedia Commons and licensed under the Creative Commons License Attribution Share Alike 2.0
10. Conclusion

The disposable polymer target that was the focus of this work proved to be suitable for all applications that it were tested for, in some aspects even outperforming the metal target. From trial and error during the design phase of the commercial product it was seen that there are certain properties that a MALDI target has to have in order to perform adequately.

The variation in the dimensions had to be very small in order to fit the target holder in such a way that a tight fit without bending was ensured. Warping or skewing of the geometry of the target introduces noticeable m/z shifts as seen in Chapter 9.1 (Polymer-based metal nano-coated disposable target for matrix-assisted and matrix-free laser desorption/ionization mass spectrometry). The right amount of carbon black in the polymer - on the other hand - was needed for conductivity and for the proper surface properties (see Fig. 11, Chapter 5.6), without which the sample spots did not crystallize properly and were unusable for MALDI mass spectrometry.

These are just a few of the challenges that were met by the development team at Sony DADC. From feedback on the performance of the mass spectrometric performance of the target, adaptations were made that finally yielded a marketable product. While most benefits of using such a disposable device were already laid out in the publications in chapter 9, the work done in the publication Long time storage (archiving) of peptide, protein and tryptic digest samples on disposable nano-coated polymer targets for MALDI in chapter 9.3 simply would not have been possible with standard metal targets due the number of targets required. Affordable disposable targets, in this case, allowed testing various archiving conditions (temperature, vacuum or atmosphere) of diverse sample types and studying the degradation over time.

The devices proved to be suitable for the whole range of analytes that MALDI mass spectrometry is used for as can be seen in Chapter 9. Substances varied from relatively small molecules such as protein digests to very large ones such as immunoglobulins and samples that require special attention such as the oligonucleotides due to their high salt content or microorganisms such as bacteria and fungi. It has to be said that the sample preparation required by MALDI MS is highly adaptable, allowing to analyze even difficult samples that might not yield perfect crystallization by tweaking the solvent the matrix is dissolved in.

Commercially the targets are being marketed by Shimadzu as a standalone product and as part of their microbial identification solution Axima-iDPlus as well as by BioMerieux in the Vitek MS system that are aimed at pharmaceutical, healthcare and food industry. More than 1 million
targets have so far been produced for the two above mentioned companies. Applications include identifying microorganisms that have infected patients, clean room monitoring where it is necessary to trace where contaminations stem from, cell culture monitoring and more. Impressively, the system only requires basic sample preparation skills without deeper knowledge of mass spectrometry or mass spectra interpretation.

As a further development it is possible to see micro/nano-structuring of the targets to add additional functionality, e.g. hydrophilic anchors as the AnchorChip by Bruker or a patterned sample spot area to facilitate better crystallization. Polymeric nanorods might enhance LDI performance. Another possibility might be selective binding of analytes via anchored biomolecules, gold particles,... to achieve enrichment of the analyte on the surface or to remove salts by washing. Some of these ideas would require a metal target to be prepared freshly every time with mixed results. Disposable targets on the other hand can be pretreated by the manufacturer with the intended functionality in mind such as there exist commercial ELISA kits for example.

The disposable polymer targets as they were tested exhibited surprisingly few downsides. The only disadvantage that may be mentioned is that very high laser irradiance tended to impart so much energy on the target that the metal nano layer was destroyed exposing the polymer beneath – a feat only achieved while experimenting with LDI using irradiance levels not usually required by experimentation.
11. Curriculum vitae

MAG. RER. NAT. STEFAN BUGOVSKY

Matznergasse 40/16, 1140 Wien | Tel. 0676/472 65 65 | stefan.bugovsky@gmail.com

PERSONAL INFORMATION
Date of Birth: April 17th 1981
Place of Birth: Vienna
Nationality: Austria

EDUCATION

Vienna University of Technology
PhD Study Technical Chemistry 2008-2012
PhD thesis „Polymer based sample holders for MALDI (matrix assisted laser desorption/ionization) mass spectrometry of biomolecules and intact cells“

University of Vienna
Diplom Study Chemistry with focus on Biochemistry and Bioanalytics 2000-2007
Diplom thesis „Adsorption capacities for biodegradable nanoparticles“

Higher technical institute for chemistry industry (Rosensteingasse, 1170 Vienna)
Thesis: „Fermentation of Trichoderma reesei on various media“

DISTINCTIONS

3. Place „Dr. Wolfgang Houska Preis 2009“
„Development of a polymer based, microstructured MALDI mass spectrometric sample holder for the life sciences“
together with Prof. Günter Allmaier, Dr. Wolfgang Winkler, Dr. Werner Balika and Dr. Peter Hausberger

WORK EXPERIENCE

Baxter BioScience/Baxalta/Shire, Vienna, Department Material Qualification
Manufacturing Engineer 2012 – to date
Qualification of materials (process aids, raw materials, ..) used in pharmaceutical manufacturing of drug products relating to compatibility for manufacturing process, safety to patient (Extractables and Leachables and adherence to regulatory requirements (esp. for excipients & final container) for production plants in Europe and Singapore.

Vienna University of Technology, Institute for Chemical Technologies and Analytics
Project Assistant 2008-2011
Dissertation und cooperation with Sony DADC Austria AG
Development and quality control of a polymer based MALDI target, autonomous planning of experiments and feedback to Sony DADC, trips to business partners in France and Germany for troubleshooting as well as measurements on site, establishing of numerous applications for the product (e.g. peptide mass fingerprinting, determination of microorganisms with MALDI MS, sample archiving including stability study), presentation of results on two international conferences in the US (ASMS 2010, ASMS 2011)

Commercial Photographer
Self employed 2008 – to date
Mostly event photography (Viennese ball season) and portraiture e.g. for Ballguide

Youth Centers of Vienna, Department Organisation
Civil service 2007 - 2008
Transport of materials, event setup and support

Baxter BioScience, Vienna, Department QCAS
Internship July 2005
Environmental Control, Determination of cleanroom quality (particle size/count) and microbial sample taking

Baxter BioScience, Vienna, Department „Fractionation”, Production
Internship July 2004
Manufacturing; Industrial fractionation and lyophilization of blood clotting factors

Baxter BioScience, Vienna, Department “Receiving and Inspecting”
Internship 07/2003, 08/2002
Quality control of incoming goods; wet chemical determination of reagents, optical control of small parts

sBausparkasse, Vienna, Department IT
Internship July 2002
End user support, network services, system maintenance and repair July – August 2001, July 2000

OMV, Schwechat, Department Quality Control
Numerous analytical determinations of crude oil and process products from crude oil fractionation

ADDITIONAL EDUCATION
„Practice oriented project management“ – 7 weeks course (150 units) finishing with SystemCert certification Sept. - Nov. 2011
PUBLICATIONS

*Polymer-based metal nano-coated disposable target for matrix-assisted and matrix-free laser desorption/ionization mass spectrometry*

*Intact Cell Mass Spectrometry (ICMS) on polymer-based and metal-coated disposable targets*
Molecular and Cellular Probes 28 (2014) 99-105 2014

*Long time storage (archiving) of biological relevant samples on disposable polymer targets for matrix-assisted laser desorption/ionization mass spectrometry*

*Quality control of oligonucleotide synthesis by means of matrix-assisted laser desorption/ionization linear time-of-flight mass spectrometry on a nanocoated disposable target*

*Long Term Storage of Protein/Peptide Samples on Polymer Based Nano-Coated MALDI MS Targets for Archiving of Biological Relevant Materials*
Poster presented on the 59. ASMS Conference on Mass Spectrometry and Allied Topics 2011, Denver, Colorado, USA 2011

*Intact Cell / Intact Spore Mass Spectrometry (MALDI TOF mass spectrometry) of Microorganisms on Single-Use Polymer Targets*
Poster presented on the 58. ASMS Conference on Mass Spectrometry and Allied Topics 2010, Salt Lake City, Utah, USA 2010

*Development of Disposable Polymer Targets for MALDI Mass Spectrometry as QC Tool for Oligonucleotide Synthesis and Intact Cell Mass Spectrometry*
Poster presented at the 6th ASAC JunganalytikerInnen Forum 2010 2010

*Wiener Walzer, Tango, Cha Cha Cha*
Bugovský, Weber, metro Verlag 2006

LANGUAGE SKILLS

German—native speaker
English—fluent in conversation, reading and writing on a high level
Hungarian—basic knowledge in reading, writing and speaking

EXTRACURRICULAR ACTIVITIES

Commercial photographer (primarily event photography, portraiture)
Tournament-style fencing (epee, membership with the Academic Sports Club – Section Fencing) since 2000
Study of the Hungarian Language (approx. 2005-2009) at the Sprachenzentrum (Language Center) of the University of Vienna and 2x one monthly summer school in Szombathely, Hungary