Activated Surfaces for Laser Desorption Mass Spectrometry: Application for Peptide and Protein Analysis

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Abstract: Thanks to the development of matrix assisted laser desorption/ionisation (MALDI), laser desorption based mass spectrometry became an essential method for the analysis of biomolecules. This review will discuss the various surface modifications used in combination with laser desorption mass spectrometry and their application for the analysis of peptides and proteins. In the first hand, some modified surfaces are designed to enhance the laser desorption/ionisation process; this includes the use of carbon, porous silicon surfaces and also immobilised matrix. In an other hand chemical and biochemical modified surfaces developed to isolate species with more or less specific interactions can be used for on-slide sample clean-up before MALDI-MS analysis. In addition, different experimental devices as mass spectrometers and microfluidic devices used for such a purpose will be presented.

INTRODUCTION

Very early, desorption/ionisation mass spectrometry was started by the introduction of field desorption (FD) [1] applied for analysing polar, non-volatile and thermally fragile organic compounds as well as bio-molecules in vacuum. Through the 70's, the study of a large variety of polar analytes was possible by using FD as well as particle bombardment modes such as: (i) MeV particle bombardment (plasma desorption mass spectrometry, PDMS) [2] and (ii) KeV ion bombardment (SIMS) [3] for a less extent. Until end of the eighties, these methods were supplanted by fast atom bombardment mass spectrometry (FAB/MS) [4] using liquid matrix. During the latter period, this mode has been considered as a softer alternative method for a better control of dissociation of the desorbed quasi-molecular ions and was widely used for analysing large classes of polar biological compounds. Nitrocellulose was used as matrix latter for PDMS but unfortunately without no more success. Alternative to the bombardment methods, pulsed infrared laser desorption (LD) appeared first for laser microprobe (LAMMA 1000) for direct irradiation of the analyte [5] and latter, was applied for desorbing glycoconjugates [6]. Following this landmark, the Cotter's group develops various applications of LD on bulk sample and re-introduced the *time-lag focusing* in TOF/MS for increasing peak resolution by lowering energy spread of ions [7] as well as decreasing the observed ion fragmentations [8]. Actually, laser desorption methods applied to analyse relatively high mass molecules (up to 10000 u) yield the deposition of a relatively large amount of energy on the ions and to many decompositions.

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More recently, *extraction delay* has been introduced for improving peak resolution of desorbed ions. Pulsed UV-laser desorption using various matrices has been introduced in 1988 by K. Tanaka [9] and by Hillenkamp and Karas [10]. Respectively, the used matrices were either colloids dispersed in glycerol to desorb high mass proteins such as cytochrome C (called as soft laser desorption, SLD) or organic compounds co-crystallized with the analyte (as matrix assisted laser desorption/ionisation, the famous MALDI) allowing a dramatic improvement of laser/desorption efficiency. Let us remember that from a long pathway, this ultimate step permitted the SLD and MALDI emergences, which were saluted by attribution of the chemistry Nobel price (October 2002) to K. Tanaka. This price was being shared with J. Fenn for electrospray (ESI) and K. Wüthrich (for 2D-NMR for biomolecule structures). The matrix acts as an intermediate by absorbing the energy of the laser and is generally used in association with a pulsed UV laser. MALDI allows desorption/ionisation of very large molecules but is limited in the case of compounds with a relatively low molecular weight because their produced ions often overlap with matrix ions in the mass spectra. Recently a revival of matrix free laser desorption is emerging through the development of new surfaces such as porous silicon which appears to be particularly useful for the analysis of low mass molecules. This approach takes advantage of the UV adsorbing properties of porous silicon, which offers an indirect excitation of the analyte.

Now, mass spectrometry is considered as a method of choice for analysing biomolecules. Indeed, a veritable analytical arsenal for biomolecule investigations grows up by introduction of performing ion analysers (MS) such as TOF (time of flight), Q (quadripole filter), IT (ion trap), FT/ICR (Fourier transform ion cyclotron resonance), IM (ion mobility) and new sector instruments as well as their corresponding tandem systems (hybrid or not) for structural determination by MS/MS or MS^n. With collisional activation of selected precursor ion, tandem mass spectrometry allows

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to investigate structures of biomolecular ions since, desorption methods give rise to very stable quasi-molecular ions, which under conventional experimental conditions, do not yield numerous spontaneous fragmentations.

The fact that laser desorption is performed generally on a flat metal surface led several groups to investigate various kind of surfaces and their modification. The nature of these modifications can be either chemical or biochemical and depending upon their properties they can be used in different application. In particular such surfaces can allow different desorption/ionisation processes or be used for on slide sample clean-up.

This review will present and discuss the various surface modifications used in combination with laser desorption mass spectrometry and their application for peptides and protein analysis. In the first part, modified surfaces designed to enhance the laser desorption/ionisation process will be presented. This includes the use of carbon, porous silicon surfaces and also immobilised matrix. In the second part, chemical and biochemical modified surfaces developed to isolate species with more or less specific interactions will be introduced. In addition, the different experimental devices as mass spectrometers and microfluidic devices used for such a purpose will be presented.

1. LASER DESORPTION USING MODIFIED SURFACES FOR ANALYSIS OF PEPTIDES

Various materials have been investigated in the purpose of enhancing the laser desorption without the need of matrix addition. In particular carbon and porous silicon surfaces have been introduced for the study of polar analytes of low molecular weight. The properties of these target materials allow enhancing the desorption/ionisation yield and/or avoiding ion decompositions by absorbing the laser irradiation energy. Moreover, others studied the potentialities of immobilised matrix on gold or on a polymer layer.

1.1. Carbon Surfaces

Surface Assisted Laser Desorption Ionisation (SALDI) is a laser desorption ionisation method using carbon powder to couple the laser UV energy into a liquid solution. This technique is inspirited from the work of Tanaka *et al*. [9], which used cobalt particles (diameter of 300 Å). Particles used for SALDI are in the µm size range and are constituted by activated carbon powder. This technique only yields few desorbed ions at low m/z ratios compared to those produced by MALDI, and it is also well suitable for the analysis of a wide range of polar and/or thermally labile bio-organic compounds like diuretics, phospholipides, amino acids, as well as peptides and small proteins [11] such as myoglobin and cytochrome c. Conventional matrix free UV-LD appears to be less reproducible and may give rise to fragmentations, which can confuse the mass spectra compared to those recorded by SALDI.

SALDI was also interfaced with thin layer chromatography (TLC), by covering the gel with activated carbon particles. Thin-layer chromatography (TLC) is a rapid separation technique, which allows running several samples in parallel. For example, it is used for the screening of urine samples for drugs. Compared to GC-MS, a preliminary extraction or chemical derivatisation was not required for SALDI. The direct application of mass spectrometric techniques on the TLC plate can be applied to a very wide range of analytes such as small oligosaccharides and nucleic acids, toxins, peptides, lipids etc.

A direct analysis of compounds is possible directly from the intact TLC plates by SALDI/MS [12]. A TLC plate cut-out is attached to the sample target, and then a suspension of activated carbon was deposited on the analyte TLC spot, followed by the addition of glycerol. It is also possible to scrape off the silica gel in the sample spot and the powder obtained is mixed with activated carbon suspension and followed by MS analysis. In SALDI-MS study, cationisation by sodium and potassium is the dominant process in positive mode. The composition of the activated carbon suspension can orient the ionisation processes toward protonation or cationisation. In fact, by using a lower ratio of activated carbon, the protonated form is favoured compared to the sodiated form. Moreover the extent of protonated species is related to the gas phase thermochemical propertie (e.g., proton affinity) of the analyte.

Addition of sucrose, which acts as an adhesive sample, is generally performed to reduce ion source contamination. TLC-SALDI-MS analysis is a simple preparation method, which leads to few background ions. A high pumping capacity is required when liquid glycerol is applied over a large area of the target. To enhance the signal resolution, an electrical contact has to be assured between the TLC plate and the metallic target. By scanning the overall surface of the sample plate, it is possible to record an image of the TLC eluates. This is an imaging technique, which needs a uniform carbon suspension depot.

SALDI-MS is characterised in practice by a relatively low sensitivity and leads to ion source contamination due to the use of the carbon particles. Actually, thin layer of carbon particles [13] was developed in order to avoid such problems. This technique is known as graphite plate laser desorption ionisation (GPLDI). A weak volume of analyte solution is directly deposited onto the surface. To improve peak resolution, an electrical contact between the activated carbon layer and aluminum foil target was ensured. Sunner and co-workers [13] successfully studied different compounds like lysine, caffeine, glucose and bradykinin. These bioorganic compounds appear mainly, as protonated species and were observed together with a few background ions constituted by alkali cations and various cationised forms of glycerol.

This laser desorption ionisation method presents a high sensitivity (detection limit from amol to fmol range) within a good resolution and leads only to few background ions at low m/z. Analysis of low molecular weight compounds, like small polymers (e.g., poly(methylsilsesquioxanes)) [14] and fatty acids [15] was possible by GPLDI. In the latter case, no preliminary chemical derivatisation such as the saponification or transesterification processes is required. Finally, the GPLDI mode can be considered as a rapid qualitative analytical method, which does not yield artefact due to carbon atom interferences presenting only few background ions at low m/z range.

1.2. Porous Silicon Surfaces

In 1999, Wei *et al*. [16] introduced a new matrix free approach, called desorption ionisation on porous silicon (*DIOS*), allowing the analysis of small mass compounds [16, 17] using silicon surfaces prepared by a "galvanostatic etching procedure" [17, 18] on a Si wafer. The preparation method is the following: the silicon wafer is sandwiched between Teflon surfaces. The cathode, a Pt wire, is placed in the etching solution above the Teflon, whereas the anode, constituted by a gold foil, is on the other side of the wafer. The surfaces were etched in a solution composed by 25% HF/EtOH with a white light illumination through a mask. After an oxidation process with ozone, the surfaces are chemically etched in a 5% HF/EtOH solution (1 minute) and finally washed in EtOH. The efficiency of the etching process and therefore the silicon morphology depend on different parameters such as the dopant type, HF concentration, light intensity, current density and etching time.

The physical properties [19, 20] of silicon (high area surface, UV absorption) are crucial for DIOS-MS analysis, but the desorption/ionisation can also be optimised by chemical [21] or structural [22] modifications of the active surfaces. The main drawback, directly linked to the silicon material, concerns the storage of these chips. In fact, the termination groups can be oxidised and for this reason, the properties, like surface wetting, are unfortunately modified. Furthermore, the chips can be contaminated by hydrocarbons or alkali cations from the glassware. Experimental factors contributing to ionisation efficiency [23] and influence of the preparation and storage [24] of the chips on the efficiency for small peptides analysis by DIOS-MS, were studied. Especially, Siuzdak and co-workers [24] showed that a pre-analysis treatment, soaking overnight the chips in solvent like isopropyl alcohol (IPA) or ethanol, contributes to eliminate the contaminations coming from the chips, the target preparation or its storage conditions.

To improve specificity, the surface's activity can also be modified by derivatisation with receptors, allowing ligand identification [25]. Moreover, DIOS technique allows to give information about the fragmentations of small molecules by combination with tandem mass spectrometry [26].

DIOS-MS presents as a useful tool in proteomic for the functional characterisation and identification of small proteins. Thomas *et al*. described DIOS as a platform for protein identification [27]. The protein activity is identified by monitoring the interaction of an enzyme with a substrate molecule (formation of enzyme reaction products) in presence (or not) of inhibitors. An on-plate proteolytic digestion combined with DIOS-MS can allow protein identification. Furthermore, unlike MALDI, low mass proteolytic fragments can be observed thanks to the absence of matrix ions in the low m/z range. For this reason, PSD can be used from low m/z ratio ions generated by DIOS giving rise to sequence information and also identification of possible posttranslational modifications without chemical or enzyme addition. DIOS-MS has been applied to serotype identification of virus.

Another possibility for the study of digest samples is the use of atmospheric pressure laser desorption/ionisation (method introduced by Laiko *et al*.) in combination with porous silicon (*AP-DIOS*). This technique allows an easier sample preparation together with a good sensitivity (e.g. subpicomole for mixture of standard peptides). AP-DIOS can be interestingly associated with ion trap instruments, which give access to MS^n investigations. This matrix-free approach offers a broader coverage of the digest products in the m/z ratio range up to 1000 Th, because of the absence of matrix interferences. Laiko *et al*. [28] studied BSA digests with AP-DIOS and mass spectra were compared to those recorded in AP-MALDI (Fig. **1**). Huikko *et al*. [29] demonstrated quantitative analysis by using AP-DIOS in the case of drugs study, such as midazolam quantification. The authors showed that the ionisation efficiency is directly related to the analyte gas phase properties (e.g. enhanced by higher proton affinity) in the AP-DIOS analysis.

Silicon chips were already used as support in the field of neuron analyses. Fromherz *et al*. mounted leech neurons (*Retzius* cell) directly on a silicon microstructure, with insulating silicon oxide layer, forming a neuron-silicon junction. The authors showed the ability of a capacitive-coupling from silicon to neuron by a stimulation spot [30] or from neuron to silicon by metal-free field-effect transistor [31]. Kruse *et al*. [32] described the use of DIOS-MS for biological samples studies, like tissues (e.g. *Aplysia Californica*) or cultured invertebrate neurons directly onto the porous silicon. This requires a preliminary treatment of the DIOS chips by using MeOH/ammonium citrate, which favoured release and relocation of peptides from tissues into the porous silicon. They confirmed that the ammonium citrate did not act as a matrix. The study of *Aplysia atrial* gland onto a treated porous silicon allowed the observation of known and unknown peptides, and also presented a good correlation with results provided by MALDI-MS analysis (2,5-dihydroxybenzoic acid matrix, DHB being the matrix). The results show the ability of DIOS/MS to detect peptides of molecular weight higher than 4000 Da by increasing the extraction delay in the TOF source. However, the ionisation efficiency of DIOS is limited by the penetration of analytes into the porous silicon but also by the possibility of the laser light to penetrate the tissue disposed on the porous silicon. With DIOS, the use of an adhesion-promoting reagent like polylysine is not required. Furthermore, DIOS-MS presents a high salt tolerance (up to 2 M NaCl). The authors studied also different techniques to favour sensitivity and to avoid background ions (coming from adsorption from solvents and plastics) like a heat treatment. Studies with surfaces presenting different morphologies will allow optimisation of biological sample analysis by DIOS.

Bhattacharya *et al*. [33] used a mid-IR laser (λ 1.45 to 4.0 μm) for desorption ionisation (IR-LDI) on silicon. Compared to the silicon target prepared for UV-DIOS, the initial silicon wafer is used directly without an initial etching or roughening step, because of the silicon porosity (or also roughness) is not needed for favouring the desorption/ ionisation process. In fact in the UV-DIOS mode, the silicon surface acts as a matrix transferring energy to the analyte, whereas in the IR-LDI mode on silicon (transparent in this wave length range), the water (or the other solvent molecules) play the role of matrix allowing ion production process. The authors studied small biomolecules (e.g.,

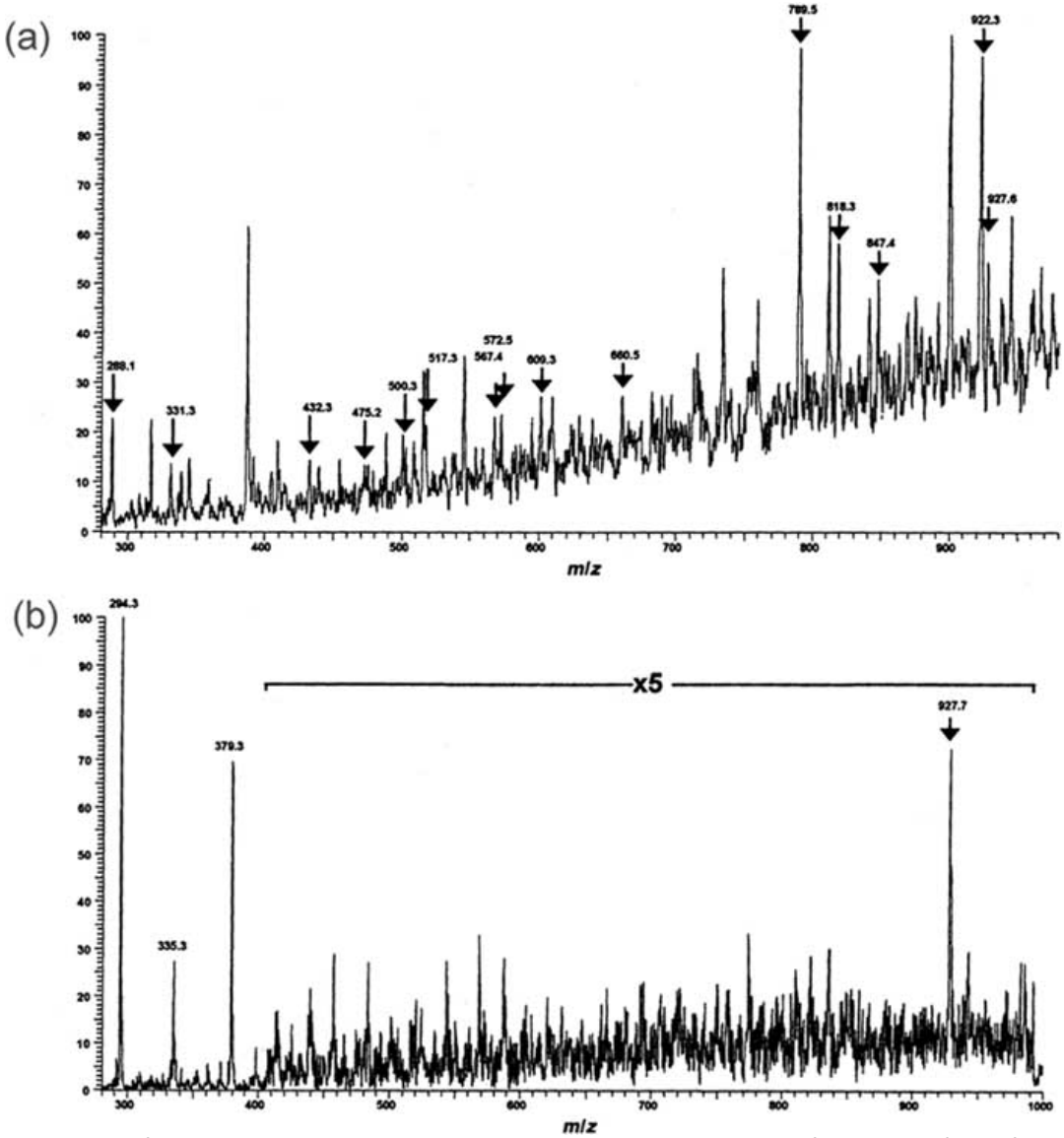

Fig. (1). Low mass region of BSA digest spectra recorded by (a) AP-DIOS and (b) AP-MALDI (250 fmol) adapted from ref. [28].

riboflavin and ascorbic acid) and also larger size molecules such as the bovine insulin. Being transparent to the IR laser beam, an irradiation of the sample from the back in transmission geometry will favour analysing samples like tissues or deposited aerosol particles, achieving spatial resolved information. Kruse *et al*. [32] described the analysis of cells and tissues directly on the porous silicon, whereas the Murray's group proposed to use IR-DIOS technique for these applications. In fact, they applied earthworm tissues (*Eisenia foetida*) and bacteria such as *E. Coli* and *B. Cereus* [34]. The samples present peaks in the 6000 Th to 14000 Th range for earthworm, whereas bacteria's signals are in the m/z 600 to m/z 900 ratio range. Furthermore, the differences observed between both these IR-DIOS spectra allow distinction of the studied bacteria.

Siuzdak and co-workers [35] developed a new deposition technique, based on the electrospray, introducing the analyte by a syringe pump into a fused-silica column. A potential difference (in order of 1.5-1.7 kV), applied between the capillary and the DIOS chip leads to an analyte layer on the DIOS chip. The authors analysed the *thymopentin* peptide (RKDVY) and two amino acids such as phenylalanine and tyrosine. The internal standards were respectively *splentin* peptide (RKEVY) for the studied peptide and deuterium labelled analogues for the studied amino acids. Compared to the conventional dried droplet technique in MALDI, a homogeneous deposit is obtained leading to a better mass spectra reproducibility and sensitivity. Moreover, the calibration curves obtained by the electrospray deposition (introduced long time ago for PDMS experiments and more recently, for MALDI by the Derrick's group) present a better linearity. This technique can be combined with LC-DIOS-MS avoiding interferences (or ion suppression) for complex samples studies. For example, it provides enhanced sequence coverage of protein compared to the conventional LC-ESI-MS-MS [36]. In the future, this technique could present a real potential for study of small molecules, coming from biological mixtures like in human plasma or urine.

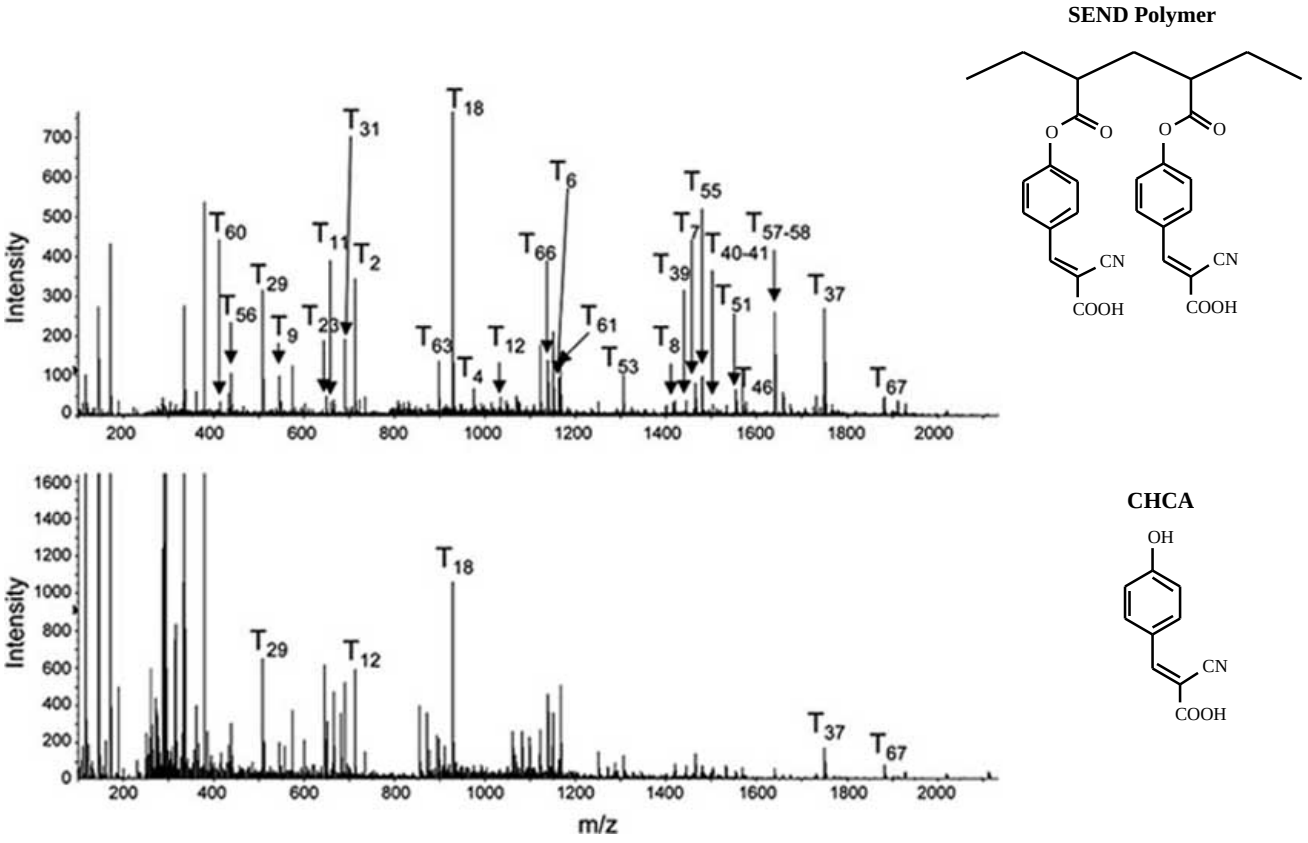

Fig . (2). Mass spectra of BSA tryptic peptides recorded using (a) immobilised matrix (SEND polymer) and (b) conventional MALDI matrix (α-cyano-4-hydroxycinnamic acid) adapted from ref. [58].

1.3. Chemically Modified Surfaces

Matrix addition presents some drawbacks, such as heterogeneous incorporation of analytes into matrix crystals, which makes difficult the localisation of analyte on the spot (presence of "sweet spots"). More homogeneous crystals were observed by Weinberger *et al.* [37], using an analyte-matrix mixture, dried in vacuum. Speir *et al*. [38] studied small peptides from a layer of analyte electrosprayed on an initial electrosprayed layer of matrix (sinapinic acid), obtaining more homogeneous samples but limited to peptides composed by less than ten amino acids. This approach is the premise in fact to the "surface enhanced neat desorption" (called as SEND mode). It was also shown that the sensitivity can be improved with an initial depot of nitrocellulose [39, 40] as used in PDMS. The concept of an initial matrix layer led to the development of other approaches, such as self-assembled monolayers (SAMs) [41-44]. SAMs is based on monolayer formation by reaction of gold surface with sulphur atoms from monolayer solution composed by thiol groups (similar method was used for MS/MS experiments with SID mode). Different monolayer types were studied [45-47] and in particular SAMs constituted by aromatic compounds like MALDI matrices [48-53] were explored. Mouradian *et al*. [54] studied different SAMs composed by UV absorbing aromatic molecules (similar to a matrix) bounded to the gold surface through thiol groups. The authors studied LHRH peptide, insulin, cytochrome C, $d(ACGT)_2$ and dT_{10} with each monolayer and showed that only MMPC [methyl N-(4-mercaptophenyl)carbamate] monolayer allows observation of the analyte signal.

Zhang *et al*. [55] used silicon powder or also derivatised silica gel as possible inorganic matrices. The silicon powder, compared to the porous silicon surface used for DIOS, is suspended and sonicated in water/acetonitrile, allowing clean-off of the contaminants and therefore allowing ion observation without large background. The authors showed that the structure and the size of the pores are crucial criteria (better signal with higher pore size). For this reason, silica gel represents another possible support for these studies. Whereas experiments on silica gel with small peptides lead to alkali cationised species, in the MALDI analysis, the protonated species are favoured but accompanied by an important ion background at low m/z ratio range. Moreover, the signal intensity can be enhanced by covalently bounding MALDI matrices such as DHB and CHCA on the silica gel in the presence of 4,4' diphenylmethane diisocyanate.

Surface Enhanced Neat Desorption (SEND) [56, 57], introduced by Hutchens *et al*. [56], is a similar approach, based on the use of a polymer, composed by terminal groups similar to α-cyano-4-hydroxycinnamic acid matrix (CHCA)

covalently attached to a chip array. This modified polymer, acting as a matrix by its terminations, allows reduction of the chemical noise in the low m/z range. Lin *et al*. [58] studied the feasibility of SEND-MS analysis to detect peptides from tryptic digestion, or from a limited acid hydrolysis. With a conventional MALDI study of BSA tryptic digest, matrix ions dominate the spectrum in the low mass range (m/z 100-1100) whereas in the SEND analysis, a better sequence coverage was obtained (Fig. **2**). The same study for tryptic lysozyme peptides shows differences between MALDI and SEND analyses. With SEND, new T17-18 and T10 peptides were detected. Coupling data obtained with a limited acid hydrolysis and SEND-MS analysis can give additional information for protein identification. In fact, in the case of myoglobin, the terminal sequence can be attributed to 18 out 25 ions compared only 11 out 25 with conventional MALDI.

The insertion of C_{18} chain into the polymer of SEND arrays allows a selective retention and purification of membrane and highly hydrophobic peptides [59]. For example in the case of the analysis of BSA digest, 38 tryptic digests were detected (from 500 Th to 4000 Th) and after an additional wash step only 23 with a negative Bull and Breeze index value, showing the selective retention of hydrophobic peptides using SEND/MS analysis. Moreover, in the case of a double digest trypsin and AspN of cytochrome b_5, a very hydrophobic peptide (residue 109-113) at m/z 2810.4 was barely detected, and after binding and washing steps its signal became dominant in mass spectra.

By a Schotten-Baumann reaction, CHCA matrix gives rise to a monomer α-cyano-4-methacryloxycinnamic acid (CHCMA) able to co-polymerise with acrylic acid, stearyl methacrylate and trimethoxysilylmethacrylate leading to a polymer used for the new Hydrophobic Interaction Chromatography (HIC) SEND arrays. Weinberger and co-workers [60] used these arrays to study *E. Coli* lysates and *nasopharyngeal* (NP) swab samples of patients with severe acute respiratory syndrome (SARS). The sensitivity is improved using HIC SEND arrays, because of the presence

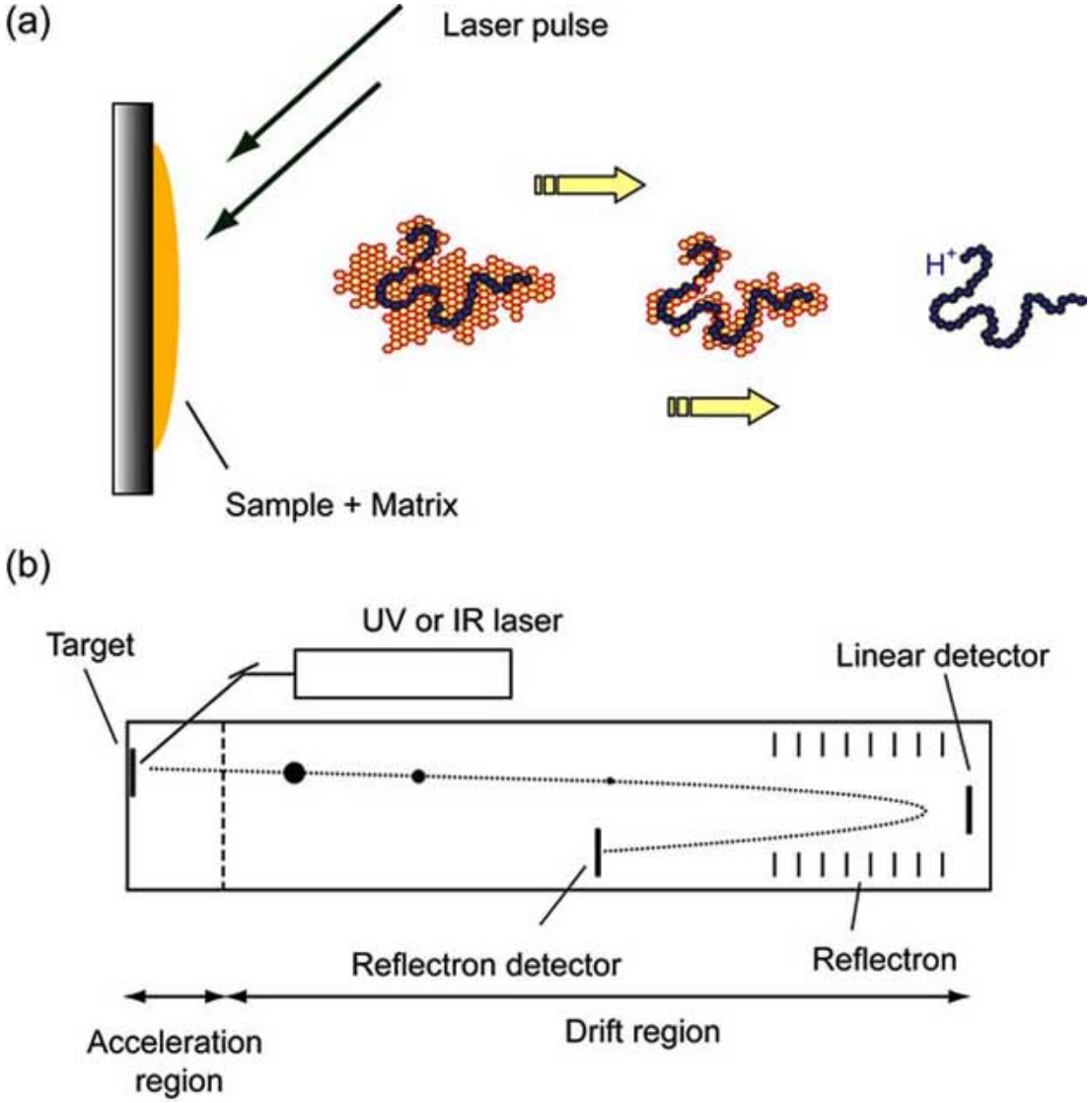

Fig. (3). Schematic representations of (a) the MALDI desorption process showing the formation of protonated molecules from precursor aggregates and (b) a reflectron time of flight mass spectrometer.

of photo-responsive monomers and a reduction of the chemical noise compared to conventional MALDI. The results show identification of biomarkers such as an antiviral group of peptides known as human neutrophil defensin. These techniques are very promising and their exploration merits to be grown up.

2. AFFINITY PURIFICATIONS WITH MASS SPECTROMETRY READOUT

Activated surfaces have been used extensively in microarray technology allowing simultaneous detection of various experiments. Specific affinity molecules (e.g. antibodies etc.) can be easily covalently bond to various kinds of supports including glass or gold. Different samples can be incubated on different spots and after a washing step, used to remove contaminants and unspecifically bounded compounds, the readout are traditionally performed using fluorescence, chemiluminescence or radioactivity detection. These techniques allow a fast and sensitive reading of the slides. Nevertheless, they usually required the use of labelling (e.g. fluorescent tag), which may reduce the specificity of the interactions. In the same way, the proteins may be labelled to a different extent leading to variable signal detection (in abundance and shape). Moreover, this approach required the use of very specific interactions, which can lead to the non-detection of some biological compounds. Indeed, antibodies may not recognise all the protein isoforms as well as its modifications. In the same way, the investigators give no information concerning the post-translational modification of the studied protein.

The use of mass spectrometry for the reading such arrays allows a label-free detection. The structural capabilities allow avoiding false positives. It is possible by this way to differentiate between a metabolites and its precursor drug although they may have the same affinity towards a receptor. Moreover, this approach permits the use of compounds with broadband affinity such as lectins (which target polysaccharides) or chromatographic support (C_{18}, ion exchange etc.). This method is particularly useful to target a large class of compounds as demonstrated by the Fenselau's group for bacteria identification [61, 62] or for differential profiling in order to identify biomarkers. Fine structural characterisation can be reached through tandem mass spectrometry experiments or/and on-slide proteolytic digestion. For sample characterisation, multiple biomarkers can be used if the protein quasimolecular ions are resolved in the recorded mass spectrum.

Numerous applications have been developed from this technology in various fields from drug discovery to proteomics [63]. In particular, proteomics [64] can be divided between protein profiling (or comparative proteomics) and functional proteomics. All these approaches may be improved by the combination of mass spectrometric detection and characterisation with affinity purification. This technology offers high throughput, reproducibility, and robustness and is easily amenable to automation.

2.1. Surface Chemistry for LD Mass Spectrometry

DNA microarrays have been shown to be very powerful for the study of transcriptional regulation [65]. In the post-genomic, the study of protein function in a high throughput approach has been of particular interest. Gene function is indeed revealed by the activity of the resulting protein. In this aim, it was necessary to develop techniques allowing proteins immobilisation in a way that preserve their specific activity. The linkage must not hide the recognition site or change binding parameters. Polyvinylidene fluoride (PVDF) and also nitrocellulose membranes have been commonly used as supports for the attachment of proteins in biochemical studies such as immunoblot, but are not appropriated for protein microarray production. In fact, using these materials, the protein density is not enough and the sample can spread on the surface. Thus other materials allowing the attachment of protein at high density *via* a possible derivatisation have been considered. MacBeath used microscope glass slides functionalised using aldehyde containing silane reagent [66]. This chemical functionalisation allows protein immobilisation through the formation of a Schiff's base linkage with primary amines.

Different immobilisation processes were developed based upon the reactivity of sulfhydril groups. For instance, the same author [67] uses the reaction process between the thiol and maleimide groups to immobilise small molecules (such as biotin, FK-506, tetramethylrhodamine) to glass slides. A similar immobilisation process was developed by Houseman *et al*. [68] using self-assembled monolayer (SAMs) of alkanethiolate on gold, composed by maleimide and penta (ethyleneglycol) groups. This process is based upon a reaction between maleimide groups and thiol ligands. The implicated immobilisation is rapid and selective in the presence of functional groups (e.g. amines, thiols, carboxylic acids, alcohols). The presence of penta(ethyleneglycol) groups prevent non-specific adsorption at the surface.

Different techniques can be used to characterise the immobilisation such as fluorescence spectroscopy, surface plasmon resonance (SPR), MALDI-TOF mass spectrometry, electrochemistry and radioisotopic labelling. The authors analyse different types of chips for instance, for studying the lectin binding properties in the case of carbohydrate thiol immobilised, the enzymatic phosphorylation of the immobilised ligand IYGEFKKKC by the tyrosine kinase c-src and so the inhibition of the enzyme. Another point of interest concerns the cell adhesion and migration, which can be performed by the immobilisation to monolayer of the peptide CGGRGDS-NH_2, playing the role of ligand for cellular integrin receptors. In fact, the RGD sequence, present in extra cellular matrix proteins (ECM), plays an important role in cellular growth. The immobilisation of peptides containing this same sequence allows specific surface cell interaction, which favours the cell organisation. Furthermore, the cell adhesion depends directly upon the density of immobilised ligands, so using this immobilisation process; the density of immobilised protein can be controlled. Xiao *et al*. [69] have yet described the binding of peptides containing the sequence Arg-Gly-Asp (as example RGDC and GRGDSPC) on a titanium surface modified with maleimide groups. Moreover, capture agents can be immobilised to the chips covalently or non-covalently [70]. Hodneland *et al*. [71] describe the possibility of a selective and irreversible immobilisation of protein to self-assembled monolayer (SAMs) of alkanethiolate on gold, taking place in different

steps and based on the use of a capture protein , acting as a covalent link. The authors fused the protein of interest to a capture protein, here cutinase, which is in fact a 22 kDa serine esterase and has the property to bond covalently to phosphonate ligand. They study the cutinase-calmodulin fusion, because of its ability to bind the *calcineurin* protein in presence of calcium cations.

The use of sensor chips presenting a carboxymethylated dextran layer covalently bounded have been developed based on gold-coated glass surface in order to limit non-specific interactions. Different covalent coupling chemistries can be applied, based on chemical functions such as amines, aldehydes or thiols. Several sensor chips, available commercially, have been developed for immobilisation and can be divided into two groups: chemical and biochemical surfaces (Table **1**). Among the chemical surfaces, immobilised metal ion affinity chromatography (IMAC) allows a selective purification of target proteins from biological samples *via* a specific affinity separation. A chelating ligand is pre-immobilised on the surface and consequently the specific metal ion. Various ligands are available such as nitrilo-triacetic acid (NTA) for Ni^{2+} ion. The protein can be reversibly bonded to the metal ion by the amino acid side chain (e.g., histidine). The biochemical surfaces allow specific affinity interaction using immobilised compounds such as streptavidin, DNA, antibody or receptor.

2.2. Microfluidic Devices

Several micro fluidic devices have been developed for the material transportation within miniature channels, allowing reactions and purifications. Compared to the micro arrays, these devices allow a better control of various processes such as the transport of the sample on the chip without the requirement of an external system. Two biosensor chip systems, available commercially have been combined with mass spectrometry detection. The first, called SELDI (*surface enhanced laser desorption ionisation*, from Ciphergen) presents a direct coupling between the initial preparation of the array and the direct analysis by MALDI-MS. Biological samples (serum, urine, cell lysates etc.) are deposited on the adequate array, followed by a washing step in order to remove the unbounded compounds. After the addition of the matrix (small molecule absorbing in the UV range), the array is directly analysed by conventional MALDI time-of-flight mass spectrometer. Identification of the protein can be performed through direct digestion on the array. The second system, BIA-MS [72] (*biomolecular interaction analysis-mass spectrometry*, Biacore) was developed. This system was originally designed to study biomolecular interactions, based on biosensor technology optimised for monitoring interactions between biomolecules in real-time. The ligand is immobilised on the biosensor chip, constituted by a glass support covered by a thin gold layer and a reticulated carboxymethylated dextran hydrogel. The sample of interest is injected with a constant flow rate through a microfluidic circuit in contact to the chip. BIA uses a non-destructive detection system based on the surface plasmon resonance phenomenon (SPR) [73] to characterise interactions implicated between the analyte of interest and the ligand immobilised on the sensor chip. The SPR provides a multidimensional detection approach in association with mass spectrometry. The implicated interaction can be characterised by following the evolution of the resonance signal as a function of time. The resonance signal corresponds in fact to the SPR angle (value implicated in the SPR phenomenon) and its change traduces directly the variation of concentration of the introduced biomolecules. However, no information about the structure of the molecule bounded to the sensor chip is available by this way. So the recent association of this technique with mass spectrometry appears promising in various fields such as in proteomics but also for micro-organisms or identification of molecular targets like toxins.

In some case, micro-recovery of the compounds isolated by affinity purification can be useful for more complex structural characterisation [74, 75]. Here, the isolation and clean-up of the compound of interest is performed as usual but instead of being directly analysed on-chip by MALDI, the sample is eluted and collected within a minimal volume. This allows further purifications, concentration of the analyte of interest and gives also more flexibility in terms of analytical procedure. These purified compounds can then be studied, using nanoelectrospray ion source coupled with ion analyser with MS/MS capabilities but also by conventional MALDI. This micro-recovery approach is particularly useful

Table 1. List of the Available Chemical and Biochemical Surfaces

	Chemistry	Interaction Properties	Applications
Chemical Surfaces	· Hydrophobic	· Reverse phase, alkyl chains immobilised such as C_{18}, teflon	· Membrane biochemistry
	· Anion exchange	· Quaternary amine group	· low pI proteins, peptides
	· Cation exchange	· Carboxylate group	· high pI proteins, peptides
	· IMAC	· Metal affinity	· Histine-tagged molecules, phosphorylated protein
Biochemical Surfaces	· Streptavidin	· High streptavidin-biotin affinity ($K_d \approx 10^{-15}$ M)	· Biotinylated molecules (DNA, proteins, peptides)
	· Antibody	· Specific	· Complementary antigen
	· DNA	· Specific	· Transcriptome analysis
	· Receptor	· Specific	· Complementary ligand

as it avoids the destruction of the chips through the laser irradiation and allows a more efficient structural characterisation if needed. Lopez *et al*. [76] used this technique to characterise the protein SHP2 tyrosine phosphatase, which interacts with an immunoreceptor tyrosine-based inhibitor motif sequence of the sst2 somastin receptor.

2.3. Interfacing with Mass Spectrometry

Mass spectrometry is one of the most efficient and specific techniques for the identification and characterisation of proteins thanks to the development of two complementary techniques: ESI and MALDI. Their respective capabilities (facilitating the analysis of intact, polar, non-volatile and thermally labile species) have been the primary driving force behind the dramatic growth of biological mass spectrometry.

Matrix assisted laser desorption ionisation (Fig. **3a**) is the method of choice for the desorption/ionisation of compounds specifically bounded on surfaces. Activated surfaces can be indeed easily adapted on MALDI plates [77]. The addition of a UV absorbing matrix solution in acidic conditions allows the release of the specifically bounded material which is transfer in the gas phase through irradiation by a pulsed UV-laser. Ions are then separated depending on their mass to charge ratio in the mass analyser. In the SELDI systems the purification is directly performed on a sample target, which can be directly introduced into a mass spectrometer, equipped with a specifically designed target holder. Most MALDI targets can, however, be modified in order to handle different surfaces. This can allow the adaptation of systems not originally designed for mass spectrometry detection. MALDI offers many advantages: short analysis time, high sensitivity (low volume of analyte with concentrations in the nM-range), no constraint on analysis time and laser repetition rate can be varied, tolerance to contaminants such as salts and detergents and the ability to simultaneously detect different components in a mixture. The MALDI process can be miniaturised and fully automated for high throughput analysis. Unlike electrospray, MALDI produces mainly singly charged ions, which are of a great importance for the analysis of polypeptide mixture. With MALDI it is easy to pause the acquisition and process the data for instance, to carry out a sequence TAG search before deciding on the next experiment to perform. MALDI TOF allows the use of standard 96- and 384-well plate formats in MS proteomics research. To improve the speed and efficiency of MS analyses, techniques must be developed to accommodate robotics and facilitate automatisation to allow high throughput.

By its nature, conventional MALDI is a pulsed technique carried out in high vacuum and is traditionally coupled with linear or reflectron time of flight instrument (TOF) (Fig. **3b**). MALDI-TOF is sensitive, robust and can be run using relatively impure samples. Such features are particularly important for the characterisation of unknown biomarkers. Because of its virtually unlimited mass range, time of flight mass analyser is so far the most used in combination with MALDI as this source produces mainly singly charged ions. During the desorption/ionisation process, MALDI usually deposits more internal energy than ESI. The amount of internal energy deposited on the ions depends in particular on the physicochemical properties of the used matrix. Matrixes can be divided between ”hot” matrixes like CHCA (α-cyano-4-hydroxy-cinnamic acid) and “cold” matrixes like DHB (2,5-dihydro-benzoic acid) which partially suppress MALDI-induced decomposition.

But MALDI was also coupled to other kind of mass analyser as hybrid quadrupole time of flight [78], known generically as QqTOF, combining a quadrupole and an orthogonal acceleration time of flight. With time of flight analyser alone, MS/MS is realised with post source decay (PSD). By this technique, it is difficult to separate ions in a narrow mass range and it provides a weak control of fragmentation. With MALDI-QqTOF, a quadrupole is used to isolate ions within a selected range, which are fragmented in a collision cell and analysed by TOF. By this way, low-energy multiple collisions that are common with triple quadrupole mass spectrometry [79] are obtained. MALDI-QqTOF has high sensitivity, resolution and mass accuracy. The automatic sequence prediction can be improved by taking into account the high mass accuracy of the QqTOF and the specificity of CID fragmentation of singly charged ions. The high resolution and mass accuracy of the QqTOF remain unchanged in the MS-MS collision induced dissociation (CID) mode. This is drastically different from conventional MALDI post source decay (PSD) fragmentation where the mass accuracy typically goes down to about 1 Da. Full control of the degree of fragmentation is another significant advantage of the CID in comparison to the PSD technique. MALDI-QqTOF may become an instrument of choice for high throughput protein identification and first results of successful use of MALDI-QqTOF for protein identification/ sequencing have been presented in 1999 [80]. On the other hand, QqTOF can be more flexible for further analysis with respect to the choice of mass spectrometric technique such as ESI (electrospray ionisation) and MALDI, which can be easily combined in one instrument. MALDI or ESI-QqTOF mass spectrometer can provide MS/MS spectra of comparable quality. A substantial advantage of the QqTOF design is the decoupling of the ionisation method from the mass analysis. This allows rapid switching between different matrixes and different laser fluences with no alteration of TOF calibration.

Recently, the novel ionisation source that combines atmospheric pressure ionisation and MALDI [81] (AP-MALDI) allows the transfer of the ions from the atmospheric pressure ionisation region to the high vacuum by using pneumatically assisted by a steam of nitrogen. Comparatively to “vacuum MALDI”, source pump-down is eliminated, reducing analysis time. The AP MALDI source can be equipped with UV or IR laser and the sample preparation is the same that conventional vacuum MALDI, using the same organic matrices but allow also the use of liquid matrixes. This ionisation method is able to produce protonated molecular ion for small proteins but favours formation of cluster ions with the matrix. AP-MALDI source can give successful combination with TOF, ion trap and QqTOF mass spectrometers with particular advantages for the high-throughput screening analyses because the sample arrays would be located outside the vacuum and it may simplify the interface to robotics systems. The coupling of AP MALDI with ion trap mass spectrometer, which is a relatively inexpensive instrument with outstanding structural capabilities, brings sequential MS^n capabilities.

MALDI was more recently coupled with recent time-of-flight/time-of-flight (TOF/TOF) high-resolution tandem mass spectrometer [82]. The components of the instrument can be divided into three sections based on their functionality: (i) ion selection at high resolution in the first TOF (typical resolving power for the precursor ion is on the order of 5000); (ii) collision cell region including the collision cell itself and the ion optics necessary to transfer ions between both the mass analysers; and (iii) fragment ions detection in a second pulsed TOF mass analyser. So, this instrument offers high sensitivity for peptide analysis and comprehensive fragmentation information obtained by collision-induced dissociation (CID) under high-energy regime conditions. However, in addition to recording MS/MS spectra, this instrument can still be operated in all conventional modes of a high-quality MALDI TOF mass spectrometer: i.e. linear, reflectron and PSD. A TOF/TOF tandem mass spectrometer permits improved ion selection in comparison to conventional ion gates employed for PSD precursor ion selection, as well as collisional activation of the selected ion and recording of full fragment spectra at a single mirror voltage. The energy of the ions in the first TOF defines the nature of the collisions between the precursor ions and the target gas in the collision cell and this instrument was designed for high-energy collisions (keV range). The nature and extend of fragmentation can be controlled by the choice of matrix and collision gas. The sensitivity of this instrument is very high, comparable to that of conventional high-performance MALDI-TOF instruments.

3. APPLICATIONS IN THE PROTEOMIC FIELD

3.1. Protein Profiling

The comparison of experiments from different samples (e.g. cancer *vs.* healthy cells) allows the observation of variation as proteins expression in the so-called "comparative proteomics" or protein profiling [83, 84]. This permits to understand biology at cellular level and to identify biomarkers of diseases. Protein profiling is traditionally performed by the combination of two-dimensional gel electrophoresis and mass spectrometry [85, 86]. This powerful approach allows the characterisation of known or unknown proteins. Phosphorylations are in particular easy to identify by this approach [87]. However, the handling of two-dimensional gel electrophoresis is relatively complex and limited mainly to high abundance proteins. Other approaches use liquid chromatography coupled with mass spectrometry. Stable isotope labelling may then be used to differentially tag the proteins [88, 89]. All these techniques are very powerful but several applications as the search of biomarkers in cancer research require higher throughput. Such application can indeed require the analysis of hundreds of samples, which is possible through the use of array technology. Fast analysis is essential in order to diagnose patient at early stages of cancer progression [90]. Affinity surfaces combined with mass spectrometry offers also access to all kind of proteins, which are difficult to analyse using 2D-PAGE as hydrophobic and low molecular weight proteins.

Chips technology [91-93] for these approaches uses often unspecific chemistry as reverse-phase, ion exchange or metal binding (Table **1**). Yuan *et al*. investigated the use of Teflon as sample support for MALDI-MS of proteins [94]. The use of such surfaces allows retaining different groups of proteins depending on their physicochemical properties. The separation of the proteins captured by these surfaces will be performed, thanks to the high resolving power of the mass spectrometer. The method is reproducible allowing the comparison of profiles from diseased and normal samples in order to identify differences in the expression patterns. Over or under-expressed proteins can be identified when abundances differ significantly between samples. The abundance difference needed to validate these ions as biomarkers depend on the number of analysed samples in order to obtain statistically reproducible data.

This approach was used successfully in biomarker discovery for various kinds of cancers [90, 95-98] and for Alzheimer's disease [99]. It allows the identification of multiple proteins, which is essential to establish signature patterns. This can be performed using different samples as serum, urine and also cell or tissue lysates. Sato *et al*. used successfully serum-free conditioned medium for peptide differential display [100] (Fig. **4**).

Adam *et al*. used such a methodology for differentiating prostate cancer from benign prostate *hyperplasia* and healthy men [101]. Serums from various patients including 167 prostate cancer, 77 benign prostate hyperplasia and 82 healthy men, were tested in order to identify biomarkers. After the test of non-specific chip chemistry, it was demonstrated that metal ion binding with Cu provided the best serum profiles. All samples were processed and analysed under the same conditions and data treatment was carried out using peak alignment. Statistical analyses were then performed in order to identify peaks with the highest discrimination potential. From this, a pattern constituted by nine proteins mass, was used to classify correctly 96% of the samples. The obtained specificity was much higher than the standard screening approach using a serum test for prostate specific antigen. The multiplexing capabilities of mass spectrometer permit to compare differential expression of several biomarkers. This provides a significant improvement of the detection rate. The enormous amount of data provided by this technology demonstrates also the need of effective bioinformatics algorithms [102, 103]. In the same way, Tockman and co-workers [104] demonstrated that distinct protein profiles can be obtained between lung tumours and pre-malignant lung lesions. Such results may be useful for the identification of populations at high risk for lung cancer. The rapidity of the analysis may permit the monitoring of the response of a patient to a treatment.

Once a biomarker is detected, the next step is the identification of the protein. This requires usually the isolation of the biomarker followed by proteolytic digestion and characterisation using tandem mass spectrometry [104]. On-chip purification and digestion can be directly performed using a micro fluidic device. Identification of the biomarkers has to be performed in particular if the purpose of the profiling is to understand biological processes. This can be performed either by direct on-chip purification and proteolytic cleavage or by a more classical chromatographic purification. Diamond *et al*. used a combination of both approaches [104]. A differentially expressed biomarker was

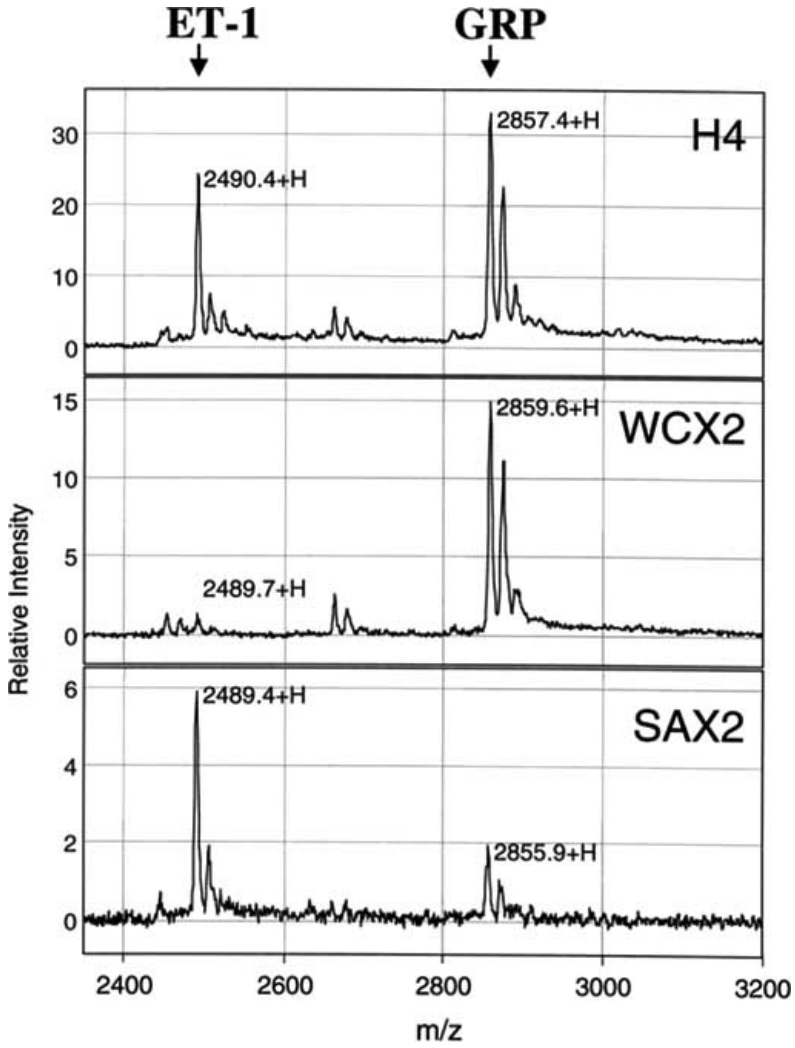

Fig. (4). Selective ion binding on various chip chemistries. From ref. [100].

detected by array/MS combination. Purification of this biomarker (thymosin β-4) was performed by reverse-phase liquid chromatography. Fractions containing the mass of interest were pooled and analysed further using an array/MS system. On-slide trypsin digestion was performed followed by MALDI-TOF detection. The peptide mass pattern is then used for the identification of the protein by a protein database search. The use of a mass spectrometer with MS/MS capabilities may then be used in order to obtain sequence information.

Hogstrand *et al*. [105] studied the response of rainbow trout to zinc exposure. In conjunction with cDNA array, protein profiling was performed using affinity/MS technology, here metal binding, cationic and anionic surfaces were used. The exploration of such different chemistries provides a large range of binding conditions allowing the selective capture of proteins as a function of their metal binding properties or isoelectric point (pI). By this way, seven proteins unique to zinc exposure were identified, whereas four other proteins were observed only on the control and eleven additional proteins were either up or down-regulated by Zn exposure. The obtained results confirmed the role of Zn in a wide range of cellular functions.

3.2. Protein Function and Target Discovery

The aim of functional proteomics [106, 108] is to define the function of proteins in a given organism. This includes the identification of interaction between enzyme/substrates or protein/protein. Molecular recognition plays indeed a major role on all living organisms [109]. The activity of most macromolecules depends on their interaction with other molecules. Functional proteomics required that proteins are in their native state. Protein micro arrays have the potential to detect hundreds of interactions simultaneously. This approach is very useful for the screening of drug candidates, ligand fishing, or identification of novel receptors. With antibody array it is difficult to detect isoforms of a particular protein and its post-translational modifications. The MS detection provides the information required to confirm the structure of the molecules captured on the chip. However, MALDI requires the addition of a UV absorbing matrix on the chip to permit the desorption/ionisation processes therefore the chip cannot be re-used.

It should be pointed out that the use of affinity MS for the characterisation of non-covalent interactions is very different to other mass spectrometry study using ESI which

required that the complex is stable in the gas-phase [110]. All the processes of isolation and purification are here performed using physiological conditions. The method is therefore analogue to conventional ELISA (enzyme-linked immunosorbent assay) technique. The mass spectrometric detection offers in addition the identity of linked compounds and the ability to detect multiple compounds with no need of indirect detection by enzyme-linked secondary antibody. It may also reveal the existence of non-specific binding. Such multiple affinity detection was recently demonstrated by Nedelkov and Nelson [111]. In this study six antibodies were immobilised on the chip surface and were tested using diluted human plasma. It has also been shown also that the combination of mass spectrometry with SPR detection offers complementary information on the stability of the associations in term of binding constant [72] providing a two-dimensional analytical technique.

The identification of a ligand for an orphan receptor [112] is a particularly interesting application of affinity/MS [113]. In this case, the receptor is immobilised on the surface and incubated with a biological fluid of interest and specifically bonded compounds can then be identified by mass spectrometry. This approach is very promising for drug discoveries and can be applied to test mixtures of compounds obtained by combinatorial chemistry. Affinity MS techniques were successfully used for the analysis of protein/protein interaction and complex mixture of peptide-ligand by Rüdiger *et al*. [106]. In this study the affinity purification was performed using magnetic beads followed by MALDI-TOF analysis. Neubert *et al*. [114] demonstrated the use of recombinant protein G with affinity capture MALDI-TOF. In this approach an Fc receptor was covalently immobilised on a MALDI gold target in order to orient an immunoglobulin G with Fab domains directed towards the target surface. This allowed a ~3 fold increase in the MALDI signal comparatively the use of randomly immobilised antibodies.

Flad *et al*. [115] describe the application of the combination of affinity array with mass spectrometry detection for the characterization of a novel antimicrobial peptide DCD-1, derived from the *Dermcidin* (DCD) gene and secreted by sweat glands, and other *dermcidin*-derived peptides directly from micro litre amounts of human sweat. Profiling of human sweat from various donors revealed that in addition to DCD-1, other DCD-derived peptide species were also present in significant quantities. Four of five identified peptides were DCD-1 related, while the fifth corresponded to a portion of the DCD protein outside the DCD-1 core. This provides clues as to how the novel protein is processed to its active form, though further work remains to elucidate this fully. All of the DCD-1-related peptides detected in the individual samples exhibited cleavage at the ends, possibly due to the action of trimming proteases.

3.3. Mass Spectrometric Immunoassay

The mass spectrometric immunoassay approach is based on the microscale immunoaffinity capture of target antigens followed by mass-specific identification and quantification using MALDI-TOF. This approach allows the generation of protein profiles of specific target protein [116]. Immunoaffinity capture of antigens effectively overcomes signal suppression effects typically encountered during traditional MALDI analysis of complex biological mixtures while simultaneous concentrating into a small volume. By this way, comparative profiling can be performed for the identification of variations within individual proteins. Mass spectrometry detection of antigens is unambiguous, as antigen signals are observed at characteristic mass-to-charge value in the mass spectrum, offering a high level of immunity to artefacts due to non-biospecific retention of mixture components. However, the most important aspect of such mass-specific detection is the ability to use a single assay to screen biological systems for the presence of multiple, mass-resolved antigens. Analyte quantification is possible by using a single antibody to capture both the antigen and an antigen variant, which has been chemically modified to have a distinct mass. Nelson *et al*. [117] used this technique for the rapid, selective and quantitation screening of human blood for the presence of myotoxyn a, and Mojave toxin from the venoms of the prairie rattlesnake, *Crotalus viridis viridis*, and the Mojave rattlesnake, *Crotalus scutulatus scutulatus*. Nelson *et al*., demonstrated that attomole amount of beta-2-microglobulin from urine can be specifically captured and analysed [118].

The analysis performed by biomolecular interaction analysis mass spectrometry (BIA/MS), is able to distinguish the number and the nature (specific or non-specific) of species bound to a sensor chip. Furthermore, unknown species retained through specific interaction with the immobilised receptor can be identified using mass spectrometric database searches. In all, analytical approaches based on the real-time optical viewing of binding events, determination of the number of participants in the biomolecular interaction, and mass spectral characterisation of interacting species is enabled by coupling SPR-BIA with MALDI-TOF. Much effort has been devoted to interfacing SPR-BIA with MALDI-TOF for the express purpose of multiplexed biomolecular characterisation [113, 119-123]. The application of BIA/MS can be increased even further when used in conjunction with gene-tagging techniques. Nelson *et al.* [124] used the biomolecular interaction analysis mass spectrometry to isolate, detect and characterise epitope-tagged peptides present in total cell lysates of unfractionated *Escherichia coli*. The presented experiments were performed to investigate the use of BIA/MS. Epitope-tagged tryptic peptides were captured *via* affinity interactions with either chelated Ni^{2+} or monoclonal antibodies and detected using surface plasmon resonance biomolecular interaction analysis (SPR-BIA). The tagged peptides can be eluted from the biosensor chips in high amounts for nanoelectrospray mass spectrometry analysis coupled to a variety of tandem MS instru-ments (Q-TOF, ion trap, triple-quadrupole) to partially sequence the tagged peptides [125]. Alternatively, lower amounts can be analysed directly from the biosensor chip using MALDI-TOF, postsource decay (PSD) and in-source decay (ISD). Protein database searches were performed using the masses of the tagged tryptic peptides [126], resulting in the identification of the protein into which the epitope tag was inserted. Detection limits for both SPR-BIA and MALDI-TOF were at low-femtomole to sub-femtomole level. Fig. **5a** shows the MALDI-TOF mass spectra of the trypsinised *E. Coli* lysate eluted from the NTA sensor chip.

The mass spectrum taken of the material eluted from the Ni^{2+} activated flow cells shows ion signal at m/z 3371.4, 3612.7, 4213.7, 4303.1, 4641.8, 8205.7 and 8594.0. The third spectrum shown in Fig. **5a** is the background-substrated spectrum resulting from substraction of the Ni^{2+}-free spectrum from the Ni^{2+}-active spectrum. Background signals are observed to cancel out leaving four ion signals, at m/z 3371.4, 3612.7, 4213.7 and 4641.8, unique to the Ni^{2+}-active SPR-BIA analysis (Fig. **5b**) which are retrieved from the unfractionated, trypsinized *E. coli* lysate. These mass values were used in database searches identifying the native protein as glutathione-S-transferase (GST).

Nedelkov *et al*. [127] applied BIA-MS for the detection of bacterial toxins in food samples. This approach used surface plasmon resonance (SPR) to detect the binding of the toxin(s) to antibodies immobilised on a surface of a sensor chip. SPR detection is then followed by identification of the bound toxin(s) by MALDI-TOF. *Staphylococcal enterotoxin* B (SEB) was readily detected in milk and mushroom samples at the level of 1 ng/mL. In addition, non-specific binding of food components to the immobilised antibody and to the sensor chip surface was detected. SEB and toxic-shock syndrome toxin-1 (TSST-1) were successfully and simultaneously detected through the utilisation of multiaffinity sensor chip surfaces.

A sensitive assay using affinity-MS systems has been developed to measure all the variants of Aβ in culture supernatants [99], which will be of great value in screening inhibitors of these proteases. A highly selective anti-Aβ antibody, anti-NTA4 raised to the ten N-terminal residues of

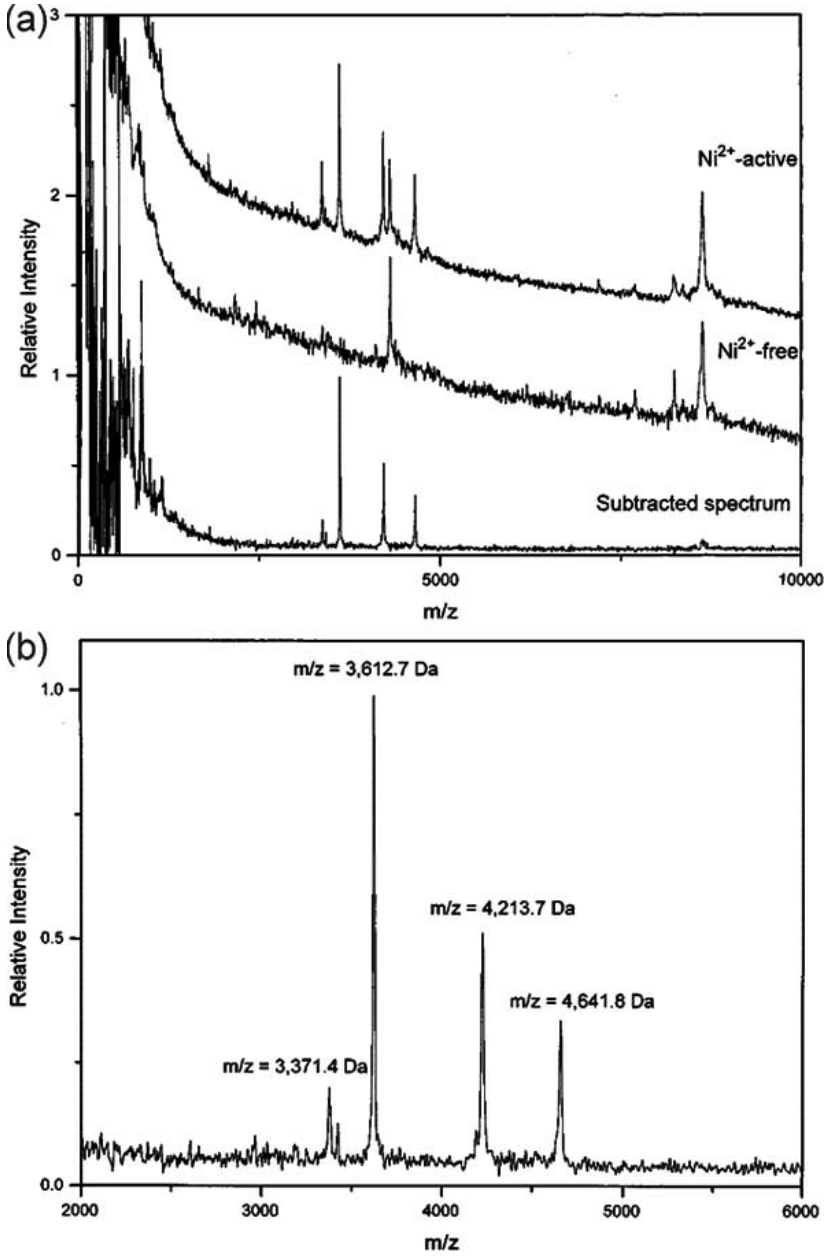

Fig. (5). (a) MALDI-TOF mass spectra of recovered material from the NTA sensor chip and (b) Expanding view of the subtracted spectrum. From ref. [124].

Aβ was coupled covalently to a pre-activated sensor chip array, forming a biologically active surface for the micro-purification of immuno-reactive amyloid species directly from crude biological samples. Once captured and purified by washing on the chip surface, the bound peptides are detected by using a time-of-flight mass spectrometer. With this assay, it has been shown that increasing intracellular cholesterol increases the activities of both β-*secretase* and γ-*secretase*-42. Moreover, changing the intracellular targeting of amyloid precursor glycoprotein (APP) yields increased α-*secretase* cleavage, and increases the amounts of oxidised/nitrated forms of Aβ.

Dare *et al*. [128] have investigated the potential of affinity-MS for the identification and characterisation of a novel protein biomarker in rat urine following the induction of target organ toxicity. Skeletal muscle, a tissue with large amounts of cellular and structural proteins of varying molecular weight, was chosen as the target site. 2,3,5,6-tetramethyl-p-phenylenediamine (TMPD) is used to induce toxicity through the generation of unusually stable radical cations (Wurster salts), superoxide and hydroxyl radicals, and hydrogen peroxide. The skeletal muscle toxicity was monitored using established endpoints such as increase in serum aldolase (Aldol), aspartate aminotransferase (AST), histopathology, and also using on-chip chromatography purification followed by mass spectrometry detection of urine samples. Clear differences in urinary protein patterns between control and TMPD-treated animals were observed on the chip surfaces. Additionally a specific urine marker protein of 11.8 kDa was identified in TMPD-dosed rats, and the detection of the marker was related to the degree of skeletal muscle toxicity assessed by recognised clinical pathology endpoints. The 11.8 kDa protein was identified as *parvalbumin*-α, a specific, non-invasive, and easily detectable biomarker for skeletal muscle toxicity in the rat. This illustrates the potential of affinity-MS technology for biomarker detection and identification in toxicology studies.

Levamisole is widely used for the control of gastro-intestinal parasites in many animal species due to its broad spectrum action. Crooks *et al*. [129] have developed an immuno-based screening method to detect *levamisole* residues in liver and milk using affinity-MS. It was derivatisated into *aminolevamisole*, conjugated to a carrier protein. The generated immunogen was used to raise a polyclonal antibody in a rabbit. The same drug derivative was immobilised onto a CM5 sensor chip. Binding of the antibody to the sensor chip surface was inhibited in the presence of *levamisole*. The chip surface was stable for many hundred of regenerations. The good agreement between results found by this procedure and LC-MS show that not only is the biosensor assay suitable for qualitative analysis.

Forde *et al*. [130] have studied the *Escherichia coli* Lac I protein or *lac* repressor. This transcription factor that controls expression of proteins functioning in lactose metabolism can be captured from total *E. coli* protein lysate on a DNA affinity chip surface bearing the *lac* promoter sequence and detected by MALDI mass spectrometry. The promoter binding sequence for LacI and a scrambled version of the same DNA sequence were prepared on two affinity chip surfaces. The *E. coli* protein lysate was applied on the two surfaces followed by MALDI-MS analysis. By this way, a 38.2 kDa protein was captured on the chip surface containing the binding sequence for LacI but not on the surface containing the scrambles sequence. The protein was identified following one-step, small affinity capture and peptide mapping. This work demonstrates an efficient method to screen for regulatory proteins using DNA affinity chip surfaces and MALDI-MS.

Nedelkov *et al*. [131] explore the detection of both the free and bound IGFs using sensor chip with both SPR and MALDI-TOF-MS detection. SPR is employed for protein quantification due to its non-destructive nature whereas MALDI-TOF-MS is utilised to obtain structural characterisation of the analysed biomolecules. The insulin like growth factors IGF-1 and IGF-2 were assayed from human plasma via biomolecular interaction analysis-mass spectrometry, utilising antibodies for affinity retrieval. Detection of both targeted and non-targeted IGFs in the mass spectra indicated possible protein complex retrieval by the individual antibodies. Plasma samples were treated with detergents to disrupt the putative protein complex and release its constituent proteins. An SDS-treated plasma yielded IGF signals in a different ratio than the one observed in the mass spectra from the non-treated plasma, suggesting disruption of the protein complex and its retrieval from non-treated plasma. In all mass spectra, novel truncated IGF-2 variant, missing its N-terminal alanine, was detected.

It was shown recently that glass slides are suitable sample supports for MALDI mass spectrometry analysis of bacteria [62]. Two lectins, concanavalin A and the galNac lectin from *Helix pomatia*, were immobilised on commercially available surface-activated glass slides and their affinities were evaluated using MALDI. The activated surface was exposed to a mixture of the two spores types *B. cereus* and *B. subtilis* (Fig. **6**). Lectins immobilised on glass slides with activated surfaces are used to concentrate and purify agglutinated spores. It is expected that such slides will provide a rapid, inexpensive way to evaluate and implement new strategies involving MALDI-MS readout.

Boyle *et al*. [132] have used the sensor chip technology combined with mass spectrometry detection to characterise the secreted cysteine protease of *Streptococcus pyogenes*, SpeB, which has been implicated as a virulence factor in group A streptococcal infections. This method allowed rapid identification of both the zymogen form of the protein (Mr ~ 41000 Da) and the fully active enzyme (Mr ~ 28500 Da) by focusing on the use of affinity-MS. SpeB production in culture supernatants was demonstrated to be growth-phase regulated and SpeB positive and negative variants of a blood passaged *S. pyogenes* isolate could readily be distinguished. The methods enabled enhanced speed, use of lower sample volume and concentrations and also a more complete molecular characterisation of Spe B that allowed by existing methods of analysis using SDS-PAGE and Western immunoblotting.

Tubbs *et al*. [133] have analysed β-2-Microglobulin (β2m) present in human biological fluids by using mass spectrometric immunoassay (MSIA). During the screening, MSIA was able to distinguish between wild-type and glycosylated forms of β2m, which made possible the accurate

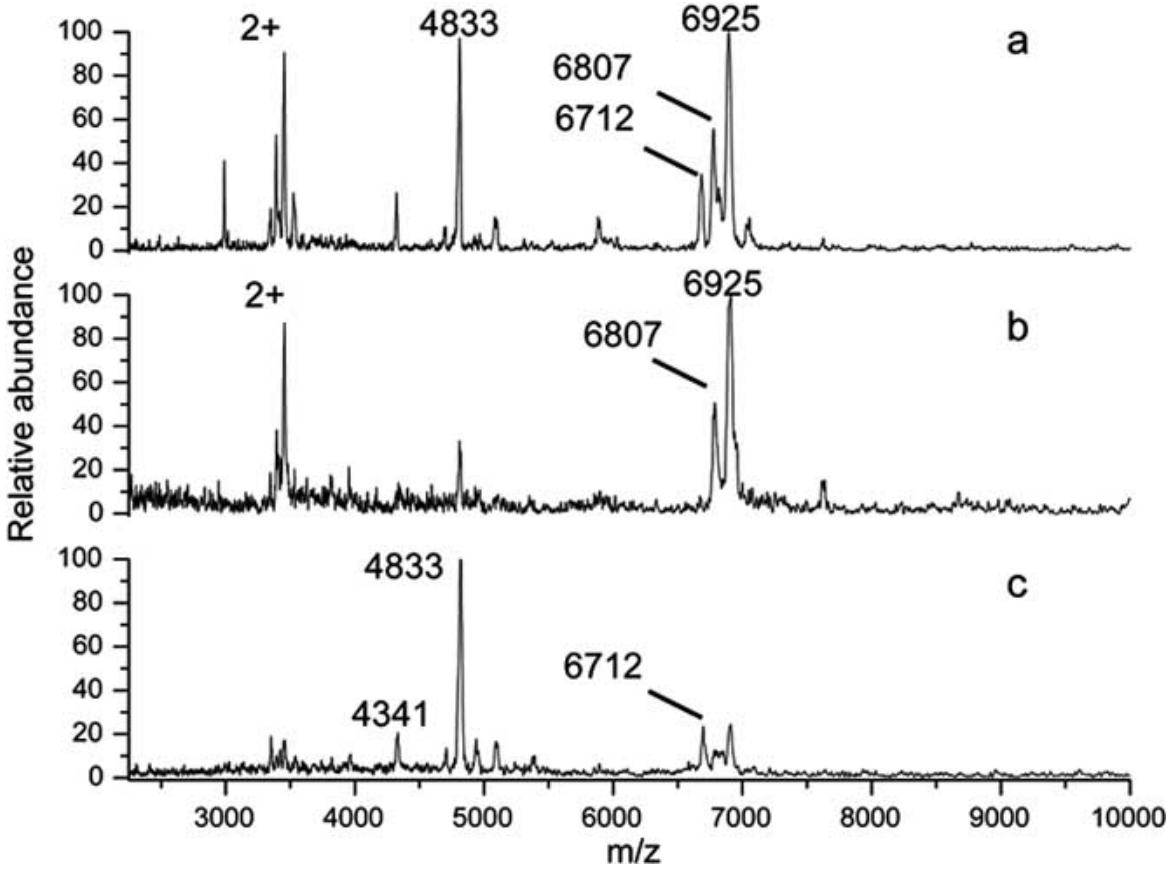

Fig. (6). MALDI-TOF mass spectra recorded on linear mode of (a) mixture of *B.cereus* spores and *B.subtilis* spores on stainless steel; (b) the mixture exposed to concanavalin A immobilised on glass and washed; (c) the mixture exposed to *H.pomatia* lectin immobilised on glass and washed. From ref. [62].

quantification of wild-type β2m without interference from glycosylated versions of the protein. A new approach to the rapid and accurate detection/quantification of β2m present in biological fluids is demonstrated.

CONCLUSION

Laser desorption based mass spectrometry is extending its range of application, thanks to the development of modified surfaces used either to enhance the desorption/ionisation processes (with or without the use of a matrix) or for affinity purification prior to analysis. On-chip purification is essential for high throughput analysis of complex mixtures allowing a large number of applications based on specific or unspecific affinity surfaces. Porous silicon and carbon surfaces may also be useful to the analysis of low molecular weight compounds without matrix interference. One very promising applications of these matrix free approaches is mass spectrometric imaging that can be used for instance to locate drugs activity in tissues. The absence of matrix crystals can in particular improve spatial resolution. All these surface chemistry in combination to mass spectrometry gives access to new applications that required multiplexing, specificity and high throughput. The future development of the laser desorption techniques based upon various chemically or biochemically-modified surface as well as the use of matrix must be influenced by the evolution of IR-laser. Indeed, it should allow (i) to produce quasi-molecular ions carrying a lower internal energy and (ii) to use OH-functionalised solvent as matrix, yielding a possible coupling with "on line" separative methods. The use of the atmospheric pressure source for MALDI (or SELDI and SEND) and LDI (included DIOS, and SALDI) should permit a direct imaging analysis without particular experimental manipulation as occurring in vacuum for preserving integrality of the cell. On the other hand the development of new chips could give a decisive advantage to the surface laser desorption applied to analysis of biological compounds.

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