CHAPTER 17

LC-MALDI MS AND MS/MS – AN EFFICIENT TOOL IN PROTEOME ANALYSIS

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Abstract: Liquid chromatography-matrix-assisted laser desorption/ionization mass spectrometry represents a sensitive, hyphenated MS- and MS/MS-technique with a broad range of

E. Bertrand and M. Faupel (eds.), Subcellular Proteomics, 355–380.

applications in all areas of proteome analysis. Whereas a number of interface types have been developed for coupling MALDI MS and liquid chromatography, in this chapter selected on-line and off-line types and techniques will be discussed with respect to their individual properties and performance. The technique is especially attractive in off-line mode where LC-separation and MS analyses are decoupled and each step can be performed at its individual optimum. Different speed of chromatographic separation and achievement of S/N criteria in MS or MS/MS mode can be optimized independently by individual adjustment of specific operating parameters. This flexibility makes LC-MALDI MS attractive for the analysis of peptide mixtures from low to medium complexity. Using sequential MS analysis of parallel LC runs (multiplexing), even highly complex samples can be handled. Quantitation at the MS and MS/MS level can be accomplished by a variety of labeling techniques, where the predominant formation of singly charged ions in MALDI alleviates the assignment of isotopomers. After discussing the level of complementarity between LC-MALDI and LC-ESI MS, selected applications of LC-MALDI MS are presented. Examples of membrane protein analysis applying 1D SDS PAGE are discussed in detail as well as applications in protein interaction analysis. These application examples clearly show that in all respects LC-MALDI MS and MS/MS are flexible and sensitive techniques which can be adapted to a wide range of different workflows.

1. INTRODUCTION

In proteome analysis the application of multidimensional separation methods to deconvolute the highly complex analyte mixtures is a prerequisite for the identification and characterization of individual components by mass spectrometric techniques. To achieve these objectives two main, but quite different workflows are currently pursued. While in the protein-centered approach extensive separation is performed at the protein level and peptides are generated only in the last step, the peptide-centered approach starts with the conversion of protein mixtures into highly complex peptide mixtures, which are then separated accordingly. The classical 2-dimensional gel-based approach combines protein separation according to pI (isoelectric focusing, IEF) and molecular mass (polyacrylamide gel-electrophoresis, SDS-PAGE) and the protein mixtures are generally well resolved. The resulting individual protein spots are of low complexity and the corresponding peptide mixtures can be analyzed directly by MALDI peptide mass fingerprinting (PMF) and MALDI MS/MS partial sequencing. By reducing protein separation to one dimension, the remaining complexity of the mixture requires at least one additional high resolution separation step at the protein or peptide level (e.g. after SDS-PAGE). In the latter case, reversed-phase microbore and nano-HPLC are the methods of choice because they provide high separation power and reasonable loading capacity. In the purely peptide-centered approach to proteomics, RP-HPLC is combined as second step with ion exchange chromatography as the first dimension of separation. In this workflow peptides are traditionally analyzed by on-line electrospray LC-MS and -MS/MS. State-of-the-art equipment allows to perform peptide mass detection and partial sequencing of a small number of precursor ions in seconds but depth of analysis is of course restricted by the number of experiments which can be performed on-line during elution of a chromatographic peak. Already on this basis, decoupling of liquid chromatography and MS analysis

would be advantageous. This can be achieved by depositing LC-effluents as traces or spots together with matrix on a suitable carrier material before they are analyzed off-line by MALDI mass spectrometry. The chromatogram is thus frozen and with minimal precautions (exclusion of humidity and light) stable for at least days. In a first step MS analysis of the complete trace or spotset provides now time-resolved masses and intensities of all peptide ions, and candidate precursor ions for MS/MS can be selected from positions with highest intensity. This is not only important for protein identification but also for optimal quantitation in MS as well as in MS/MS mode. With current instrumentation (tandem time-of flight instruments, MALDI-ion traps and other MS combinations) partial sequence information can thus be obtained for selected peptide ions in the higher attomole range.

In this chapter, the interfacing of LC and MALDI-MS and -MS/MS will be described, performance will be discussed and selected applications in the proteomics field including analyses of membrane proteins will be presented.

2. INTERFACING LC AND MALDI MS

For interfacing liquid chromatography and matrix-assisted laser desorption-ionization mass spectrometry several MALDI-specific criteria have to be considered (for reviews see Murray 1997; Gusev 2000; Orsnes and Zenobi 2001). In contrast to electrospray, the desolvation process of LC-effluents should not just provide analyte ions in the gas phase but, ideally, analytes fully incorporated in matrix crystals suitable for MALDI ionization. Since standard MALDI matrices can not be directly added to LC solvents without compromising chromatographic performance, special techniques were developed to achieve this goal. For on-line LC-MALDI, desolvation and matrix/sample crystallization need to be perfectly time-controlled and have to proceed without interference with the high vacuum (and in selected cases high voltages) in the MS systems. Therefore LC methods with low flow rates like microbore- or nano-HPLC are most suitable. Moreover, they also provide the most concentrated analytes, which is of prime importance for detection sensitivity in ESI-MS as well as LC-MALDI MS techniques.

2.1. On-line Interfaces

As a result of the technical constraints summarized above, only a small number of on-line LC-MALDI interfaces have been described in the literature. In aerosol LC-MALDI (Murray et al. 1994) column effluent was mixed with matrix solution and passed through a heated nebulizer; the resulting aerosol beam was further desolvated in a heated tube and laser desorption was performed "on the fly" from the airborne particles. Because of limited focusing of the neutral particles and the high flow rates (0.5–1 mL/min) sensitivity was only in the nanomole range. In continous flow MALDI (Nagra and Li 1995) a low flow (5 μL/min) of a mixture of 3-nitrobenzyl alcohol as liquid UV adsorbing matrix, ethylene glycol and TFA was admixed to 1–5 μL of LC effluent. At the tip of the capillary located in front of the repeller of the MS ion source,

laser desorption was performed and ions were extracted perpendicularly. With microbore HPLC and reduced split ratios (ca 1:100) small proteins could be detected in low picomole amounts. In a modified version with a porous stainless steel frit at the capillary tip and well balanced matrix and LC solvent conditions continous analyte and matrix co-crystallisation was achieved (Zhan et al. 1999). Solvent flushing and laser ablation were applied for interface regeneration. In two interfaces primarily developed for CE-MALDI, the area of sample and matrix crystallisation was separated from the point of laser desorption. Matrix was added to the sheath flow used in capillary electrophoresis and the combined eluent mixture was then vacuum deposited on a rotating quartz wheel located directly behind the repeller of the MALDI ion source (Figure 1, Preisler et al. 1998). The quartz wheel was later replaced by a 80 m long Mylar® tape, which allowed up to 24 h of continuous operation (Preisler et al. 2000). Vacuum deposition provides very homogenous amorphous crystalline matrix layers resulting in attomole MS sensitivity for test peptides and in a significant reduction of suppression effects in peptide mixtures. To prevent freezing and concomitant formation of ice crystals, solvents had to contain at least 30% methanol. This interface-type was later modified to accept up to eight parallel traces which could be interrogated by a 5 kHz laser positioned by a fast-scanning mirror (Preisler et al. 2002). Good performance was also achieved with an interface using a rotating stainless steel ball (Orsnes et al. 2000).

Despite the high performance of selected interface types, analyte deposition and MS analysis have to be synchronized which restricts the available time window, for

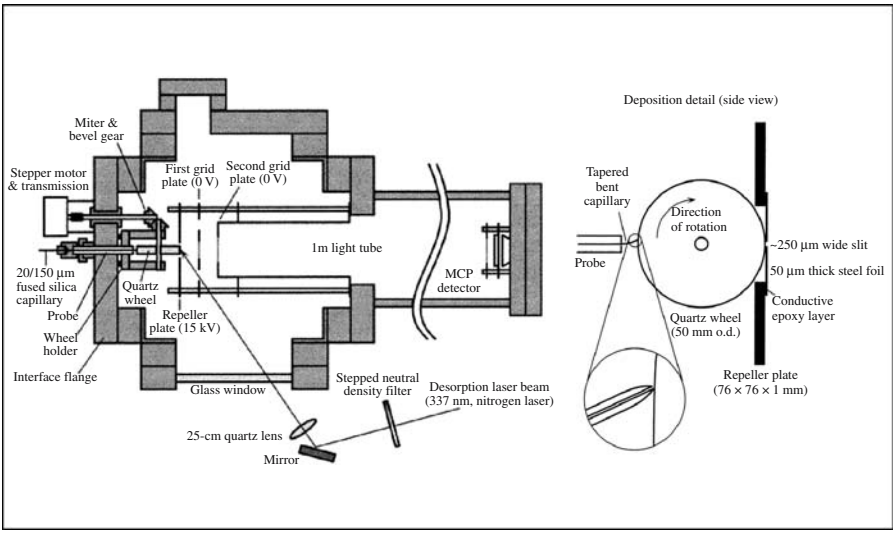

Figure 1. Rotating Wheel Vacuum Deposition Interface. Schematic of the on-line MALDI-TOF MS instrument (left) and details of the deposition process (right). Reprinted with permission from Preissler et al. (1998). Copyright ©1998 American Chemical Society.

example for MS/MS experiments in protein identification. In addition, all on-line approaches are technically demanding and were – to the best of our knowledge – not commercialized.

2.2. Off-line Interfaces

In the off-line approach, analyte and matrix deposition are decoupled from MS analysis and only instrument-specific plate formats have to be considered in interface design. In this case interface design focuses on the development of techniques for a perfect transfer of LC-effluent and matrix to the surface from which laser desorption takes place at a later stage. The development of suitable techniques started very early in conjunction with capillary electrophoresis (CE) and some of the methods which are applied nowadays for low-flow LC-MALDI are included in the following compilation.

2.2.1. Continous deposition

Continous deposition of a CE trace on a MALDI probetip using post-column addition of 2,5-DHB matrix (T-piece) was already reported in 1992 (Castoro et al. 1992) and detection limits in the picomole range were obtained for small proteins. By addition of matrix to the CE sheath flow selected peptides deposited on a moving target could be detected down to high femtomole amounts (Van Veelen 1993). When the CE trace was deposited on a conducting cellulose membrane precoated with alpha-cyano-4-hydroxycinnamic acid, detection limits for peptides like neurotensin could be extended to the medium attomole range using linear UV-MALDI MS (Zhang and Caprioli 1996). For LC traces blotted onto PVDF membranes, infrared MALDI with succinic acid as matrix could be successfully applied for molecular mass determination of peptides at the low picomole level (Eckerskorn et al. 1997). Continous vacuum deposition, first described in on-line mode (cf. Section 2.1.) is also applicable off-line in a separate vacuum chamber; trace deposition on metal MALDI targets allowed to use low percentage of organic solvents owing to better heat dissipation (Rejtar et al. 2002). In combination with CE, electrophoretic resolution was perfectly preserved and not only MALDI MS but also MS/MS spectra were obtained at the 200 femtomole level. For higher flow rates (up to 3 μL/min) like in nanoLC or fast LC with monolithic columns, the MALDI plate was coated with nitrocellulose to prevent uncontrolled droplet formation (Chen et al. 2005b). In order to reduce fast depletion from amorphous thin matrix layers especially in MS/MS mode (elevated laser power), deposition conditions (solvent, pressure) were adjusted in such a way that fine micro-crystalline layers were formed (Chen et al. 2005b). Another method for solvent removal is the application of heat. In a heated nebulizer interface nitrogen gas and LC-gradient-dependent heating was applied to the tip of the interface capillary and a fine mist of droplets was sprayed onto targets precoated with alpha-cyano-4-hydroxycinnamic acid (Wall et al. 2002). For flow rates up to 10 μL/min, MS sensitivity in the high attomole range was reported for testpeptides and, for protein digests, femtomole detection limits were achieved. A similar approach was applied

for spot deposition and is discussed in the next section. Aerosol deposition can also be achieved without heating for flow rates up to 3 μL/min with an oscillating capillary nebulizer (Fung et al. 2004). A high gas flow between two coaxial capillaries leads to the oscillation of the inner capillary and, with optimized dimensions, an aerosol is formed.

2.2.2. Spot deposition

In spot deposition the continuous LC-trace is divided into fractions which are collected on a MALDI sample plate. Continous information is thus "digitized" and, in order to preserve the chromatographic resolution, spotting intervals have to be adjusted accordingly. With too many sample points the analyte is dispersed over several fractions and as a consequence, analyte to matrix ratio and sensitivity are reduced. In the opposite case peaks start to merge and suppression effects become operative. Consequently, deposition of three to five fractions across a peak profile seems to be a reasonable compromise. To maintain chromatographic resolution apart from spotting frequency, any sample dissipation during droplet formation has to be suppressed. This is especially important for interface types which avoid contact of capillary and MALDI plate and which do not actively support droplet transfer. With a high content of organic solvent and a small outer diameter of the spotting capillary, liquid can easily be retracted at the outside of the tip by capillary forces. Apart from these technical details, fraction deposition in a standardized pattern presents a clear advantage compared to continous techniques with respect to ease of data management and data acquisition.

Spot deposition on a computer controlled x-y table in a pattern of 100, 400 or 1024 spots was already demonstrated in 1994 (Blakley et al. 1994) and by MALDI MS, picomole level sensitivity was achieved for a LC separation of peptides at high flow rates (2.1 mm column). For CE a similar procedure allowed to detect high femtomole amounts of smaller proteins (Walker et al. 1995). Matrix was either spotted onto dried fractions or was admixed to the sheath flow. Modified silica sleeves have been especially developed for CE interfacing (Tegeler et al. 2004). Parallel deposition from two neighbouring capillaries is also feasible; this method was applied in MS and MS/MS (PSD) analysis of peptides from a single neuron (Hsieh et al. 1998). An elegant way to transfer liquid as droplets from a continous flow to a carrier is piezoelectric droplet dispensing (Oennerfjord et al. 1998). By the action of a piezoelectric element, the volume of a flow channel is reduced and droplets are ejected from a nozzle (Figure 2). With MALDI targets precoated with a alpha-cyano-4-hydroxycinnamic acid/nitrocellulose matrix, peptide detection at the higher attomole level could be demonstrated (Miliotis et al. 2000). In a similar workflow MALDI plates with prespotted matrix/calibrant layers in hydrophilic surface areas were prepared in a first step and CE eluate was spotted in a separate second step. After recrystallisation, low femtomole amounts of standard peptides could be detected (Johnson et al. 2001). A very effective alternative for droplet transfer is the application of a short electric pulse (−2 kV, 20 ms) between the droplet hanging at a metal capillary tip and the metal target plate located at a distance of 2–5 mm (Ericson et al. 2003). Droplets are first polarized and then attracted

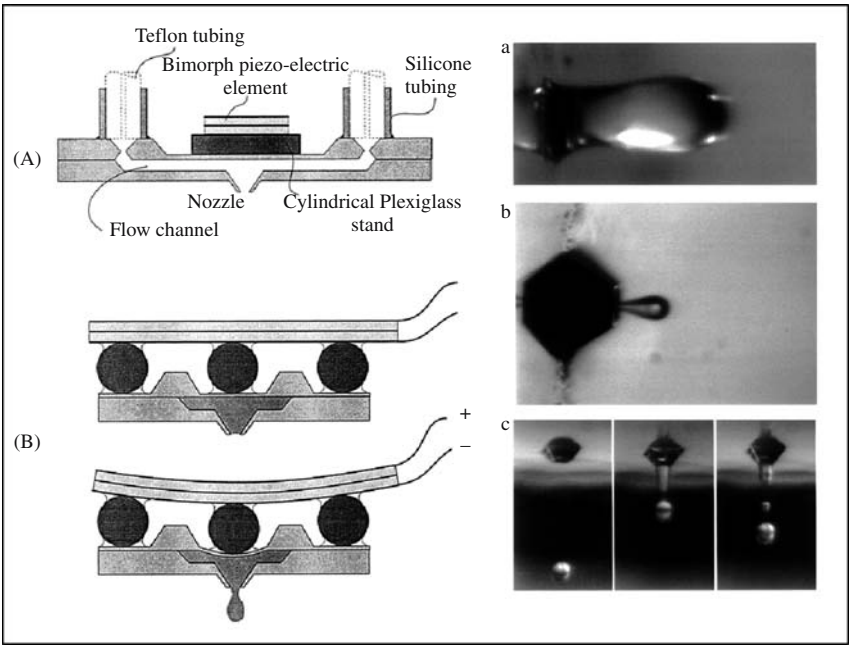

Figure 2. Piezoelectric Droplet Dispenser. Schematic of the dispenser construction (panel A, left) and actuation principle (panel B, left). Droplet formation at the dispenser tip (right, a and b) and droplet appearance at different voltages (right, c). Optimal voltage (right, c, left panel) and voltage too high (right, c, middle and right panel). Reprinted with permission from P. Oennerfjord et al. (1998). Copyright ©1998 American Chemical Society.

to the MALDI plate. This principle was implemented in a multichannel interface which allowed parallel deposition of four LC-traces. Matrix was added via T-piece or was prespotted onto plates with 384 or 1536 hydrophilic surface areas (Figure 3, Ericson et al. 2003). With prespotted alpha-cyano-4-hydroxycinnamic acid matrices (1 mg/mL) and recrystallisation after spot deposition, 50 attomoles of a standard peptide could be detected. An interface type which is adapted to a wider range of flow rates uses partial droplet desolvation. This was achieved by applying LC-gradient-dependent heating to a flow of nitrogen at the capillary tip. Aerosol formation was thus prevented and the remaining solvent was evaporated by heating of the MALDI plate to 110°C (Zhang et al. 2004). Flows up to 200 μL/min could be handled and standard peptides were detected at the low femtomole level. Only nitrogen flow assistance and careful capillary selection were used in an interface for low flow nano-LC; with precoated MALDI plates containing hydrophilic anchors and a TFA washing step after sample deposition, nine peptides of a 1 fmol BSA digest could be detected (Mirgorodskaya et al. 2005a). Another means to transfer droplets is a mechanical impulse; this principle was realized by applying a pulsed voltage to a selenoid surrounding the capillary outlet (Young and Li 2006). By careful adjustment of plate distance low femtomole sensitivity was achieved.

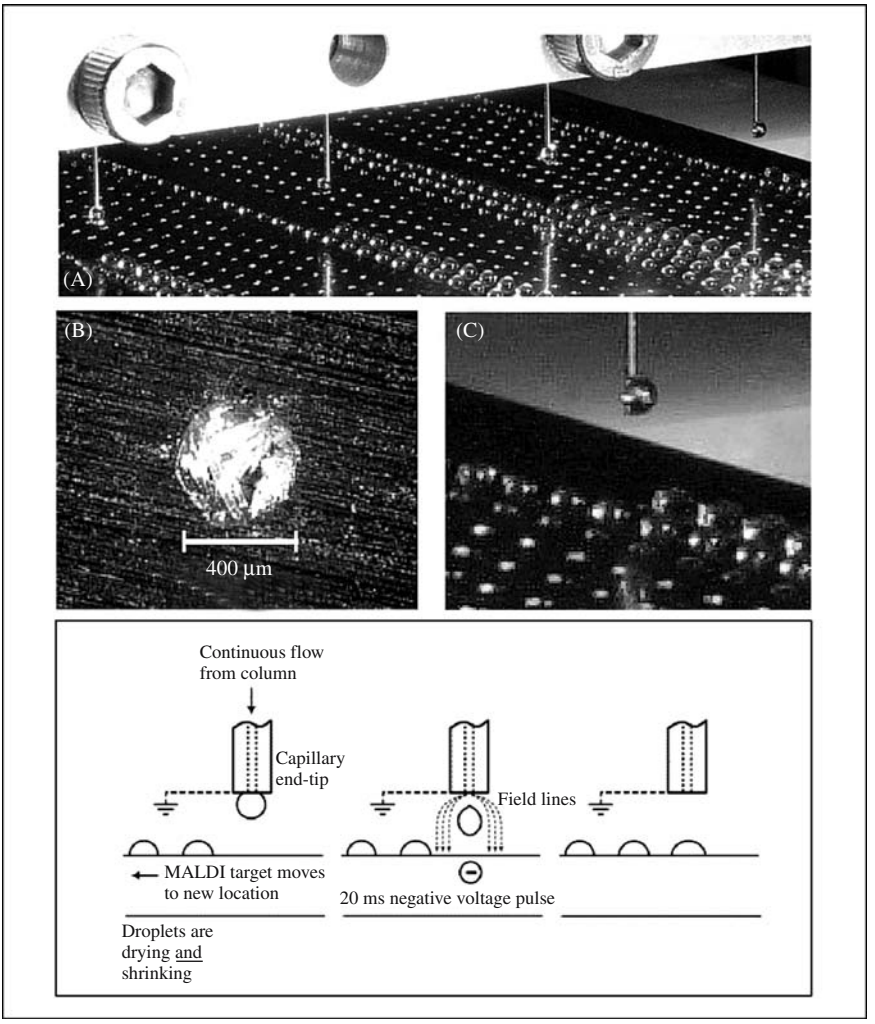

Figure 3. Interface with pulsed field droplet deposition. Principle of the electrically mediated liquid deposition (lower panel). Deposition of the eluents from 4 parallel LC separations (upper panel, A). Samples cocrystallized with matrix on hydrophilic islands with 400 µm diameter (upper panel, B) and droplets just before application of electrical field (upper panel, C). Reprinted with permission from Ericson et al. 2003. Copyright ©2003 American Chemical Society.

Most of the currently applied LC-MALDI interfaces use off-line spot deposition and a number of commercial instruments are available (Mukhopadhyay 2005). Compared to on-line techniques, this interface type is technically less demanding but only careful adjustment guarantees optimal performance.

3. LC-MALDI MS IN PROTEOMICS

3.1. Introduction

In the early days of proteomics, MALDI MS was mainly applied for peptide mass fingerprinting using time-of-flight (TOF) mass spectrometers as the major instrument type. MALDI MS/MS on commercial TOF-instruments was only available with post-source decay analysis (PSD, Kaufmann et al. 1994) and MS/MS spectra had to be assembled from 10 to 20 subspectra acquired within a long time frame. Therefore partial MS/MS sequencing of peptide ions was mainly performed on instruments fitted with electrospray ionization (ESI), for example on triple quadrupole (QQQ), quadrupole-TOF (Q-TOF) or ion trap instruments (iTRAP). The situation started to change with the introduction of (vacuum) MALDI ion sources which could be fitted for example to Q-TOF instruments (Harvey et al. 2000; Krutchinsky et al. 2000; Loboda et al. 2000; Wattenberg et al. 2002). A major breakthrough for MALDI MS/MS was however achieved with the introduction of tandem time-of-flight instrumentation (Medzihradszky et al. 2000) and the implementation of LIFT-TOF/TOF technology (Suckau et al. 2003). Interfacing of MALDI to ion trap-TOF (vacuum MALDI, Martin and Brancia 2003) and ion trap instruments (vacuum and atmospheric pressure MALDI, Krutchinsky et al. 2001, Tan et al. 2004) completed this development. With this set of technologies MALDI MS and MS/MS could be performed on the same instrument and sample without splitting. In the proteomics field peptide mass fingerprinting and MS/MS could now be merged and, for the analysis of complex peptide mixtures, MALDI MS and MS/MS could be directly combined with LC separation. Figure 4 shows a standard LC-MALDI setup as used in our laboratory; peptide mixtures are desalted and concentrated on a short trap column, separated by capillary LC and after UV detection and matrix admixture, fractions are directly spotted on a MALDI target. In the standard workflow (Figure 5) MS spectra are first acquired from all spots and subsequently precursor ions for MS/MS are selected. After MS/MS data acquisition, database seach is performed with the complete set of MS/MS spectra.

3.2. General Aspects

3.2.1. Adaptation of LC-MALDI MS workflow to sample complexity

To achieve the best performance in protein identification or quantitation, the extent of protein or peptide separation should match the capabilities of the technique applied in the identification or quantitation step. With LC-MALDI MS and MS/MS as analysis technique, the number of good-quality MS/MS spectra, which can be acquired from one sample spot (LC-fraction) represents one limitation; the number of components in one fraction should therefore not exceed this maximum. By increasing matrix concentration more laser shots could be acquired from one spot, but analyte concentration in the crystals and detection sensitivity would

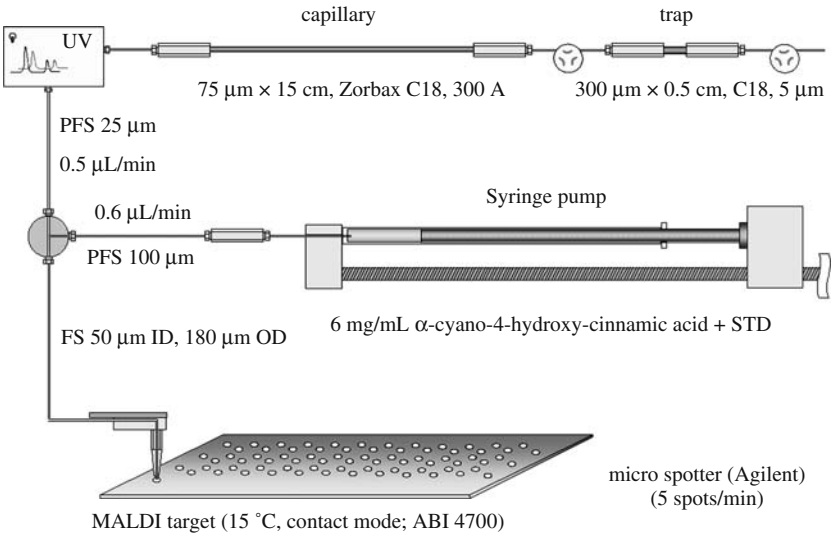

Figure 4. Standard LC-MALDI setup with sample concentration/desalting step, nanocolumn LC separation and off-line deposition of LC-eluent after admixture of matrix solution.

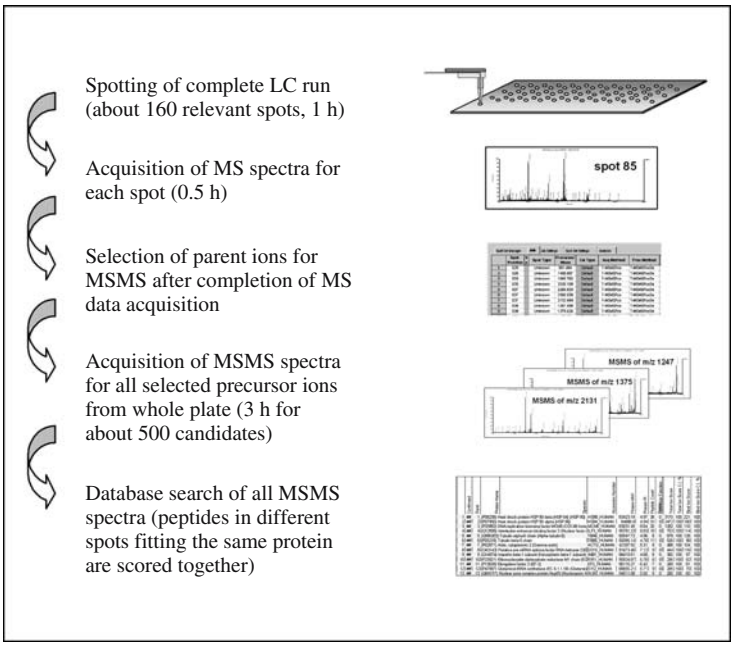

Figure 5. Typical LC MALDI-MS and -MS/MS workflow.

thus be reduced. The same dilution effect reduces detection sensitivity when analytes are distributed over too many fractions. In our setup with a LC-gradient of 50 min, a chromatographic peak is dispersed over three to four spots, tailing effects are small and with a matrix concentration of 3 mg/mL MS/MS sensitivity is good (Figure 6). The other limitation for MS and MS/MS detection is the suppression of components in peptide mixtures; this effect can be caused for example by preferential ionization of components in MALDI or by differences in the crystallisation step (e.g. Rejtar et al. 2002). Suppression cannot be quantitated in general (independent of individual peptide properties) but in a peptide mixture with more than 20 components present in different concentrations, suppression has to be anticipated. Therefore a smaller average number of mixture components would be of advantage. Depending on sample concentration and instrument performance, either the suppression effect or the maximum number of good-quality MS/MS spectra which can be acquired from one fraction is the limiting factor. On this basis the extent of separation should be adjusted to analyte complexity.

For the analysis of a complex sample (yeast whole cell lysate), three different separation schemes combined with LC-MALDI have been evaluated for identical sample amounts (Hattan et al 2005; cf. Figure 7); (i) fractionation at the protein level by RP-HPLC (12 fractions) followed by tryptic digestion and cation exchange (4 elution steps; 48 LC-MALDI runs in total); (ii) separation at the peptide level by strong cation exchange (SCX, 15 fractions, gradient elution; 10 used) or (iii) strong anion exchange (SAX, 14 fractions, gradient elution; 12 used). In the second dimension, peptides were separated by a dual solvent gradient within 40 min and spots were deposited in 5 s intervals. About 12,000 peptides were identified by approach (i) using the most extensive separation, 4000 by SCX (ii) and 9100 by SAX (iii). The number of unique peptides was 4871 for (i) 3350 for (ii) and 5700 for (iii) indicating a high redundancy for the extensive separation (4871 of 12,000), a low value for SCX (3350 of 4000) and a medium value for SAX (5700 of 9100). The most homogenous peptide distribution over the fractions was achieved by SAX in contrast to SCX, were peptides were concentrated in a few fractions. While distribution over too many fractions in the extensive separation (mostly in the protein chromatography step) reduced detection efficiency, in this example SAX provided the most homogenous flow of peptides. 1215 proteins were identified by LC-MALDI analysis of the 12 SAX fractions (iii) which is about 80% of the number of proteins identified in a similar preparation by ESI-LCMS/MS analysis of 80 SCX fractions (Peng et al. 2003).

Compared to yeast with about 5800 ORFs which code for proteins, samples from higher organisms can be much more complex and the separation scheme has to be adjusted accordingly. For very simple species as the other extreme, already subcellular fractionation can provide the appropriate pre-separation. For example only one LC-MALDI MS/MS run of a peptide mixture derived from the separated E-coli 50S ribosomal subunit allowed to identify 30 of the 33 expected proteins (Mirgorodskaya et al. 2005a).

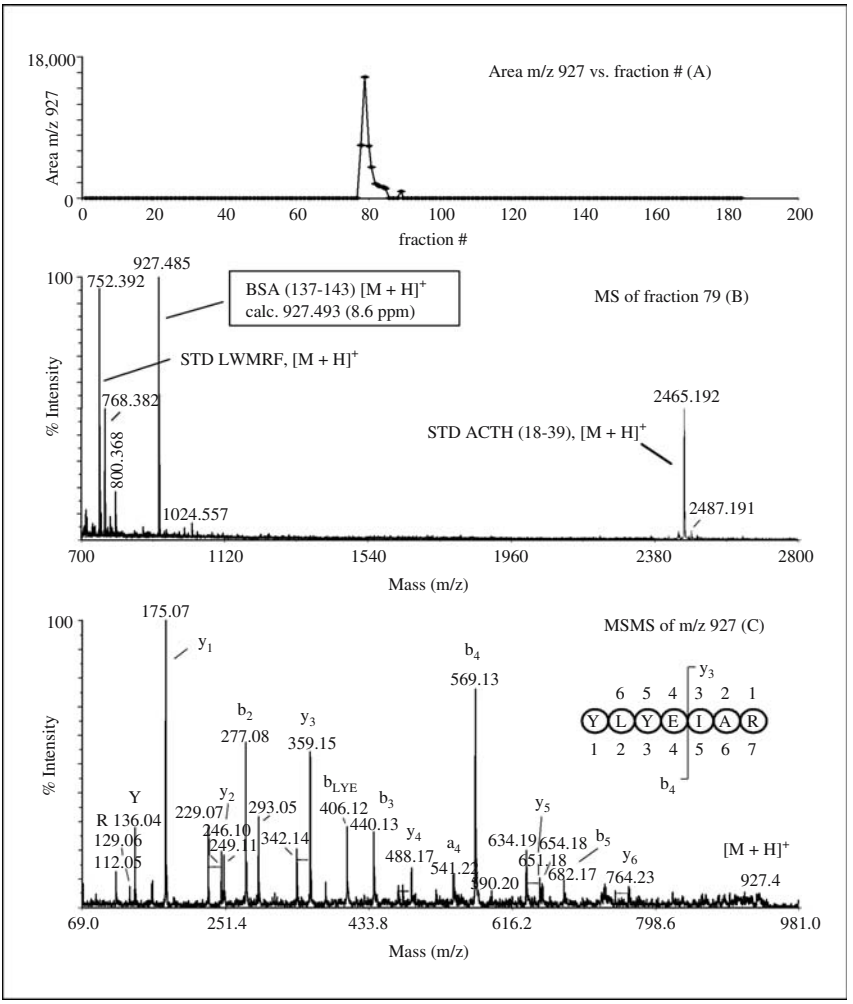

Figure 6. Chromatographic resolution, MS- and MS/MS sensitivity achieved with the LC-MALDI setup described in Fig. 4. Trace of m/z 927 ($[M + H]^+$ of peptide 137–143) from 10 fmol of a tryptic BSA hydrolysate using 12 sec wide fractions and a matrix concentration of 3 mg/mL (A). MALDI MS (B) and MS/MS spectrum (C) of the fraction with the highest intensity of m/z 927 (fraction #79).

3.2.2. Multiplexing

To cope with the high number of RP-HPLC runs required to analyze fractions in the two-dimensional LC-MS approach, multiplexed LC-separation and spotting for LC-MALDI analyses was applied very early. In quantitative analysis of differential protein expression by ICAT technology, total analysis time could be significantly reduced with four parallel separation lines (Lee et al. 2002). Prerequisite is of course

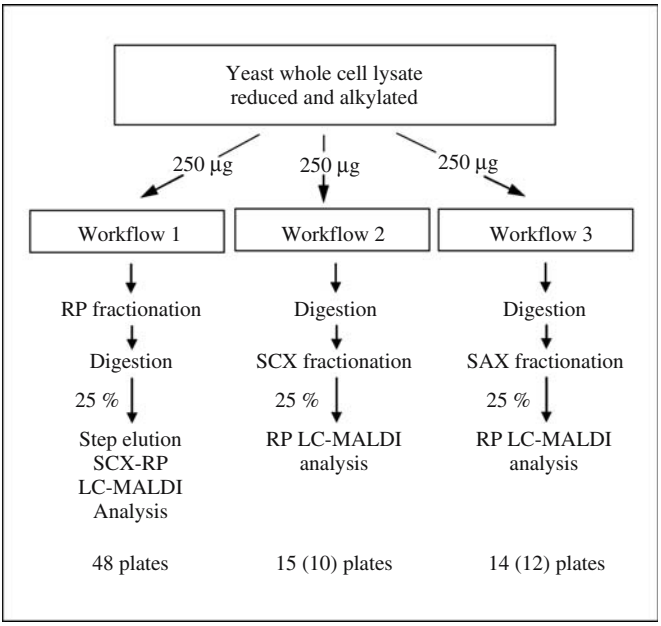

Figure 7. Comparison of different prefractionation steps in typical LC-MALDI workflows for complex mixture analysis. Adapted from Hattan et al. (2005). Copyright ©2005 American Chemical Society.

that MS and MS/MS capacity matches the output from multiplexed LC and MALDI spotting. Especially for the combination with fast LC-chromatography on monolithic columns, the implementation of faster lasers and the development of faster data systems would be of high value. Multiplexing is also advantageous when parallel LC-MALDI MS and MS/MS analyses are performed on complex (split) samples (Chen et al. 2005a). In this case peptides reliably identified by MS and MS/MS in the first LC-MALDI run were excluded from MS/MS experiments on corresponding spots of the next LC-trace (spotted at the same time). With this strategy the number of peptide identifications from an *E. coli* total cell lysate improved from 1042 to 1418 already by using two steps (1 exclusion list); by four steps 1735 unique peptides were identified in total. Proper alignment of the four LC-separations was performed by a linear correction applied to 20 chromatographic segments.

3.2.3. Quantitation and LC-MALDI MS

Relative protein quantitation is the basis of all types of differential proteome analyses. In the 2D-gel approach protein staining with either visible or fluorescent dyes provides a reliable and sensitive method to detect changes in protein expression or isoform abundance. In the multidimensional LC approach quantitation relies mostly on stable isotope labeling and ratios between light and heavy isotopomers are determined by MS or MS/MS at the peptide level. Labeling can be performed on the protein level by

incorporation of labeled amino acids in cell cultures (Ong et al. 2002), by feeding with isotope-labeled nutrients (e.g. ^{15}N labeled yeast; Krijgsveld et al. 2003) or by chemical derivatization with suitable reagents (e.g. Schmidt et al. 2005). At the peptide level a variety of derivatization methods are available which allow to introduce ^{2}H, ^{13}C, ^{18}O, ^{15}N or combinations thereof. Since the methodology was recently reviewed (Moritz and Meyer 2003), only selected examples important in the context with LC-MALDI will be presented.

3.2.3.1. Quantitation at the Protein Level In differential protein labeling with isotope-coded affinity tags (ICAT) a biotin-containing iodoacetyl derivative is used for cystein alkylation either as d_0- or d_8-isotopomer. Mixture complexity is significantly reduced by avidin affinity enrichment of cysteine-containing peptides which are then selected for relative quantitation (Gygi et al. 1999; Gygi et al. 2002). In the differential analysis of yeast grown on different media (Lee et al. 2002), separation was performed by 2D LC and a multiplexed version of LC-MALDI (cf. Section 3.2.2) was evaluated. Multiplexing reduced total analysis time but in addition, MS analysis after completed LC separation allowed to select only peptide ions displaying changes in expression level for MS/MS (expression-dependent MS/MS). A second generation of reagents with acid-cleavable biotin-moiety and ^{13}C-incorporation for quantitation provided improved MS/MS fragmentation behavior and identical chromatographic retention of the isotopomers. 2D-LC-MALDI and -ESI were assessed in a study evaluating this improved ICAT methodology (cICAT) for differential analysis of proteins attached to the prion protein (pull downs) and of low μg amounts of tracheal epithelial gland secretions (Hansen et al. 2003). In this study standard deviations below 10% were observed for quantitation of protein-specific cICAT-labeled peptides resulting in the identification of a number of proteins upregulated in cystic fibrosis (cf. also Section 3.2.5.). Compared to ICAT, labeling of lysines with differently isotope-labeled nicotinic acid derivatives increased the number of peptides which can be used for protein quantitation (Schmidt et al. 2005). This method which was originally developed for combination with the 2D gel approach is also of value for LC-MALDI MS and MS/MS, especially with expression-dependent precursor ion selection (Tebbe et al. 2005).

3.2.3.2. Quantitation at the Peptide Level At the peptide level, methylesterification (d_0/d_3; e.g. Goodlett et al. 2001) or acetylation (d_0/d_3; e.g. Hunt et al. 1980) are simple means for the introduction of different isotope labels and also trypsin-catalyzed ^{18}O incorporation has a long tradition (Rose et al. 1983). Another alternative is reductive methylation with CH_2O or CD_2O (Melanson et al. 2006). In MS/MS the N,N-dimethylated peptides show a strong transition from $[M+H]^+$ and $[M(d_4)+H]^+$ to the fragment a_1 which can be selected for quantitation by multiple reaction monitoring MS/MS (MRM). For a combination of MRM with LC-MALDI on a prototype triple quadrupole instrument with a MALDI ion source, quantitation of testpeptides was reported at the attomole level. The same labeling method (Ji et al. 2005) was previously applied for quantitation and identification of differentially expressed proteins

from E-Cadherin deficient SCC9 cells and SCC9 transfectants expressing E-cadherin. With a combination of SCX fractionation and microbore RP-HPLC-MALDI, 49 proteins were identified by MS/MS analysis of only those 320 peptide ion pairs exhibiting a more than 2-fold change. In this expression-dependent MS/MS experiment the other 5160 peptide ion pairs detected could be excluded from MS/MS analysis. Other derivatizing agents which improve detection sensitivity are reagents that convert lysines into their 4,5-dihydro-1H-imidazol-2-yl derivatives. They not only improved MS/MS-based identification but also allowed the introduction of four ^{2}H atoms for quantitation. (Peters et al. 2001). In all these methods quantitation is based on the ratio of different isotopomers detected in MS mode or in MRM. In a new class of reagents (iTRAQ) this step is performed on the MS/MS level (Ross et al. 2004). Four different isotopomers of a N-methylpiperazine reporter group provide four different MS/MS signatures for quantitation. Their mass differences are counterbalanced by a CH_2CO-linker with corresponding opposite isotope labels incorporated in such a way that in MS mode the overall mass increment of all four reagents is the same. Since in MS mode signals are not split into isotopomers and in MS/MS mode only those of the reporter ions, sensitivity is improved in both modes and quantitation is simplified. Multiplexed analyses can be performed by applying all four labels for example in time-course experiments. Quantitation of protein standards and protein-spiked depleted plasma by 2D LC-MALDI combined with cleavable ICAT (cICAT) or iTRAQ labeling as well as DIGE technology were recently assessed and all three techniques were subsequently applied to the differential analysis of HCT-116 cell lysates (Wu et al. 2006). While all methods provided similar accuracy for standards (90–110%; standard deviations at least $<15\%$) and spiked plasma (80–120% ; standard deviations $<30\%$), the best sensitivity was achieved with iTRAQ followed by cICAT and DIGE. The reduced cICAT sensitivity was attributed to losses during avidin affinity purification and the fact that proteins with few cysteines are less likely be detected. However, ICAT is a technique designed to reduce sample complexity and it has its inherent drawbacks like any other method. For the MS/MS-based iTRAQ quantitation for example, the purity of precursor ions is of high importance.

3.2.4. Derivatization and LC-MALDI MS

For non-quantitative LC-MALDI applications, derivatization chemistry is not restricted to compounds which allow a convenient incorporation of heavy isotopes. For example for improved MS/MS detection sensitivity, tryptic peptides were labeled with sulfonated coumarin-tags at the N-termini after guanidylation of lysines (Pashkova et al. 2005). Despite reduced MS sensitivity for arginine-terminated peptides (in alpha cyano-4-hydroxycinnamic acid matrix), formation of y-ions was enhanced in MS/MS by the second mobile proton provided from the sulfonic acid group. For a SCX fraction from a *E.coli* hydrolysate 50% more peptides and 30% more proteins could be identified by multiplexed LC-MALDI MS and MS/MS after derivatization.

3.2.5. Complementarity of LC-MALDI and LC-ESI MS techniques

Since the ionization in MALDI and in ESI is based on different mechanisms, peptides and proteins are protonated in a method-specific form with different efficiencies. While in ESI multiply charged ions are the preferentially observed species for peptides, MALDI leads predominantly to singly protonated molecules. Their fragmentation behavior in MS/MS is different to that of multiply charged ions and, depending on peptide sequence, one or the other method can provide more information for protein identification. Similarly, response factors in MALDI and in electrospray have different method-specific contributions, for example matrix incorporation of analyte or surface properties in solution. All these factors together lead to a different peptide-specific response in LC-MALDI MS/MS and LC-ESI MS/MS. Parallel application of both techniques has therefore a considerable potential for increasing proteome coverage and the complementarity of both methods was evaluated.

A direct comparison of both techniques was performed on the same instrument with different ion sources for the analysis of proteins derived from the 39S and 28S subunit of mitochondrial ribosomes (Bodnar et al. 2003). 21% of the proteins were identified by LC-MALDI only, 16% by LC-ESI only and 63% by both methods. In a comparison of LC-MALDI MS/MS analysis on a TOFTOF instrument with LC-ESI MS/MS on a Q-TOF, values for method-unique identifications ranged from 20 to 50% (Hansen et al. 2003). Similar proportions were obtained in membrane protein analyses on a LC-ESI ion trap instrument compared to LC-MALDI on a MALDI-Q-TOF (Zhang et al. 2004b). LC-ESI-only identified 27% , LC-MALDI-only 38% and both methods together 35% of all proteins. While in ESI more shorter peptides were observed, MALDI MS/MS preferentially allowed to identify higher mass peptides (Hansen et al. 2003; Zhang et al. 2004b). Although different instrument types and samples of different complexity were applied in the various comparisons, all studies revealed a substantial complementarity of both methods at least for protein identification. A parallel application of LC-MALDI MS/MS and LC-ESI MS/MS is therefore essential to achieve optimal coverage in proteome analysis.

3.3. Selected Application Areas of LC-MALDI MS in Proteomics

3.3.1. Membrane protein analysis

Membrane proteins play a critical role in many biological processes especially in the transfer of information and material between different intra- and/or extracellular compartments (e.g. receptors, ion channels). Their analysis represents a special challenge because of their strong aggregation behavior and the poor solubility of hydrophobic sequence stretches. These characteristics are the reason for their underrepresentation in protein identification based on 2D PAGE, most likely due to solubility problems in the isoelectric focusing step. One promising alternative method for appropriate reduction of mixture complexity is the combination of 1D SDS PAGE with LC-MALDI MS and MS/MS, where prefractionation is achieved by dividing the gel into slices.

In the course of a proteomics study of Haemophilus influenzae (1740 open reading frames) we evaluated this approach for establishing a map of membrane and membrane-associated proteins. In the first step proteins from a carbonate-washed membrane fraction (Fujiki et al. 1982) were separated by SDS PAGE on a (4–20%) gradient gel, which was subsequently divided into 65 slices covering the complete separation range. After in-gel digestion the 65 peptide mixtures were analyzed with our standard LC-MALDI setup (Figure 4) using a 40 min. LC-gradient. From the about 190 spots deposited in each run on average 120 contained peptides. A maximum of 6 MS/MS spectra were acquired from each individual spot (average of 4000 shots). Confidence levels for peptide identification using Mascot/GPS Explorer were determined by manual examination of a substantial number of MS/MS spectra in the critical confidence range. Based on 22067 MS/MS spectra, 1763 confident protein hits were identified in all 65 runs with 47% of all MS/MS spectra contributing to these hits (Figure 8). On average, proteins were distributed over 3.7 bands. After removal of duplicates from adjacent bands 480 unique protein identifications could be established. From these, 426 proteins (88%) were identified by more than one peptide which indicates good reliability. In general the molecular masses of the identified proteins fitted well with those extrapolated from the standard calibrants on the gel (Figure 9). From the 480 identified proteins 189 are annotated as membrane proteins in the SwissProt database, representing 42% of the 443 membrane proteins in H. flu. (SwissProt). From the 376 proteins with predicted alpha-helical transmembrane domains (SwissProt) 40% (149) could be identified. The distribution of the number of membrane domains of these 149 identified proteins matched that of the predicted 376 proteins with TM-domains (Figure 10). Only proteins with

Summary of 1D SDS-PAGE/LC-MALDI analysis

65	LC-MALDI MS- and MSMS runs
22067	MSMS spectra (mean 340/run)
10268	MSMS spectra contribute to confident hits (47 %)
1763	"confident" protein hits (BIS >= 37)
480	nonredundant proteins
3.7	"ID's" per protein indicates good separation by 1D SDS PAGE
189	"membrane proteins"
42 %	of 443 proteins with SwissProt annotation "membrane protein"
149	with predicted alpha-helical TM domains
40 %	of 376 proteins with predicted alpha-helical TM domains

Figure 8. Summary of 1D SDS-PAGE/LC-MALDI analysis of a membrane preparation derived from Haemophilus Influenzae.

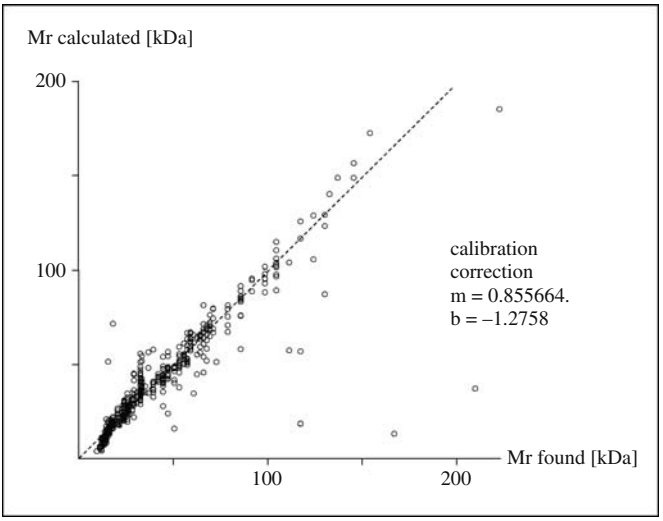

Figure 9. Comparison of calculated and observed molecular masses of all proteins identified by LC-MALDI in a membrane preparation derived from Haemophilus Influenzae.

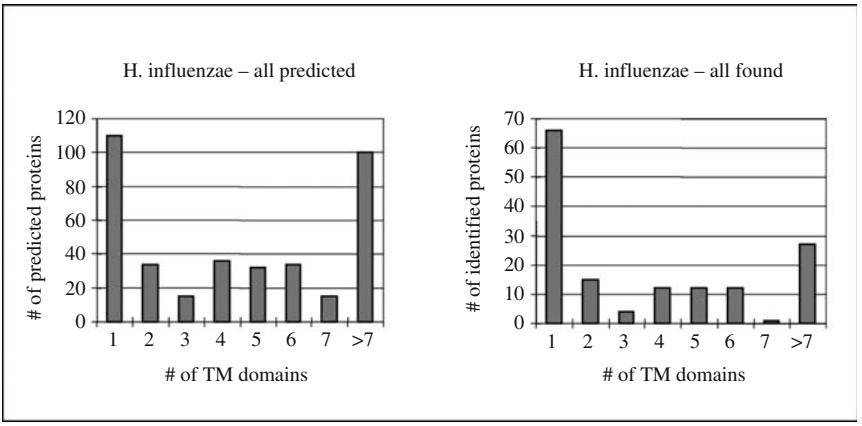

Figure 10. Comparison of the transmembrane domain distribution of all proteins identified by LC-MALDI in a membrane preparation derived from Haemophilus Influenzae with that of all H. flu. proteins predicted to contain alpha-helical transmembrane domains.

more than seven transmembrane domains were still underrepresented. The study presents currently one of the most comprehensive maps of membrane proteins of Haemophilus Influenzae and it serves as a basis for further quantitative differential analysis.

1D PAGE combined with LC-MALDI was also applied for membrane protein analysis of staphylococcus aureus (strain N315, 2596 ORFs) in a study correlating transcriptomic and proteomic expression profiles (Scherl et al. 2005). Total protein extracts were analyzed by 2D LC-MALDI (SXC as first step) and the standard 2D PAGE approach; with these methods 245 and 211 proteins were identified, respectively. After phase partitioning, the two different membrane fractions were first separated by 1D SDS-PAGE, gels were divided into 30 slices, and after in-gel digestion hydrolysates of each slice were subjected to LC-MALDI MS and MS/MS ($75\mu \times 150$ mm columns). 291 proteins were identified in the soluble part and 269 in the pellet. 24% of the expected proteins with one transmembrane (TM) domain were detected and 5% or less of the classes containing 2–5, 6–10 and >10 TM-domains. With 2D gels only 1% of the expected membrane proteins could be identified.

As an alternative to the application of protein separation methods which are compatible with membrane protein characteristics, direct in-solution digestion of SDS-containing extracts was applied in the analysis of lipid rafts of THP-1 monocytes (Li et al. 2004). A SDS-guard column was used to reduce the SDS concentration and with LC-MALDI, which is less sensitive to trace amounts of detergents, 71 proteins were identified. By adding cation exchange chromatography as pre-separation step, the number of proteins identified with high confidence could be increased to 126. For the analysis of membrane proteins of human HT 29 cells, in-solution digestion and direct LC-MALDI was even superior to 2D LC-ESI MS and MS/MS; 50 membrane or membrane-associated proteins were identified compared to 24 (Zhang et al. 2004a). A possibility to avoid the detergents used in solubilization is to apply microwave-assisted TFA hydrolysis in suspension (25% TFA) instead of enzymatic digestion (Zhong et al. 2005). When this approach was combined with LC-MALDI MS/MS analysis, 119 proteins (including 41 membrane proteins) with gravy indices from -1.135 to 0.992 were identified in a membrane preparation derived from human MCF7 cells. Most enrichment techniques for membrane proteins rely on several stages of differential centrifugation and washing. As an elegant alternative for mapping of membrane proteins cell surface proteins were derivatized with different biotin-containing sulfo-succinimidyl derivatives and captured on streptavidin beads (Scheurer et al. 2005). Best overall performance was achieved with a SS-containing biotin-linker; after reductive cleavage from beads, in-solution digestion of the released derivatized proteins and LC-MALDI MS/MS analyses, 549 proteins could be identified. 28% of these 549 were either membrane or extracellular matrix proteins.

3.3.2. Protein interaction analysis (pull downs)

For the understanding of protein function in a complex cellular environment the analysis of interaction partners is of high importance. In many essential protein complexes these partners are proteins, mRNA or DNA. Another class of interacting entities is represented by small molecules. They are very often involved in regulatory events or in cell signalling (e.g. via receptors) and many pharmacologically active compounds

belong to this class. Knowledge about their interacting partners is essential to understand their mode of action. For interaction analysis, the protein (or small molecule) of interest is normally attached (as bait) with a linking element to a carrier (e.g. beads) and the potential complex partners are selected from a suitable protein mixture, for example from a cell lysate. Low affinity binders are removed by washing steps and only the remaining complex partners are analyzed. They can form relative complex mixtures and in general, at least one separation step (1D PAGE or LC) is applied before MS and MS/MS analysis. To assess the potential of LC-MALDI in this application area, ampicillin was attached as bait to a carrier and treated with solubilized membranes of *E. coli* with the aim to detect penicillin receptors (Zhen et al. 2004). All known Penicillin-binding proteins were identified despite the fact that they were expressed in the range between 5–10 copies/cell up to thousands of copies/cell. This wide dynamic range could only be covered by incorporating LC-separation. For more complex pulldowns (e.g. double-tagged GRB2) 2D LC separation (SCX, RP-HPLC) was necessary to reduce mixture complexity. LC-MALDI analysis required significantly fewer SCX-fractions compared to LC-ESI mainly because of the much longer analysis time available for MS/MS experiments (Zhen et al. 2004). Differentiation of true and unspecific binders in affinity pulldowns was achieved by differential ^{18}O-labeling of tryptic peptides and quantitative analysis by LC-MALDI (Figure 11) (Mirgorodskaya et al. 2005b). Proteins attached to beads with GST tag but without bait were trypsinized with $H_2^{16}O$, beads with GST tag and bait (but without protein mixture) with $H_2^{18}O$ and both mixed in a 1:2 ratio. The pulldown was trypsinized with $H_2^{16}O$ and added to give a 3:1:2 ratio of all three samples. In MS analyses tryptic peptides from true interactors with the bait appear as singlets, unspecific binders as doublets with changed ^{16}O:^{18}O ratio and peptides observed only in controls present the original 1:2 ratio. Nontryptic peptides can be sorted out by reversed labeling (Wang et al. 2001).

3.3.3. Other applications areas

With the high performance of LC-MALDI, application areas come now into reach which were formerly a domain of on-line ESI-MS/MS. One such area is the analysis of major histocompatibility complex (MHC) Class I-associated peptides. After prefractionation on RP-HPLC, MHC-associated peptides eluated from human leukocyte antigene complexes were recently identified by LC-MALDI (Hofmann et al. 2005). The prefractionation step allowed to reduce (adjust) sample complexity and, with MALDI MS screening, MHC-peptide-containing fractions were specifically selected. By a combination of automated LC-MALDI MS and MS/MS with standard database search and de novo sequencing, 30 novel HLA-presented peptide ligands were identified including species presented at very low levels. Another application with high sensitivity requirements is the analysis of tear-derived proteins (Li et al. 2005). In a study of such proteins from small amounts of clinical samples (5 μL), LC-MALDI analysis was applied to three ZipTip derived peptide fractions and 44 proteins could be identified. Especially ease of detection and in-depth analysis of phosphorylated

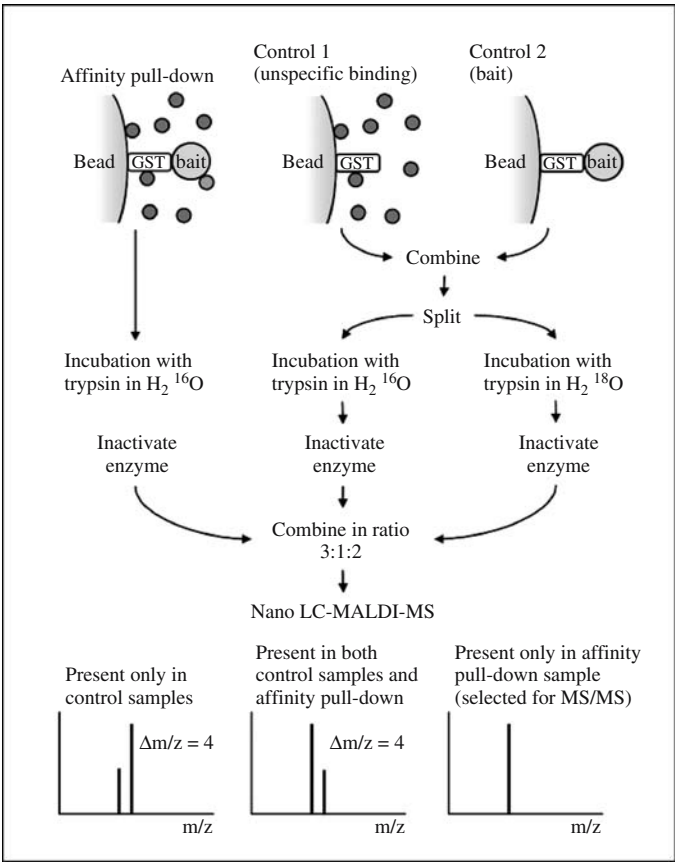

Figure 11. Strategy for Affinity-Pulldown. Differentiation of true binding proteins and unspecific binding background by ^{18}O-labeling (for details cf. text). Reprinted with permission from Mirgorodskaya et al. (2005b). Copyright ©2005 American Chemical Society.

and glycosylated peptides was reported as advantage in the LC-MALDI setup. Phosphopeptide detection in LC-MALDI and MALDI in general could be significantly improved by using 2,5-DHB matrix containing 1% phosphoric acid (Kjellström and Jensen 2004).

4. SUMMARY

In the last years off-line LC-MALDI has developed into an independent and sensitive technique which is now applied in all areas of proteome analysis. By decoupling separation, MS and MS/MS analysis, each step can be performed at its individual optimum. For example, different speed of chromatographic separation can be adapted by different spotting frequencies and spectral quality in MS and MS/MS mode can be

optimized without time constraints by setting signal to noise criteria. Since MS/MS is performed only after MS data acquisition for all fractions is completed, precursor ions can be selected from spots where they show maximum intensity. The number of precursor ions is only limited by sample depletion or overall analysis time, which can probably be reduced by higher frequency lasers and faster data acquisition in the future. All parts of the spots which remain after one round of analysis are still available for further experiments, which is a major advantage for the characterization of post-translational modifications. In all respects, LC-MALDI is a very flexible technique which can be adapted to a wide range of different workflows.

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