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Review

# Differential polypeptide display: the search for the elusive target

Stefan Wittke [a], Thorsten Kaiser [a], Harald Mischak [a,b,*]

[a] *Mosaiques Diagnostics and Therapeutics AG, Hannover, Germany*
[b] *Department of Nephrology, Medical School of Hannover, Germany*

**Abstract**

Proteomics, as a tool to identify proteins in biological samples, is gaining rapidly importance in the postgenomic era. Here we discuss the current and potential role of different techniques in the field of proteomics such as two-dimensional gel electrophoresis off-line coupled to MALDI-MS (2D-PAGE-MALDI-MS), high performance liquid chromatography mass spectrometry (HPLC-MS), surface enhanced laser desorption/ionization mass spectrometry (SELDI-MS) and a newly developed technique, capillary electrophoresis mass spectrometry (CE-MS). The developments of the last years are presented discussed.


*Keywords:* Reviews; Proteomics; Data processing; Polypeptides

**Contents**



## 1. Introduction

In the post-genomic era emphasis of research shifts from accumulating sequence data towards the identification of the functional significance of the gene products, the proteins. Several approaches have emerged to identify proteins, their regulation, post-translational modifications and interactions. Proteomics is the new field of large-scale analysis of proteins and their function.

Proteomics can be separated into three major categories [1]:

(i) *protein micro-characterization*: large-scale identification of proteins and their post-translational modifications;
(ii) *differential display proteomics*: comparison of the levels of protein expression under various circumstances; potentially applicable to diseases (e.g. nephropathies, cancer, etc.);
(iii) studies of protein–protein interactions.

It is evident that proteomics gains increasing importance as a new tool for the identification of therapeutic targets [2,3]. One of the major challenges is to gain insight into the function of polypeptides found in body fluids like urine or blood to obtain information on the state of health of an individual [4]. Polypeptides regulate a vast number of physiological functions of complex organisms, like men. Hence, a deeper knowledge of their presence or absence in certain

* Corresponding author. Tel.: +49-511-55474413; fax: +49-511-55474431.
*E-mail address:* mischak@mosaiques.de (H. Mischak).

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(patho)physiological conditions would consequently lead towards a thorough understanding of their function and, in turn, would enable a better diagnosis based on molecular mechanisms as well as the identification of therapeutic targets. The need for methods to identify disease markers is evident e.g. from the survival-rate of patients diagnosed at earlier disease stages of cancer.

In this paper, we review currently available technologies, such as two-dimensional (2D) electrophoresis (2D-PAGE), surface enhanced laser desorption/ionization– (SELDI–), high-performance liquid chromatography– (HPLC)– and capillary electrophoresis–mass spectrometry (CE–MS) aimed towards the identification of differentially expressed proteins. In the last section, most recent developments will be presented, including bioinformatic approaches to comprehend the large amounts of data resulting from these analyses. However, due to the explosive growth of the field this review cannot be entirely comprehensive.

## 2. Analytical methods for proteomics

### 2.1. 2D-electrophoresis

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) has been the main method available to routinely separate thousands of proteins, and thus is the dominant technique in the field of proteomics [5–13]. The proteins are separated in two steps:

(i) isoelectric focusing (IEF), separates proteins due to their isoelectric point (pI);
(ii) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), separates the proteins according to their size.

As evident, there are certain limitations when utilizing this technology [14–16]. First, it is not possible to detect all the proteins present in a sample within a single 2D-PAGE. Protein spots (each ideally represents one distinct protein) are visualized by different staining methods. Silver staining requires the presence of approximately 0.1 pmol protein [17]. Immunoblotting—a combination of high affinity antibodies and enhanced chemiluminescence (ECL)—allows the detection of proteins with concentrations above 2 fmol. Thus, the proportion of a protein—extracted from a biological tissue—which can be detected by 2D-PAGE depends on the protein quantity loaded onto the gel, and the method of detection. Aiming towards the identification of proteins differentially expressed in patients with Alzheimer disease, Ueno et al. [18] used silver staining and immunodetection by antibodies. Out of the five plasma samples of Alzheimer patients, three contained apolipoprotein E4 and another showed apolipoprotein L and complement factor H. Apolipoproteins and their receptors are the main controllers of lipid metabolism and, therefore, have a major impact on the development and degeneration of the central nervous system [19].

A major advance in proteomics was the implementation of mass spectrometric analysis as a method of protein identification [15,16,20–23], resulting on the development of so-called soft-ionization sources as electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI). Further details about the capabilities and mechanisms of ESI–MS [24–27] and MALDI–MS [27–29] can be found in several reviews.

This evolution enabled not only the separation and detection of thousands of proteins in a single 2D-PAGE, but also their identification [14,20]. This revolutionary advance led to the step-by-step identification of hundreds of proteins in a single gel via sequential analysis of the peptide–protein mixtures generated by digestion of individual gel spots, and to the display of a polypeptide pattern [5,6,14].

For the identification of proteins in complex mixtures, several search algorithms can be used, e.g. the peptide fingerprint database search and the amino acid sequence database search [30]. In the first case, the masses of peptides obtained from proteolytic digests are compared to predicted masses from theoretical digests of proteins in a database. Several databases and search algorithms are currently available, e.g. PepSea, PeptIdent, MS-Fit, MOWSE and ProFound [25,30–32]. Disadvantages of this approach are peptide mass redundancy (GIVLY has the same mass as VGYIL), mass accuracy (post-translational modifications of proteins) and, especially, the complexity of protein mixtures which increases the complexity of the peptide mass fingerprint [30]. A more advanced way of protein identification is the amino acid sequence database search. A partial amino acid sequence of the peptide is obtained from $(MS)_n$-spectrometry and searches against databases with programs, such as Mascot [31], SONAR [30] and SEQUEST [30,32], are used to identify the parental protein.

2D-PAGE–MALDI–MS is commonly used for global proteome analysis, although several limitations have to be accepted [14,16,23,33–36]:

- certain classes of proteins are known to be absent or under-represented in a 2D-gel pattern (e.g. very acidic or basic proteins; very large and small proteins and membrane proteins). In general, only proteins of molecular weight between 10 and 100 kDa with isoelectric points (pI) between 4 and 10 migrate well in 2D-gels. Usually, around 20% of the loaded proteins are actually visualized;
- especially in body fluids, such as urine or blood, a large proportion of polypeptides with molecular weight less than 10 kDa cannot be analyzed using 2D-PAGE;
- low abundant proteins are generally not detectable without preconcentration;
- true relevant differences in protein patterns between differentially treated proteomes can be validated only with great difficulties since 2D-gels show high variability;
- finally, the creation of a total protein pattern by 2D electrophoresis with MALDI–MS is a time consuming

process because each gel spot has to be digested and subsequently analyzed by mass spectrometry.

To overcome the most severe problem, the evident lack of several important polypeptides in a typical 2D-PAGE, the simple approach of increasing the amount of sample is not beneficial. This results in overloading of the gel and a severe loss of resolution.

Several papers describe advances in staining procedures as well as preconcentration or enrichment of low abundance proteins, which are not detectable without preconcentration. Butt et al. [37] reported the preconcentration of proteins from *Escherichia coli* by non-denaturating anion exchange chromatography. The successive fractions were then analyzed by 2D-PAGE and the selected gel-spots identified and quantified by MALDI–TOF-MS. This resulted in an up to 13-fold increase in sensitivity. Marshall and Williams [38] describe a method for concentrating urine from low to intermediate protein content (0.02–0.5 g/l) utilizing dye precipitation. The authors emphasize that the positional coordinates of the polypeptides were unaffected by the treatment of the sample with dye. Lopez et al. [39] and Steinberg et al. [40] describe sensitive fluorescence staining methods with a linear signal response over a wide dynamic range [41]. Tonge et al. [42] introduced a two-color fluorescent labeling system which allows the simultaneous electrophoresis of two differentially labeled protein samples in the same 2D-gel. This technique was evaluated by Gharbi et al. [43] using breast cancer cells as a model system.

To ease data evaluation, Egelhofer et al. [44,45] developed a sequence database search program to introduce a new strategy in protein identification by MALDI–TOF-MS peptide mapping. The strategy does not rely on an excellent mass accuracy as an attribute for distinction of false or positive results. The authors emphasize that a search engine is developed that renders internal spectrum calibration unnecessary and adapts to the raw data without interference by the user. The performance of this strategy is demonstrated by the identification of a set of human cDNA expression products—20 proteins were identified—without applying any molecular weight restrictions. Marvin et al. [46] evaluated the potential of ESI–MS by the identification of two unknown proteins from cellular cultures of ‘mammary epithelia’ separated with one-dimensional (1D)-PAGE. Whereas, the mass mapping failed with MALDI–MS, the identification was achieved by LC–ESI–Q-TOF-MS/MS.

2D-PAGE maps have been used as a tedious, but, within limits, reliable method for comparison of polypeptide patterns from different disease states. Seow et al. [47] separated the proteins of a human heptacellular carcinomia cell line—heptacellular carcinomia is one of the most common cancers in Africa, Southeast Asia and China—with 2D-PAGE (silver staining) and after digestion with trypsin, peptide fragments were identified with MALDI–TOF-MS. Approximately 400 gel-spots were analyzed and proteins, such as alcohol dehydrogenase, α-enolase, asparagine synthetase, isocitrate dehydrogenase, and glucose-6-phosphate 1-dehydrogenase were identified. In addition, proteins with expression patterns that have been postulated to be related to the process of carcinogenesis were identified. These include 14-3-3 protein, annexin, prohibitin, and thioredoxin peroxidase. Smolka et al. [48] described a strategy to separate proteins labeled with isotope-coded affinity tag reagents by 2D-electrophoresis and the identification and quantification by MALDI–MS. Changes in the proteome of the yeast, *Saccharomyces cerevisiae*, were investigated and the authors accentuated that this method quantifies accurately changes in protein abundances even if proteins co-migrate. Chen et al. [49] isolated human brain proteins and analyzed 33 common spots by ESI–MS, MALDI–TOF-MS and Edman sequencing. These identified proteins were compared to protein databases and included enzymes and regulatory proteins. Along the same line, Tsuji et al. [50] used nano-ESI–TOF-MS for a quantitative proteome analysis of Alzheimer’s disease brains. More than 100 protein-spots were detected and 35 were identified with tandem mass spectrometry using a high-resolution quadrupole time-of-flight (Q-TOF)-MS. Vejda et al. [51] identified protein alterations in plasma of prostate-, lung-, and breast-cancer patients with 2D-PAGE–MALDI–MS. The authors demonstrate that characteristic alterations of plasma protein composition occur in patients with neoplastic disease.

Bienvenut et al. [52] and Muller et al. [53] describe a novel approach towards protein identification in a highly automated manner utilizing 2D-PAGE as a molecular scanner. The separated proteins are digested and simultaneously transferred onto a polyvinylidene-difluoride membrane. Next, the membrane is sprayed with matrix solution and inserted into a MALDI–TOF-MS, which measures a comparable peptide fingerprint.

Taken everything into account, the coupling of 2D-PAGE and MALDI-TOF is a powerful tool in proteomics, but several limitations of 2D-PAGE and the time consuming method confirm the need for proteomic approaches that offer more information in a single, time-limited step. This statement is further supported by a recent study of Gygi et al. [54], who showed that only the most abundant proteins were detected by 2D-PAGE combined with MALDI–MS.

Along the same line, Anderson and Anderson [55] published a list of 289 proteins as a “steady-state” list of proteins, whose detection in plasma or serum is documented in the literature and emphasize that this list is restricted due to limitations of the used conventional proteomic method. Although 2D-MALDI–MS was a breakthrough for proteomic research, the limitations of the method provoke the investigation of additional approaches.

### 2.2. SELDI–MS

SELDI uses ProteinChip arrays. Proteins with a specific affinity to a certain matrix bind more or less selectively to the

ProteinChip depending on several parameters like hydrophobicity, Lewis acid–basis interaction, charge, etc. Matrices for the chips are either ion-exchange, reversed-phase, etc., or more specific: antibodies, receptors, enzymes or DNA. Subsequently, the unbound proteins are washed off the chip surface, matrix solution is applied onto the ProteinChip array and the sample is directly analyzed by MALDI–TOF-MS [56–59].

The SELDI technology is currently starting to be used for the discovery of biomarkers for diseases such as cancer or neurological disorders. One approach utilizing SELDI to identify ovarian cancer in human serum was recently presented by Petricoin et al. [60]. Diagnostic markers for prostate cancer in blood were investigated by Eggeling et al. [57], Srinivas et al. [61], Paweletz et al. [62,63], Adam et al. [64] and Wright et al. [65]. Differentially expressed proteins were found by comparing the protein patterns (generated by SELDI–TOF-MS) of tumor tissues with tissues of healthy volunteers. Further characterization of these proteins is still needed. Wu et al. [66] reported the identification and validation of metastasis-associated proteins in head and neck cancer cell lines (HNSCC) using SELDI ProteinChip technology. In the metastatic cell line UMSCC10B proteins were identified by database search and two membrane-associated proteins, annexin I and annexin II and glycolytic protein α-enolase were found to be upregulated, and a calumenin precursor was down-regulated. Rosty et al. [67] identified a biomarker for pancreatic ductal adenocarcinoma. Using a ProteinChip immunoassay, the differentially expressed protein hepatocarcinoma–intestine–pancreas/pancreatitis-associated protein (HIP/PAP-I) was identified. Hampel et al. [68] applied the SELDI technique to assess its applicability for protein profiling in urine. They detected proteins in the range of relative molecular masses ($M_r$) from 9.75 to 66.4 kDa. As a proof of principle, the peak at $M_r$ 11.75 was identified as $\beta_2$-microglobulin. The authors emphasize the capability of SELDI for the detection and characterization of trace amounts of proteins in urine. Dare et al. [69] used this technology to identify urinary parvalbumin-α a biomarker of compound-induced skeletal muscle toxicity in rats.

As evident, the ProteinChip technology allows the protein profiling from several complex biological samples such as urine, blood, cell lysates etc. Especially for the profiling of low molecular weight proteins the ProteinChip system appears suitable. Other advantages are the small sample volume and the sensitivity for the selected proteins. Furthermore, this technology is quite fast and simple to use, hence well suited for high throughput analysis [56,58]. However, limitations have to be taken into account. The most severe limitation might be the reproducible loss of the majority of proteins and peptides present in the sample. This in turn leads to the establishment of rather low-resolution patterns, which represent only a minority of proteins and peptides present. Therefore, the SELDI technology, similar to the 2D-PAGE–MS system, does not appear suitable to establish an all-embracing polypeptide pattern for the investigated biological samples. This pattern, however, would be essential for diagnostic purposes and for a complete differential display of (two) different proteomes.

The limitations of the currently used approaches to proteomics have promoted the development of new as well as the rediscovery of old chromatography-based methods for the separation and identification of proteins in complex mixtures. HPLC and/or CE in combination with MS were employed to analyse polypeptide patterns. In these cases, the sample is fractionated and subsequently on- or off-line ionisation sources of mass spectrometers have been used.

### 2.3. HPLC–MS

The separation of a complex protein mixture is certainly not an easy task because human body fluids, such as urine or blood, contain several thousands of proteins and peptides. Recent publications (reviewed in [14,16]) lead to the impression that the separation of such complex samples—containing several thousand different polypeptides—is not possible in a single liquid-chromatographic run, due to a lack of resolving power of 1D-HPLC and limitations in the dynamic range of mass spectrometers. In addition, larger polypeptides can frequently not be separated by HPLC and might also require higher resolution than currently available by the widely used quadrupol or ion-trap mass analyzers.

One approach to circumvent some of these problems is the tryptic digestion of the proteins prior to chromatographic separation. This results in the generation of peptides, which are easier separable and more soluble than the parent proteins, particularly, hydrophobic and membrane proteins [14].

With great success, one- or two-dimensional chromatographic approaches for proteomics such as cation exchange followed by on-line RP-HPLC–MS have been used to identify proteins in complex mixtures after tryptic digestion. Song et al. [70] used LC–ESI–MS for routine determination of salmon calcitonin in rat serum. Adkins et al. [32] performed a proteomic analysis with submilliliter quantities of serum and increased the measurable concentration range for blood-proteins. First, immunoglobulins were removed from serum with protein A/G and the remaining proteins were digested with trypsin. Four hundred and ninety proteins in serum were detected by on-line RP-microcapillary chromatography coupled to an ion-trap mass spectrometer. Some low-abundant serum proteins in the nanogram per milliliter-range were found, including human growth hormone and interleukins. Valianpour et al. [71] studied cardiolipin (CL), an indicator of the Barth syndrome (BTHS), with HPLC–ESI–MS and suggested that the proposed method allows identification of BTHS more rapidly than gene analysis or analysis of CL in cultured skin fibroblasts. Chong et al. [72] detected several hundred proteins in cell lysates of human breast cancer cell lines using on-line non-porous-RP-HPLC–ESI–TOF-MS. The non-porous separation uses C18-coated hard-sphere silica beads, with a

separation range from 5 to 90 kDa. Several hundred proteins were detected and the authors claim that 75–80 proteins were more highly expressed in cancer cell lines than in human breast cell lines. Wang and Dass [73] developed a method for the analysis of bioactive peptides in bovine adrenal medulla, utilizing a combination of fast-HPLC and ESI–MS, also using non-porous silica-based C18 columns. Several peptides, including the opioid peptides methionine–enkephalin (Met–Enk) and leucine–enkephalin (Leu–Enk), were identified with MS–MS. An elegant approach to identify urinary polypeptides was published by Spahr et al. [74]. The authors used HPLC coupled to MS–MS to analyze tryptic digests of pooled human urinary proteins and identified more than 100 polypeptides. While this technology results in the rapid identification of urinary proteins, it appears not well suited to obtain a representative pattern of the polypeptides originally present in a sample.

Lubmann et al. [75] and Kachmann et al. [76] used 2D-liqiud phase separation ESI–MS to map the protein content of ovarian surface epithelial cells and an ovarian carcinoma-derived cell line. As first dimension, isoelectric focussing (IEF) was used. The second dimension was non-porous-RP-HPLC with on-line coupling to an ESI–TOF-MS. The result is a 2D-map of pI versus relative molecular mass ($M_r$) in analogy to 2D-gels. Three pI-sections were studied, each contained more than 50 proteins, and about 40% of these proteins could be identified by database search. Olsen et al. [77] identified amino acids of human serum albumin involved in the reaction with the naproxen acyl coenzyme A thioester with HPLC–MS/MS. As estimated, the naproxen-CoA reacted preferentially with lysine 199, lysine 541 and lysine 351. Devreese et al. [78] established a nano-LC–MS–MS system (second dimension) which allows automated sequence analysis of tryptic digestion mixtures from single 2D-PAGE spots (first dimension). This system is applied in a differential display study to identify differentially expressed proteins in neuroendocrine cells of the frog, *Xenopus laevis*.

Using a combination of ion exchange and reverse phase chromatography, termed 2D-HPLC, complex peptide mixtures, but not protein mixtures have successfully been investigated. In the first dimension, peptides are separated utilizing ion exchange chromatography. Each fraction is further separated in the second RP-HPLC-dimension. The collected fractions are, finally, investigated by mass spectrometry to constitute a complete peptide map. These peptide patterns are further investigated to identify differences between normal control samples and patient samples to reveal possible biomarkers [79,80]. Raida et al. [81], Hock et al. [82], Heine et al. [83], and Kubler et al. [84] used this approach to isolate and characterize peptides from human plasma hemofiltrate (cutoff 20 kDa). No digestion was used during sample preparation and enrichment. Several thousands of peptides were detected and a LC–MS database of circulating human peptides was created.

The restriction of these two-dimensional methods lies in the limited size of the proteins that are investigated (usually $<$10 kDa) and in the required time for analysis [14,16,30].

Wang et al. [85] compared the capabilities of on-line HPLC–ESI–MS and off-line HPLC–MALDI–MS to analyze bacterial extracts. From 156 to 423 protein components in the mass range from 2 to 20 kDa were revealed by HPLC–MALDI–MS. In comparison, HPLC–ESI–MS showed only 46–59 detectable compounds. While these results are promising, the time consuming steps during sample analysis remain a major drawback.

The goal for clinical application must be to display a thorough pattern of a large number of polypeptides in a single, reproducible and time-limited step, which also enables comparison of different protein patterns. The limitations of the typical reversed-phase HPLC–MS, 2D-PAGE–MS and SELDI–MS technique [14,16,30] are summarized in Table 1 and reveal the need for an analytical technique that is fast, sensitive, reproducible, shows high resolution and allows the investigation of protein mixtures without prior digestion.

A promising approach towards this goal is the coupling of CE to an ESI–TOF–MS [11,86–88].

### 2.4. CE–MS

The combination of CE and MS allows to improve automation, speed and precision of proteome analysis [11,88,89]. Today, limitations of capillary separation techniques, like the small sample amount, are overcome [90–92], as well as the technical problems which are provoked by the combination of these two systems [86,93–95].

Waterval et al. [95–97] describe an approach for on-capillary preconcentration by using an on-capillary adsorptive phase. This technology allows multiple capillary volumes of sample solution to be injected and to increase the concentration sensitivity of CE by three–four orders of magnitude for angiotensin II and gonadorelin in plasma. Cao et al. [98] analyzed peptides, proteins and peptide digests and whole human blood with capillary electrophoresis. They identified major tryptic digest fragments of myoglobin and cytochrome *c* at attomol levels in less than 10 min. Major protein components of whole blood, such as α- and β-hemoglobin, were separated and detected at 10 fmol levels. Bateman et al. [99] characterized protein glycoforms by CE–ESI–MS and demonstrated the capability of this method to analyze complex mixtures resulting from enzymatic and/or chemical digest. Zhang et al. [100] describe the development of a microdevice with integrated liquid junction to facile peptide and protein analysis in the attomole range. The performance of the device was tested for CE–MS of proteins (cytochrome *c*, myoglobin, lactoglobulins), peptides and protein digests from BSA. The authors accentuated that the separation efficiency was comparable or better to that of conventional capillary electrophoresis systems. Liu et al. [101] used CE–ESI–MS to investigate peptides mixtures of Met– and Leu–enkephalin and peptide

Table 1
Comparative overview of the discussed technologies

| Technology | Advantages | Disadvantages |
|---|---|---|
| 2D-PAGE–MS | Established technology, multidimensional, high resolution. | Certain protein classes absent, not applicable to peptides ($M_r$ < 10 kDa), no automation (except molecular scanners), time consuming (up to days), high variability, quantification difficult, high costs. |
| SELDI–MS | Sensitivity for selected proteins, simple sample preparation, high sample throughput, automation, low sample volume required, low costs. | Reproducible loss off majority of polypeptides, restricted to polypeptides binding to selected matrix, low-resolution MS, no MS–MS capability. |
| HPLC–MS | Established technology, automation, multidimensional use possible. | Restricted mass range, time consuming, high costs. |
| CE–MS | Automation, high sensitivity, fast, low sample volume, multidimensional use possible, low costs. | Experimental technology, not standardized. |

mixtures of horse cytochrome *c* after tryptic digest and the results indicated that almost all peptides generated, were identified. Rubakhin et al. [102] analyzed cellular releases with off-line CE–MALDI-TOF. The capillary electrophoresis was used for separation and desalting. Peptides and proteins with relative molecular masses up to 11 kDa released from single neurons and neuron clusters from the neuronal model *Aplysia californica* were identified.

A promising approach towards utilizing CE–MS to obtain a complete proteome pattern was published by Jensen et al. [103]. The authors employed capillary isoelectric focusing (CIEF) coupled to a Fourier transform–ion cyclotron resonance (FT–ICR)–MS to analyze an undigested lysate of *E. coli*. The high-resolution power of the FT–ICR instrument enabled the authors to analyze up to 1000 proteins ranging from 2 to 100 kDa from about 300 ng lysate. Another advantage of this combination is that, if the accurate mass of the polypeptides does not lead towards identification, additional MS–MS experiments that utilize the high resolving power of the FT–ICR can be performed, leading to sufficient sequence information on the polypeptides analyzed. The capabilities of FT–ICR–MS for peptide mapping of proteins in human body fluids, such as blood, cerebrospinal fluid, plasma, and urine is reviewed by Bergquist et al. [104].

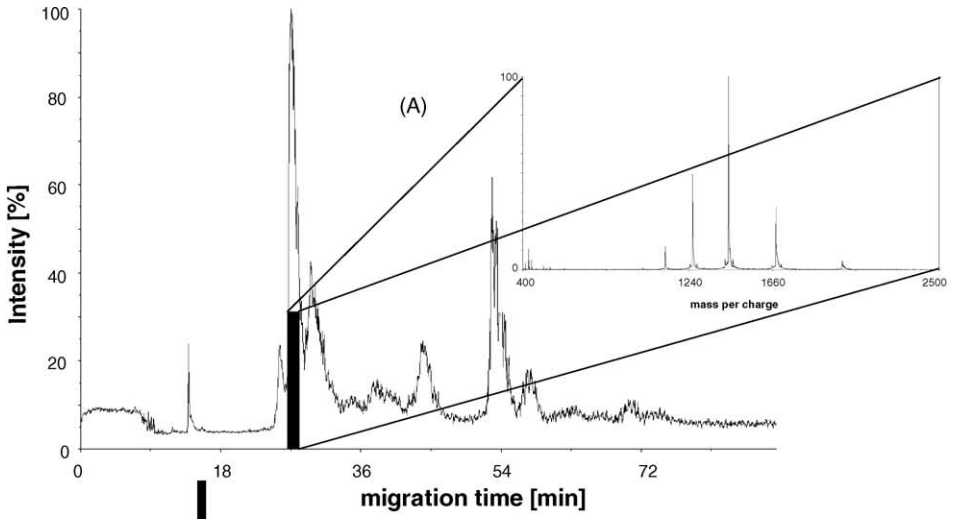
While CE is already widely used for the separation of complex protein mixtures [10,103,105], the combination of CE and MS to analyze polypeptide patterns in human body fluids such as urine and blood is not yet established. To date only a few specific proteins or peptides were investigated [98–102,106], although this technique permits the analysis of several hundred polypeptides simultaneously in a short time, in a small volume, with high sensitivity [103]. Thus, CE–ESI–MS is a powerful alternative to common proteomic technologies.

These reports led to the development of a robust CE–MS technique in our laboratory. We have developed a stable on-line coupling of CE to an ESI–TOF-MS to depict a large number of polypeptides found in body fluids in a single, time-limited process, under the assumption that this will lead to the establishment of polypeptide patterns typical for the state of health of individuals [107,108].

The CE-system was a Beckmann P/ACE MDQ system (Beckman-Coulter, Fullerton, USA) coupled to a Mariner ESI–TOF-MS by Applied Biosystems (Applied Biosystems, Farmington, USA). The sample was injected hydrodynamically (1 psi, 20 s, injecting approximately 100 nl of the sample solution) on an untreated silica capillary (Beckmann, i.d. and o.d., 75 and 360 μm, respectively, and 90 cm in length). When using a running buffer composed of 30% methanol and 0.5% formic acid in water (pH 2.4), the electrophoretic runs usually take between 25 and 60 min at 30 kV with 0.2 psi positive pressure. After each run, the CE capillary was rinsed for 5 min with 0.1 M NaOH, followed by 5 min rinse with water and another 5 min with running buffer. The capillary temperature was held constantly at 35 °C. The ESI-interface was either self-built or from Agilent technologies (Agilent technologies, Palo Alto, USA). The sheath flow was applied at 5 μl/min coaxial to the capillary and the sheath liquid was identical to the running buffer. This method can be performed fully automated without the need of any manual operation for at least 50 runs.

First experiments aimed towards analysis of examining urine and dialysis fluid revealed that up to 2000 polypeptides between 1 and 30 kDa could be evaluated using this technology. As evident from the wealth of data, it is impossible to evaluate the raw data using commercially available software. Hence, a software tool tailored to the requirements of this type of analysis was developed.

A probabilistic clustering algorithm [109–111] was employed to map charge-conjugated peaks onto ideal proteins of common mass and unit charge. In general, finding the underlying mass-defined proteins from an incomplete set of detected peaks is a non-trivial task and may often yield more than one unique solution. Each of the experimentally observed CE–MS peaks (usually >1000) may carry an arbitrary charge and thus give rise to several potentially existing proteins of distinct mass. The clustering algorithm developed attempts to find a solution to this ill-defined optimization problem by iteratively associating each CE–MS peak with possible proteins and evaluating the probability for these proteins to exist. Limiting the search algorithm to a maximal charge of 40, each peak may belong to one out of up to 40,000 proteins. By applying these algorithms, the raw data




(E)

| $M_r$ | CE-t [min] | dt [min] | amplitude |
|---|---|---|---|
| 1025.63 | 48.2 | 0.95 | 657.57 |
| 1035.29 | 41.5 | 0.36 | 174.46 |
| 1059.58 | 26.5 | 0.35 | 168.06 |
| 1060.66 | 36.9 | 0.33 | 5352.09 |
| 1081.94 | 37.6 | 0.49 | 849.51 |
| 1099.61 | 38.4 | 0.56 | 4264.87 |
| 1108.57 | 37.9 | 0.42 | 12327.76 |
| 1157.79 | 24.3 | 0.28 | 194.81 |
| 1161.50 | 37.7 | 0.15 | 733.24 |
| 1169.57 | 33.4 | 0.77 | 316.76 |
| 1170.68 | 31.2 | 0.48 | 860.63 |
| 1187.50 | 31,1 | 0.32 | 1021.39 |
| 1198.71 | 38.7 | 0.78 | 6966.09 |
| 1268.04 | 38.7 | 0.56 | 1921.15 |
| 1285.73 | 38.7 | 0.79 | 7208.83 |
| 1286.08 | 31.2 | 0.41 | 5367.66 |
| 1342.78 | 38.7 | 0.79 | 4045.97 |
| 1368.78 | 38.7 | 0.75 | 21345.51 |
| 1386.82 | 38.7 | 0.76 | 96517.89 |
| 1404.81 | 31.3 | 0.95 | 18351.66 |
| 1440.40 | 38.7 | 0.75 | 6099.45 |
| 1483.88 | 38.7 | 0.78 | 10903.59 |

Fig. 1. The MosaiquesVisu software allows the depiction of the information of a crude CE–MS analysis (A) as a three-dimensional contour plot. Here, a contour plot of urine from a patient with membranous glomerulonephritis (MNGN) is shown (B), mass per charge on the *Y*-axis against the migration time in min (*X*-axis), signal intensity is color coded. The signal to noise is calculated and the noise removed, thus leaving only actual signals (C). The software calculates the actual mass (D) based on both isotopic distribution and conjugated masses. This leads to a table of up to 2000 polypeptides defined via their mass and migration time. As an example, (E) shows 22 polypetides found in patients with MNGN.

of a typical CE–ESI–MS spectrum can be processed and a peak list can be calculated within less than 3 min (Fig. 1).

Typically, the raw data consists of 500–1500 time-sequenced mass spectra, 80,000 data points each. These spectra feature Gaussian and non-Gaussian noise as well as significant baseline offset, produced by unidentifiable analytes across the detection range (*m*/*z*) from 400 to 2500.

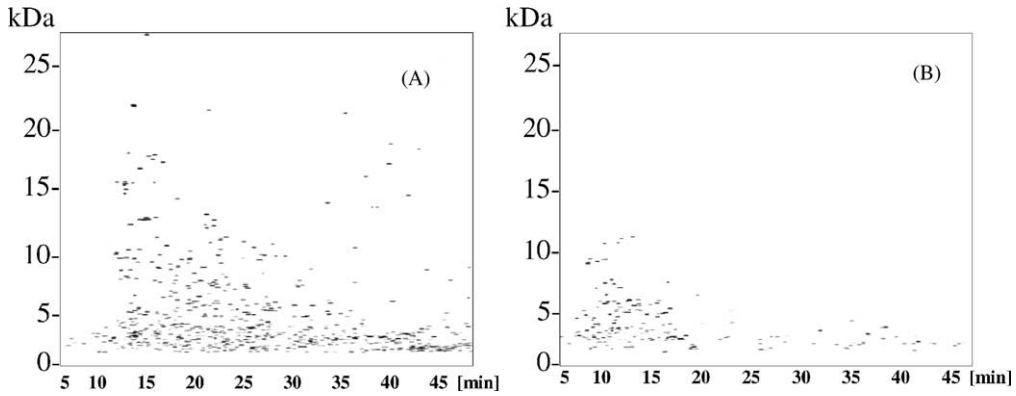



Fig. 2. Comparison of the abundance of polypeptides detected in high-flux (A) and low-flux (B) dialysates in dependence of their molecular weight and migration time. While the distribution, but not the absolute number, is quite similar in the low molecular weight range ($M_r$ < 10 kDa), a shift even in distribution can be observed in the high molecular range of the high-flux dialysates.

In a first step, the individual spectra (Fig. 1, panel A) are searched for protein-signatures to yield some 100,000 raw peaks (Fig. 1, panel B). Next, unique masses are determined for all proteins found and the CE–MS spectrum is reduced to isotope-free proteins of defined charge, i.e. well-defined atomic mass (Fig. 1, panel C). For our purpose, only masses above 1000 Da were accepted and singly charged compounds were eliminated.

Detected polypeptides were defined by the two coordinates: mass and migration time, and these (together with the amplitude of the signal) were deposited in an MS-Access database and statistically analyzed to identify common patterns. Due to limited reproducibility of the exact migration times it was necessary to allow a wider deviation range of 3 min for the CE-time, while the mass deviation could be set to 0.05%. Applying these limits, the resolution is sufficient to accommodate more than 1000 polypeptides ranging from 1 to 30 kDa in a single CE–MS run.

As a proof of concept in first experiments, the CE–MS technology was used to comparatively examine dialysis fluids from hemodialysis using two different types of membranes (low- and high-fluxes, Fig. 2). One of the goals of renal replacement therapy (dialysis) is the elimination of toxic polypeptides from serum, so-called uremic toxins. These have been theoretically defined, but mostly not yet identificated [112]. Observational studies have suggested that membranes with high porosity or flux, clear uremic toxins and larger solutes, such as 2β-microglobulin ($M_r$: 11.900 Da) [113,114], more efficiently than low-flux membranes.

In our experiments, more than 600 polypeptides could be analyzed in a single sample. As expected [113,114], the results revealed that larger polypeptides (>10 kDa) were only present in the samples from high-flux dialysates (Fig. 2A), while in low-flux dialysates (Fig. 2B) additional small polypeptides could be detected.

The results enabled us to establish typical patterns of high and low-flux dialysis membranes and compare these to the normal urine polypeptide pattern. This comparison yielded in a surprisingly low consensus, a number of polypeptides present in urine was missing. These initial results spur hopes to define dialysis based on polypeptides and consequently develop technologies that enable the specific removal of the uremic toxins. For further details about uremic toxins see Vanholder et al. [112].

In a different set of experiments, we evaluate the feasibility of the application of CE–ESI–MS for diagnostic purposes. To this end, urine samples from healthy volunteers and patients with different renal diseases and impaired renal function were examined.

Weissinger et al. [115] showed that the obtained urinary polypeptide patterns were easily distinguishable from the pattern obtained from samples of healthy volunteers. Actual masses were calculated as described and compared within the database. Since the protein content of the patient samples was much higher than that in the normal controls, the lack of certain polypeptides actually might be the result of deficient renal secretion or re-absorption. Although these data are obtained from a rather small number of patients, they indicate that evaluation of proteomic patterns in urine might serve as a diagnostic tool to further characterize diseases.

Taken together, these results clearly indicate that it is possible to analyze, in one single, time-limited step, an exhaustive polypeptide profile of human urine. The technique used allows to analyze up to 1000 polypeptides simultaneously and to interpret the raw data.

## 3. Conclusions

One of the main objectives of differential display proteomic studies is the comparison of proteins expressed under various conditions, e.g. in healthy and diseased individuals, and to find potential biomarkers for a certain disease. For this purpose, several technological approaches have been developed, on the one hand, basing on efficient separation methods, such as 2D-PAGE, HPLC and CE and, on the other hand, basing on mass spectrometry. Despite several limitations, to date, 2D-PAGE–MS still appears to

be the main method for proteomic studies (Table 1). However, the development of on-line methods such as single- or multi-dimensional HPLC–MS, SELDI–MS, and the now emerging CE–MS suggest that alternative technologies will greatly expedite the identification of therapeutic polypeptide targets and the establishment of proteomic patterns for diagnostic purposes. Especially, the data presented on CE–MS indicate that this technology might be capable to depict a comprehensive analysis of a complex proteome in a single analysis run.

As evident, all these technologies are still burdened with certain limitations (Table 1). The most severe limitation, however, might not be the technical aspect of MS and/or separation (data accumulation), but rather the following data evaluation. All these technologies create enormous amounts of data and information and suitable software to handle and properly utilize this wealth of information is mostly still in an experimental stage. Hence, it is conceivable that in the near future more emphasis will be laid on the development of data evaluation software.

The already available technologies, however, indicate that in the near future the application of this type of analysis will not only greatly expedite the discovery of disease markers and potential therapeutic targets, but also enable a thorough diagnosis of a variety of diseases based on the molecular polypeptide pattern.

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