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Review

Capillary electrophoresis–mass spectrometry: Recent trends in clinical proteomics

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ABSTRACT

The increasing attention now paid to the elucidation of human proteome strengthened the development of analytical instruments able to provide reliable proteins and peptides quantitation and characterization in biological fluids and tissues. Emerging from proteomics, clinical proteomics exclusively considers its biomedical applications. It evaluates, often by high-throughput comparative platforms, the protein and peptide variations in body fluids, cells and tissues under different physiological and pathological conditions with the aim of discovering disease biomarkers. Among the available analytical methodologies, mass spectrometry in coupling with liquid chromatography or capillary electrophoresis demonstrated to be the eligible technique for protein detection and identification. This review summarizes the most recent applications of capillary electrophoresis–mass spectrometry to clinical proteomics, focusing on capillary zone electrophoresis separation mode and ESI and MALDI ionizations, which are the most frequently applied capillary electrophoresis–mass spectrometry hyphenated techniques.

Contents

1. Introduction

The complete sequencing of human genome moved nowadays the attention of scientists to proteomics and peptidomics, defined as the identification and characterization of the complete asset (or subset) of proteins and peptides of a specific biological fluid or cell or tissue and the study of their structure, function and interactions [1,2]. Proteomics has broad applications which, starting from basic scientific research, extends towards clinical diagnosis, therapeutics and biomarker discovery associated to pathological states [3]. Together with the evaluation of the global protein expression of a tissue, a biological fluid or a cell, proteomics purposes include the characterization of protein–protein and protein–ligand interactions and the identification of protein post-translational modifications [4].

Particularly, clinical proteomics considers the biomedical diagnostic applications of proteomics and correlates high-throughput

Abbreviations: CAD, coronary artery atherosclerosis; CDK, cronic kidney disease; DEC, droplet electrocoupling; FASI, Field Amplified Sample Injection; ICAT™, Isotope-Coded Affinity Tags; iTRAQ™, Isobaric Tag for Relative and Absolute Quantitation; PTMs, post-translational modifications; SILAC™, Stable Isotope Labeling with Amino Acids in Cell Culture.

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analytical techniques and statistical approaches to epidemiology, clinical chemistry and various medicine disciplines [5]. By comparing qualitative and quantitative differences in protein expression profile under healthy and disease conditions, the purpose of clinical proteomics is the identification of specific biomarkers useful for the diagnosis or prognosis of diseases and the monitoring of patient follow-up. Although the application of clinical proteomics for biomarkers discovering possesses a great potential, several clinical and technical aspects are still challenging and need to be implemented. Pre-analytical factors, including biological variations and technical variables involving sample collection, processing (pre-fractionation) and storage, are relevant critical points differently influencing and affecting the results of clinical proteomic studies [5]. The majority of clinical proteomics efforts involves the analysis of conventional (plasma, urine) and less conventional (cerebrospinal fluid, saliva) body fluids samples. In fact, the composition of body fluids can reflect the physiological and pathological states of an organism. Compared with tissue samples, the analysis of body fluids has the advantages of easier accessibility and generally lower invasiveness for specimens collection. Among conventional biofluids, plasma did not provide the expected number of biomarkers due to the wide dynamic range of protein concentration and the complexity of the matrix, addressing clinical proteomics towards the analysis of less demanding biofluids such as urine, cerebrospinal fluid and saliva. These body fluids allow an easier access to the less abundant proteins and peptides, relevant for biomarkers or biomarker panels discovery. In fact, rather than identify single entities, proteomic analysis is now addressing its platforms towards the discovery of biomarker clusters able to simultaneously provide a selective response for diagnosis or prognosis of diseases with higher specificity [6].

A recent publication edited by Van Eyk and Dunn [5] presents an interesting overview on clinical proteomics focusing on the advances in methodologies and technologies employed and summarizing the results obtained in various relevant clinical areas. The techniques and methodologies used in proteomics are in continuous development and are strongly related each other to provide precise and reliable results in proteins identification and characterization. Among them two-dimensional gel electrophoresis (2DE), isoelectric focusing (IEF) and liquid-based methodologies such as liquid chromatography (LC, nano-LC) and capillary electrophoresis (CE) on-line or off-line coupled to mass spectrometry are the most popular. All of them exhibited advantages and limitations [3] and show high analytical potential in proteomics. However, the standardization and validation of procedures and methods is still the limiting step for routine clinical proteomics applications. Using different separation mechanisms, the current technologies can be considered complementary each other in producing a multi-approached set of data important for a full and complete characterization of the structure and function of a protein and its potential biomarkers role. Among the techniques used in proteomics, the coupling of mass spectrometry (MS) with CE showed very promising results in the field of clinical proteomics and biomarker discovering as documented in recent review papers [7–10].

This review summarizes the most recent applications (2005–2010) of capillary electrophoresis-mass spectrometry to clinical proteomics, focusing on capillary zone electrophoresis (CE) separation mode and ESI and MALDI ionizations, which are the most frequently applied capillary electrophoresis–mass spectrometry hyphenated techniques. The recent applications of CE–MS to the identification of post-translational modifications (PTMs) are also illustrated. Before their description, the procedures and methodologies used in proteomics are below briefly described particularly focusing on CE and CE–MS distinct features.

2. Proteomics procedures and methodologies: CE–MS features

A proteomic analysis involves different steps, including sample preparation, protein separation, identification and characterization, all contributing to the production of precise and reliable results [3].

The sample preparation is a relevant and critical step involving specimen collection and storage, its treatment with anticoagulants and protease inhibitors and clean-up procedures for salts and lipid removal. Depletion of highly abundant proteins is often required to access to the less abundant proteins and peptides present in the sample. Protein separation and fractionation is therefore performed by using different mono- or bi-dimensional analytical technologies, such as LC, CE or 2DE, and/or by affinity approaches, in either on-line or off-line coupling with mass spectrometry detection. With respect to 2DE, the MS coupling to LC or CE is performed by on-line interfaces and it can be successfully applied to the analysis of undigested samples and to the identification of low abundance proteins and peptides [11]. Protein characterization includes the determination of the amino acid sequence, the characterization of post-translational modification, conformational and structural studies as well as cellular localization [12].

The development of ESI and MALDI interfaces for CE–MS hyphenation widely expanded the applicability of CE in the field of protein and peptides analysis [8–10,13,14]. Detailed description of the different CE–MS interfaces can be found in several review papers [14–18].

CE technique is challenging and complementary to conventional LC, providing different data analysis based on the electromigration properties of the analytes. CE is particularly suitable for the analysis of polar and chargeable compounds. Among the several modes of separation available in the capillary electrophoresis techniques, the free zone (CE) mode, based on the differences in charge-to-size ratio of the analytes migrating under an applied electric field, has been the most popular applied to the analysis of proteins and peptides. In fact, due to their molecular structure, proteins and peptides exhibited in the majority of the cases high polarity and the possibility to migrate as positively or negatively charged compound due to their zwitterionic character. These features well match the use of CE for their analysis.

With respect to chromatography and other electrophoretic techniques, CE possesses the strong advantage to provide protein and peptide separations inside an aqueous environment, condition very close to their natural state [19] and exhibits relevant and challenging analytical features as below briefly summarized.

Miniaturization makes CE a cheap and low environmental pollution impact methodology particularly suitable for the analysis of biofluids allowing several repeated injections per few microlitres of sample. Analysis are generally fast and the high separation efficiency, that is usually 1 or 2 orders of magnitude higher than conventional LC separations, allows sample pre-treatment to minimize, contributing to a significant reduction of the total analysis time. However, for proteomics applications, the sample preparation step is fundamental for cleaning-up the matrix, obtaining protein fractionation and/or depletion of highly abundant proteins and allowing the access to the minor proteins and peptides present in the complex matrix. The use of unmodified fused silica capillaries additionally reduces costs and time of analysis with respect to the expensive LC chromatographic columns often employed in gradient elution programs involving long time preconditioning. Furthermore, uncoated fused-silica capillaries can be rapidly rinsed, reconditioned and easily replaced. As counterpart, the reduced detection path length (few micrometers, on-capillary detection) and the small amount (few nanolitres) of sample injected need the coupling of CE with sensitive detectors,

Table 1
CE–MS applications to human disease biomarkers discovering.

Disease	Biofluid	Enzymatic digestion	MS instrumentation	Capillary type	Reference(s)
Immunoglobulin A nephropathy	Urine		ESI-TOF MALDI-TOF	Uncoated	[32]
Focal-segmental glomerulosclerosis					
Membrane glomerulonephritis					
Lupus nephritis					
Diabetic nephropathy					
Renal transplantation		–			
Urothelial cancer	Urine	–	ESI-TOF	Uncoated	[33]
Bladder cancer	Urine	–	ESI-TOF	Uncoated	[34]
IgA-associated renal diseases	Urine	–	ESI-TOF	Uncoated	[35]
Autosomal dominant polycystic kidney	Urine	–	ESI-TOF	Uncoated	[38]
Allogenic hematopoietic stem cell transplantation complications	Urine	–	ESI-TOF	Uncoated	[39–41]
Acute graft-versus-host disease (aGvHD)	Urine	–	ESI-TOF	Uncoated	[42]
Obstructive nephropathy	Urine	–	ESI-TOF	Uncoated	[43]
Diabetes	Urine	–	ESI-TOF	Uncoated	[44]
Cronic kidney disease (CDK)	Urine	–	ESI-TOF	Uncoated	[44,45]
Coronary artery disease	Urine	–	ESI-TOF	Uncoated	[46]
Renal Fanconi syndrome	Urine	–	ESI-TOF	Uncoated	[47]
Anti-neutrophil cytoplasmatic antibody-associated vasculitis	Urine	–	ESI-TOF	Uncoated	[48]
Coronary artery atherosclerosis (CAD)	Urine	–	ESI-TOF	Uncoated	[49]
Ureteropelvic junction obstruction	Urine	–	ESI-TOF	Uncoated	[50]
Alzheimer's disease	CSF	–	ESI-TOF	Uncoated	[32]
Neurodegenerative diseases	CSF	x	MALDI-TOF-TOF	PolyE-323 coated	[51]
Brain injury	CSF	x	iTRAQ-MALDI-TOF	PolyE-323 coated	[52]
Appendicitis	Plasma	x	ESI-FTICR	MAPTAC coated	[53]

as MS, to overcome the depletion of detection sensitivity due to miniaturization.

The coupling of MS with CE, as well as other on-line separation techniques, provides a consistent increase of the detection sensitivity contemporaneously offering high separation selectivity and specificity and the possibility of analytes structural characterization. In fact, MS/MS fragmentation spectra give additional information for structural peptide identification. Moreover, single ion monitoring (SIM) or multiple reaction monitoring (MRM) scan modes are precious instruments for an increase of selectivity and sensitivity, avoiding the interference of isobaric contaminants and strongly reducing the noise of the background. Alternatively or in addition, several on-line pre-concentration methods can be coupled to the technique to further increase its detection capability, as recently reviewed [20–24].

It is noteworthy to underline that the constant composition of the BGE in CE is ideal for MS interfacing with respect to the mobile phase modification occurring during LC gradient elution [25].

A pitfall of CE technique is the possible adsorptions of proteins and peptides on the capillary wall.

Several types of modified capillaries can be used in coupling with MS to prevent this phenomenon. The modification of the capillary inner wall can be obtained with different types of coating procedures compatible with MS hyphenation, i.e. dynamic, static-covalent and static-adsorbed coatings, as recently reviewed [26]. In uncoated capillaries, the use of BGEs either at low or high pH can be used as strategy to overcome protein adsorption, if compatible with proteins stability and solubility.

As all the MS based analytical platforms used in proteomics, CE–MS analysis of proteins and peptides can follow either top-down or bottom-up approaches [1,5]. In the bottom-up approach proteins mixtures or proteins fractions or 2DE purified proteins (2DE spots) are digested by chemical or enzymatic proteolysis and analysed by tandem mass spectrometry detection either directly (MALDI-TOF-MS) or after chromatographic (capillary and nano-LC) or electrophoretic (CE) pre-fractionation. The obtained MS/MS fragment profiles are therefore analysed in bioinformatic databases by matching for proteins identifications (shotgun proteomics) [27]. The use of bioinformatics databases and dedicated softwares is of utmost importance for proteins identification and for the interpretation of the huge amount of mass spectrometry data obtained with these platforms. Often, for quantitative comparison, the bottom-up approaches use two (or more) forms of a reactant selective for reactive groups of specific amino acid residues (i.e. cysteine). One form is composed only by light isotopes, while the other(s) are composed by heavier isotopes (i.e. ^{2}H, ^{13}C). The light reactant is used to label one group of samples under analysis (i.e. normal), the heavy reactant labels the others (i.e. pathological). The molecules of reactant have also a bite (i.e. biotin) which allows to separate the reacted peptides from the unreacted ones by affinity-chromatography. After labelling and enzymatic digestion, the two samples can be combined and simultaneously analysed by high throughput MS/MS for comparative quantitation. In fact, the relative quantitation of the two isotopic forms of the same migrating (or eluting) peptide can disclose different amounts of the corresponding parent proteins in the pathological samples with respect to the healthy control. This strategy is at the basis of many patented platforms such as, i.e. Isotope-Coded Affinity Tags (ICAT^{TM}), Isobaric Tag for Relative and Absolute Quantitation (Tandem mass tags, iTRAQ^{TM}), and Stable Isotope Labelling with Amino Acids in Cell Culture (SILAC^{TM}).

Differently, in the top-down approach mass spectrometry analysis provides the molecular mass data of the intact proteins. The top-down approach shows several advantages, e.g. the possibility of identifying post-translation protein modifications or disclosing the presence of isoforms. When the protein dimensions are compatible with the MS apparatus available, characterization of the structure can be carried out by tandem mass spectrometry (MS/MS or MS^{n} analysis) of the intact protein. Alternatively, after purification, protein identification may be obtained by different chemical and enzymatic reactions aided by mass spectrometry analysis. Differently from the bottom-up, top-down platforms cannot take full profit by automated LC–MS/MS data elaborations, because they are suitable for the analysis of small peptides up to 5–6 kDa.

3. Recent CE–MS applications to clinical proteomics (2005–2010)

CE–MS was extensively applied to protein and peptide analysis and biomarker discovery during the last 5 years [14]. The present paper summarizes the most recent applications (2005–2010) of CE–MS with ESI and MALDI interface in clinical proteomics report-

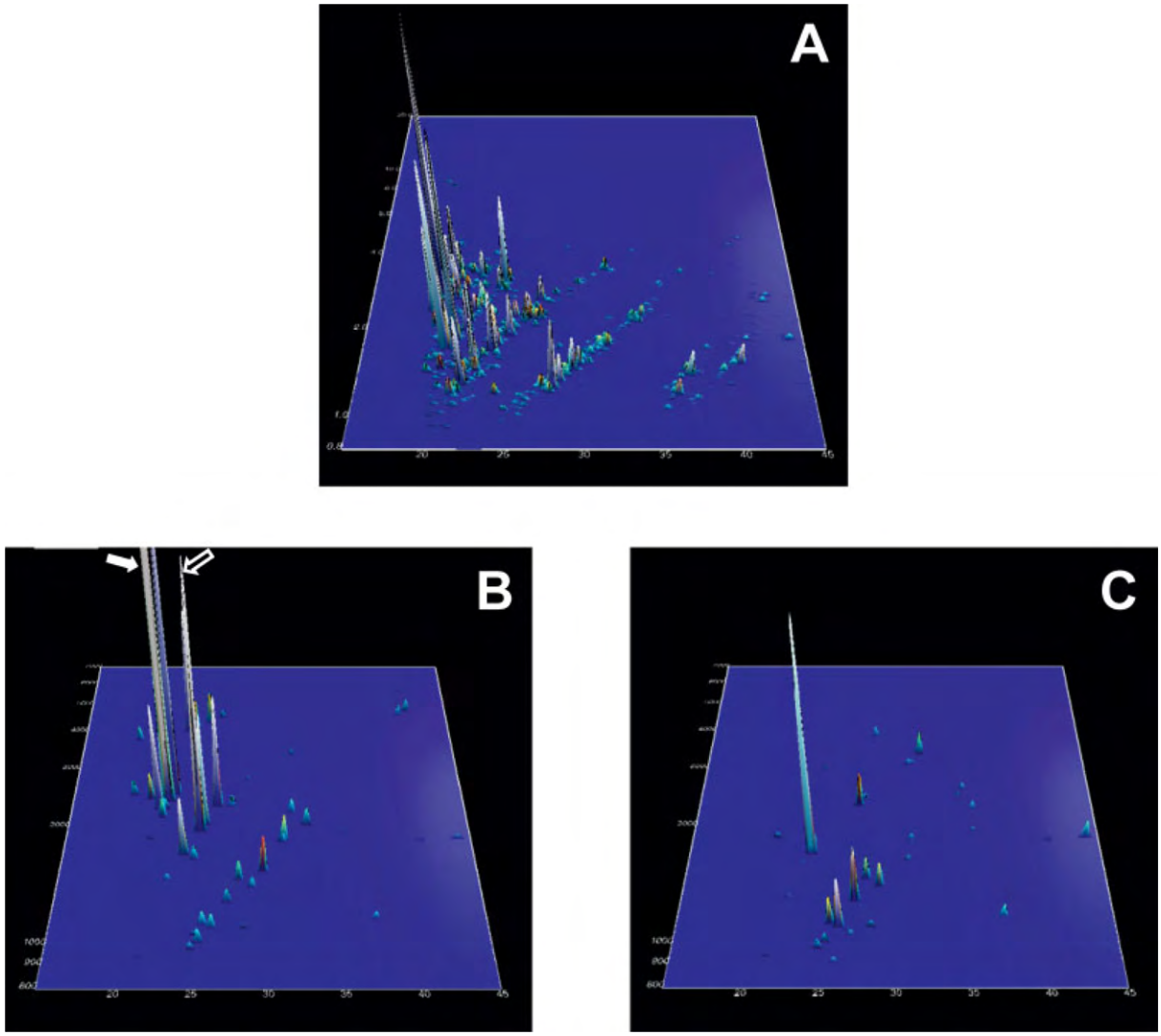

Fig. 1. 3-D digitally compiled contour plots of CE-ESI-TOF-MS analysis of 402 polypeptides in the urine of patients affected by chronic renal disease (A). The normalized migration time is plotted on the *X*-axis, the molecular mass (logarithmic scale) on the *Y*-axis, and the mean MS signal intensity on the *Z*-axis. Panels (B) and (C) show the 95 polypeptides pattern specific of renal damage (B) with respect to healthy controls (C). Reprinted from Ref. [35]. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

ing the results obtained in different body fluids. Table 1 presents a summary of the below described CE–MS applications to biomarkers discovery of human diseases. The use of CE–MS for the identification of post-translational modifications (PTMs) is also illustrated.

3.1. Urine

CE in coupling with ESI-TOF-MS was extensively applied to the identification of protein and peptide disease biomarkers in urine, as recently reviewed [28–31]. Urine is a relevant body fluid to investigate for disease biomarker discovering due to the easiness of collection and large amount accessibility by non-invasive methods. Among the different analytical techniques tested for the identification of urine biomarkers, CE–MS allowed the identification and validation of specific proteomic patterns of disease, matching the requirements for application in clinical routine analysis [29].

CE-ESI-TOF-MS with a top-down proteomic approach was applied to the analysis of specific peptide and protein patterns with the aim to identify biomarkers of immunoglobulin A nephropathy, focal-segmental glomerulosclerosis, membrane glomerulonephritis, lupus nephritis, diabetic nephropathy and renal transplantation diseases [32]. The TOF analyzer offered a suitable fast response for the identification of the sharp CE peaks with accuracy and repeatability and allowed the identification of polypeptide patterns as biomarker panel of diagnosis and therapy of the above specified renal diseases. The urine samples were purified and concentrated on C2 column before running CE in uncoated fused-silica capillary using formic acid BGE containing 20% acetonitrile. Mass spectra were collected in positive ionization mode using an aqueous solution of 30% isopropanol and 0.5% formic acid as sheath liquid. Due to several drawbacks for routine analysis, the use of coated capillaries and sheathless interfaces, even though evaluated, was discarded. Peptides sequencing was performed by MS/MS with off-line MALDI-TOF-TOF experiments by spotting the complete eluate of the CE run on the MALDI target. The identification of a large set of polypeptides as biomarker demonstrated to be robust in terms of test sensitivity, specificity and predictive value.

CE-ESI-TOF-MS was applied to the proteomic analysis of urine also in relation to cancer diseases. 22 polypeptides were identified by CE–MS as a diagnostic pattern for urothelial cancer with

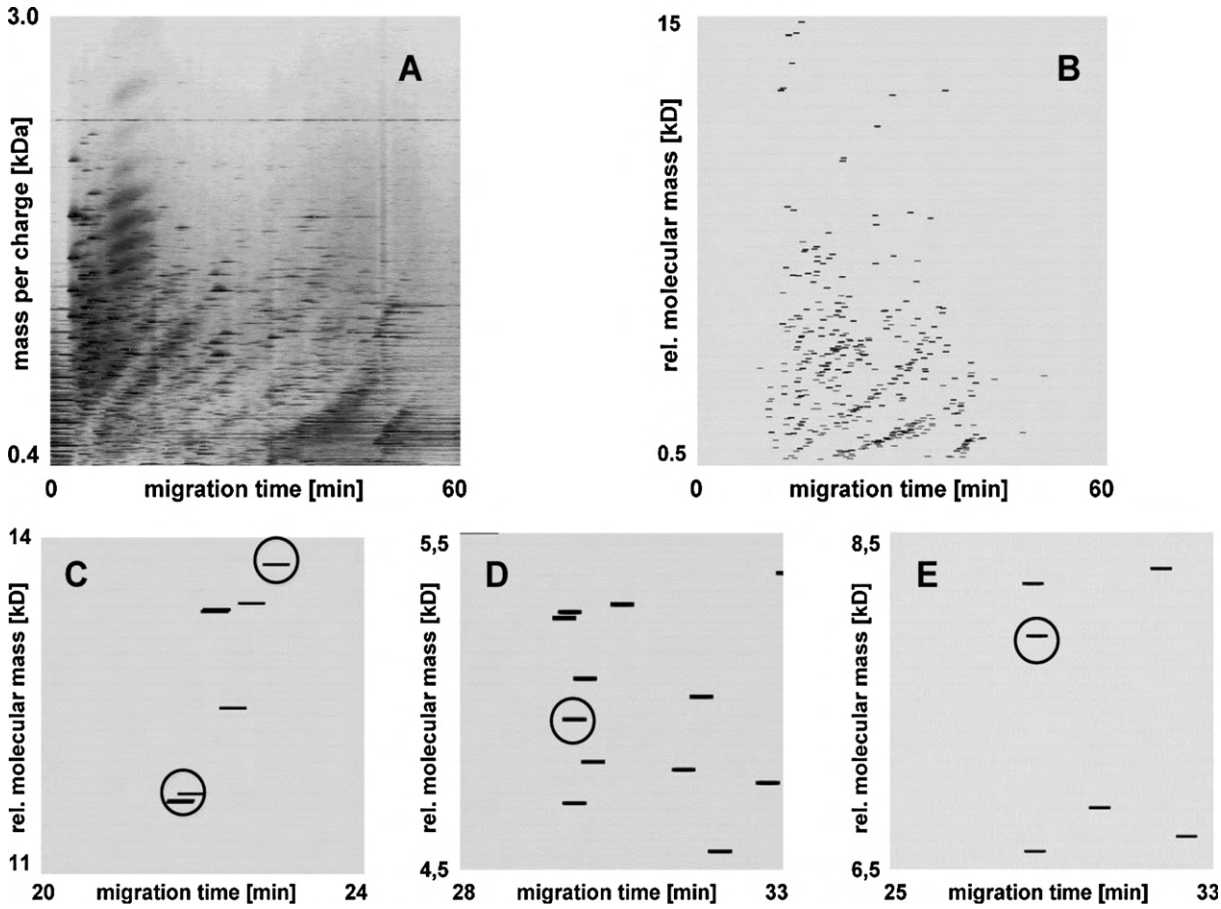

Fig. 2. CE–MS 3-D (molecular mass per charge versus migration time) electropherogram (A) and protein (mass range 0.5–14 kDa) contour plot (B) analysis of cerebrospinal fluid sample of a patient affected by Alzheimer's disease. The grey scale is indicating the signal intensity. The lower panels (C–E) show the CE–MS contour plots of selected protein mass ranges showing with a circle the proteins potential disease biomarkers. Reprinted from Ref. [32]. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

high sensitivity (100%) and specificity (100%) versus healthy controls [33]. With respect to patients affected by other malignant and non-malignant genitourinary diseases this methodology was able to correctly identify (86–100%) patients with urothelial cancer. Among the identified polypeptides of the pattern, fibrinopeptide A resulted one of the most relevant.

The CE–MS proteomic analysis of urine of 127 patients identified a panel of four sequenced biomarkers able to predict muscle-invasive bladder cancer (with a sensitivity of 81% and a specificity of 57%) and to distinguish it from the muscle non-invasive cancer form [34].

The same CE-ESI-TOF methodology used in [32] was applied for discovering biomarkers of diagnosis of IgA-associated renal diseases [35]. Different polypeptide patterns specific for renal damage and IgA nephropathy were identified, by using software elaborated 3D contour plots, and applied for a differential diagnosis (Fig. 1). Before CE–MS analysis, urine was diluted and pre-treated by desalting and precipitation of the most abundant proteins. As recently reviewed [36,37], a proteomic analysis of urine by CE–MS developed a panel of polypeptide biomarkers offering great promise for the non-invasive diagnosis of IgA nephropathy, currently performed only by renal biopsy.

In a proteomic study on autosomal dominant polycystic kidney patients, CE–MS analysis showed altered expression of 197 protein fragments, 38 resulting from collagen [38].

CE-ESI-TOF was recently applied to proteomic analysis of urine for the identification of biomarkers indicative of complications after allogenic hematopoietic stem cell transplantation, offering a clue for the development of new therapeutic targets [39–41].

The CE-ESI-TOF analysis of urine identified a proteomic pattern with potential high specificity for the early diagnosis of acute graft-versus-host disease (aGvHD) [42].

In the recent years, the proteomic profile of urine by CE-ESI-TOF-MS was studied in relation to several other diseases, such as obstructive nephropathy in newborns [43], diabetes and chronic kidney disease (CDK) [44–45], coronary artery disease [46], renal Fanconi syndrome in children [47], anti-neutrophil cytoplasmatic antibody-associated vasculitis [48] and coronary artery atherosclerosis (CAD) in symptomatic patients [49].

The proteome pattern established for the obstructive nephropathy in newborns [43] was able to predict with high specificity the need of pyeloplasty (patients younger than 1 year old) with severe ureteropelvic junction obstruction [50].

Many of the biomarkers found for diabetes [44] and CAD [49] by CE–MS analysis of urine were identified as fragments of collagen type I and α1 (I and III), respectively. Peptide sequencing was performed by CE–MS/MS, LC–MS/MS with quadrupole-TOF analyzer or nano-LC-LTQ Orbitrap high resolution MS.

In all the above reported papers, CE–MS methodology successfully provided the identification of different protein patterns of urinary biomarkers useful as easy and non-invasive tool of diagnosis, prognosis and therapeutic monitoring of renal and non-renal diseases.

3.2. *Cerebrospinal fluid (CSF)*

Few papers report the application of CE–MS to the proteomic analysis of cerebrospinal fluid (CSF). A previously reported

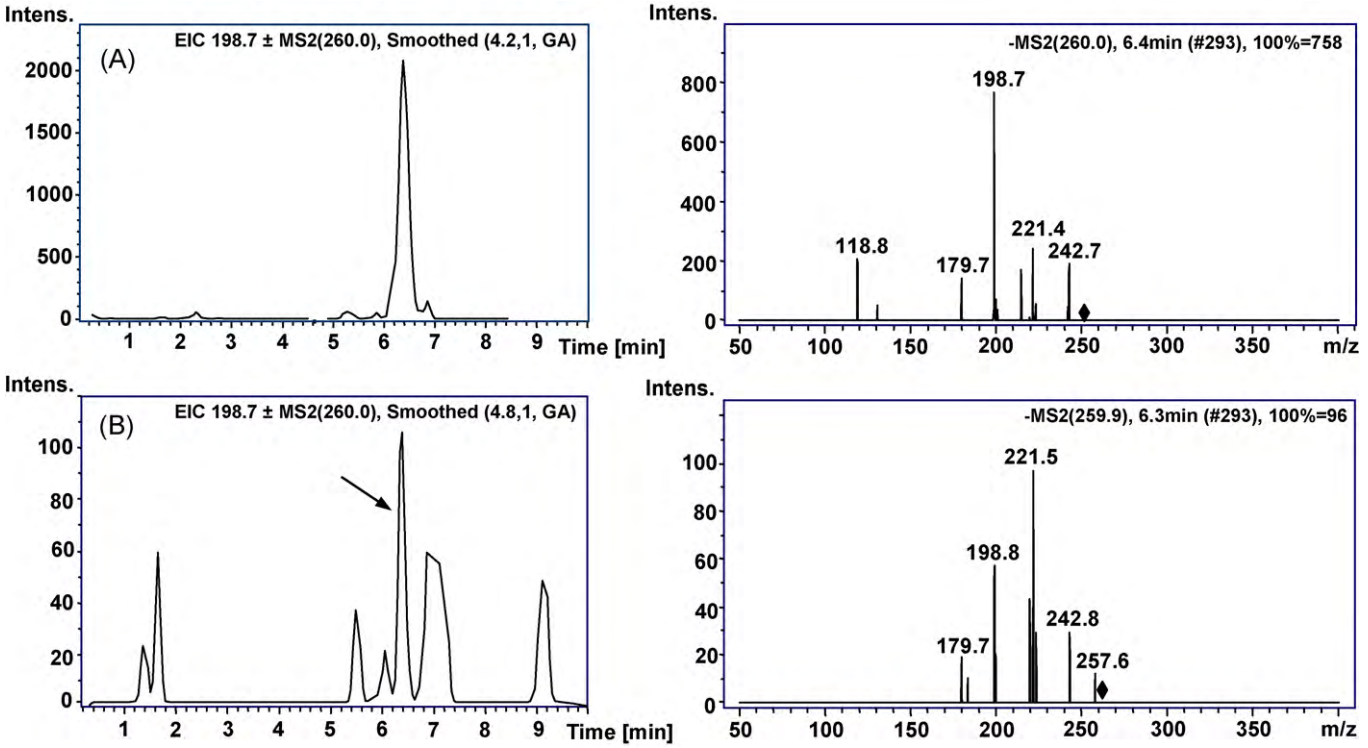

Fig. 3. CE-ESI-IT-MS detection of sulfo-tyrosine in pronase digestion mixture of an enriched preparation of histatin-1 and its derivatives (negative ESI ionization). MS/MS extracted ion current (EIC) plots and relative MS/MS spectra of (A) sulfo-tyrosine standard compound (m/z = 260.0, $[M-H^+]^{-1}$); (B) pronase digestion mixture: the arrow indicates the peak of sulfo-tyrosine. Modified from Ref. [56].

approach [32] was also applied to the proteomic analysis of the CSF in association with Alzheimer's disease and schizophrenia. Data evaluation was performed by applying specific software and 3D representations (Fig. 2).

CE coupled off-line to MALDI-TOF-TOF-MS was used in a bottom-up approach for the detection of several proteins and peptides candidates as biomarkers of neurodegenerative diseases [51] and brain injury [52]. CE–MALDI-MS was first applied to investigate the effect of immunoaffinity pre-treatment for the depletion of different serum proteins, judging it as a relevant step for the detection of less abundant proteins [51]. CSF was analysed after enzymatic digestion and sample desalting. Derivatization of the tryptic digests with 2-methoxy-4,5-dihydro-1H-imidazole reagent was also carried out to enhance ionization efficiency. CE separations were performed in PolyE-323 modified capillaries to reduce the protein interactions with capillary wall.

The use of isotope-coded tag (iTRAQ^{TM}) in coupling with MALDI-MS was applied to the quantitative proteomic profiling of CSF of one case study of traumatic brain injury [52]. The labelling was performed after tryptic digestion and desalting of the samples on C18 ZipTip®. iTRAQ^{TM} labelling prior to CE–MS analysis increased the analysis reliability minimizing the variability of the injection, analytes separation and ionization processes. CE separations were performed in PolyE-323 coated capillary, using 10 mM acetic acid both as BGE and sheath liquid. CSF was collected at days 1, 3, 5 and 8 after the injury, and the CE-MALDI-MS analysis identified 43 proteins that showed a variation of concentration at the different sampling times.

3.3. Other body fluid

Two different techniques, namely CE-ESI-FTICR-MS and capillary electrochromatography CEC-ESI-TOF-MS, were differently used with a bottom-up approach for the identification of protein biomarkers of appendicitis in pre- and post-surgery plasma samples [53]. Both the methods used capillaries coated with 3-(methacryolyamino)propyl-trimethylammonium chloride (MAPTAC) and octadecyldimethyl(3-trimethoxysilylpropyl) ammonium chloride (ODAC) in combination with N-trimethoxysilylpropyl-N,N,N-trimethylammonium chloride (TAC), for CE and CEC open tubular analysis, respectively, disclosing fast and efficient protein separations with reversed EOF in 5 mM acetic acid 25% acetonitrile. Plasma samples were digested by trypsin and desalted on ZipTip® prior to the analysis. The two methodologies used chemometric tools for data evaluation and evidenced differences either in the proteomic profile of pre- and post-surgery plasma samples or in the pre-surgery plasma of gangrenous and phlegmonous appendicitis types, leading to a list of potential biomarkers for the differential diagnosis of the disease.

CE-MALDI-TOF-MS/MS was used for characterizing the protein content of human follicular fluid with a multidimensional approach. The biological samples were pre-fractionated by isoelectric focusing (IEF) and enzymatic digested prior to the CE–MS analysis in PolyE-323 polymer coated capillary using 10 mM acetic acid as BGE [54].

3.4. Post-translational protein modifications (PTMs) studies by CE–MS

CE-IT-MS in uncoated capillary was used for the characterization of non-enzymatic post-translational modification of collagen I test protein incubated with different oxo-compounds (glucose, ribose and malondialdehyde) [55]. The separation occurred after enzymatic digestion of the modified protein with trypsin (bottom-up approach) in uncoated capillary using 3.3 M acetic acid solution as BGE and 5 mM ammonium acetate 50% isopropanol as sheath liquid. Both CE–MS/MS and LC–MS/MS contributed in the elucidation of the glycation sites of the molecule.

In our experience CE-ESI-IT tandem MS was very useful for the identification of tyrosine sulfation PTM in histatin 1 after hydrolysis at basic pH [56] (Fig. 3). CE–MS/MS analysis of sulfo-tyrosine was obtained by using 20 mM ammonium acetate pH 8 as BGE and 0.5% ammonium hydroxide (v/v) in 70% methanol as sheath liquid in negative ionization. Sulfo-tyrosine exhibited an intense fragment at 198.6 m/z, as result of the loss of both ammonia and CO_2 from the molecule.

4. General considerations and future trends

CE–MS was successfully applied to the identification of disease biomarkers in biofluids by using both the top-down and the bottom-up approaches. Relevant results have been obtained by CE–MS proteomic analysis of urine with top-down approach in association to several renal, non-renal and cancer diseases. Groups of peptides, polypeptides and protein fragments were discovered and validated as biomarker panels able to perform diagnosis or prediction of specific diseases with high specificity. These results confirm the importance of identifying sets, rather than single, proteins and peptides biomarkers. This approach requires the analysis of less complex biological matrices such as urine or cerebrospinal fluid. These biofluids were mostly analysed by top-down proteomic approaches in the above illustrated CE–MS applications. Clinical proteomics applications on plasma samples or other biofluids by CE–MS have been very poor in the recent years and all utilize enzymatic digestion prior to the CE–MS analysis.

From the described studies, a wider applicability of CE–MS to the analysis and identification of peptides rather than proteins, especially by a top-down approach, is evident. Moreover, in CE–MS hyphenation, TOF-MS analyzers were frequently used. This success derives from the fast response of TOF analyzer, compatible with the reduced peak width of CE separations, and from the accuracy of the molecular mass data produced, important requirement for the identification of biomarkers. An interesting review illustrates the usefulness of CE-TOF/MS in the analysis of biomolecules and in the identification of patterns of biomarkers in body fluids [57]. The slower response of ion trap and quadrupole-MS analyzer sometimes limits their application to protein and peptide analysis. Mischak and co-workers [58] compared and discussed the advantages and disadvantages of the use of different MS instrumentations on-line or off-line coupled to high performance separation technique (CE and LC) for biomarkers discovery and peptides sequencing. Among them, the coupling of CE with FTICR-MS resulted to be the most reliable for peptides characterization due to the possibility of combining the electrophoretic mobility and molecular mass data with direct sequencing by MS/MS analysis.

The final identification of peptides was often achieved by the use of multidimensional approaches such as off-line analysis of the collected CE eluate by MALDI-TOF-MS, combination of different peptide sequencing techniques (LC–MS/MS, nano-LC-LTQ Orbitrap high resolution MS, quadrupole-TOF-MS) or on-line coupling with pre-concentration techniques. Among the on-line pre-concentration methods used for proteins and peptides, stacking procedures, transient isotachophoresis (ITP) and on-line solid phase extraction (SPE) were the most applied [20–24]. Alternatively Field Amplified Sample Injection (FASI) and dynamic pH junction were successfully applied to peptides and trypsin digests [59,60]. The SPE pre-concentration resulted in nanomolar detection of enkefalines in spiked cerebrospinal fluid [61] and showed comparable sensitivity of nano-LC–MS [62]. Isotacophoresis (ITP)–CZE-MS possesses a strong pre-concentration power making detectable even trace amounts of peptides additionally able to provide sample precipitation and pre-fractionation [63]. A multidimensional approach based on transient capillary isotachophoresis/capillary zone electrophoresis (CITP/CZE) in coupling with nano-LC–MS was applied to the proteomic analysis of human saliva with a bottom-up approach allowing the sequencing of numerous low abundant proteins in saliva [64].

Although modified capillaries can successfully overcome the drawback of capillary wall protein adsorption, they were not extensively used recently. The use of uncoated capillaries under acidic conditions seemed to be preferred due to the easiness of rinsing and replacing procedures particularly advantageous for fast biomarkers screenings of numerous biofluids specimens. Some authors outlined to have not found any benefits in utilizing coated capillaries, observing a lower resolution power and MS detection sensitivity than the uncoated ones under the same conditions used [9].

The development of CE–MS methods for protein analysis therefore required the optimization of different parameters and conditions involving the CE separation and MS ionization. Chemometric approaches can be used with profit for the fast optimization of the different analytical options [65]. Many software tools were also developed to manage and analyse the vast amount of data generated by a CE–MS run. A CE–MS analysis produces indeed an enormous quantity of data that need the use of dedicated software to extract information from the MS spectra.

MosaiquesVisu software [66,67] is able to elaborate the CE–MS electropherograms in three-dimensional contour plots (a, migration time; b, molecular mass; c, signal amplitude) and to identify a list of peptide and/or protein peaks by analysing the isotopic distribution and conjugated mass. This approach was extensively applied to the identification of biomarkers in urine by a top-down approach [32–50] and required the use of high mass accuracy MS instrumentation such as ESI-TOF-MS. It was demonstrated that the position of the identified peptides biomarkers in 3D contour plots representations of CE–MS analysis, correlated at pH 2.0 with the number of positively charged amino acid residues in the molecules. This allowed to facilitate the assignment of peptide sequence to the CE–MS identified biomarkers or to predict the peptide migration time on the basis of its known amino acids sequence [58].

Recently, a new approach for handling CE–MS data in simplified 2D representations was presented and applied to bottom-up proteomic analysis of trypsin digest of cytochrome c deriving from diverse sources [68]. Differently from the previous described software, this approach does not require high mass accuracy and it can be therefore applied to ion-trap MS data. In the 2D representations the migration time or the mobility value of the peptides are plotted versus the m/z value and different colour shades are connected to the relative intensities of the m/z signals.

All these softwares produce 3D or 2D plots that, as a fingerprint of the biological fluid, evidence at the glance the differences associated to healthy and pathological states.

A list of tools available on-line for processing the CE–MS and CE–MS/MS data is reported in a very recent review [25].

Conclusively, this review suggests that CE–MS is a validated analytical method that can be applied in clinical proteomics for the reliable identification of disease biomarkers. Important advances have been obtained by the use of CE–MS analysis of urine for the development of validated diagnostic tests of specific diseases. However, nowadays the number of applications of CE–MS to protein and peptides analysis is not comparable to that of other proteomic platforms. The still difficult hyphenation of CE with MS and the lack of automation need technology improvements and the availability of complete CE–MS apparatus by the vendors for a full application of the technique in clinical laboratories. Its wide use is indeed limited by the analyst choice usually addressed to more standardized liquid-based analytical methods such as LC or nano-LC-ESI-MS systems.

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