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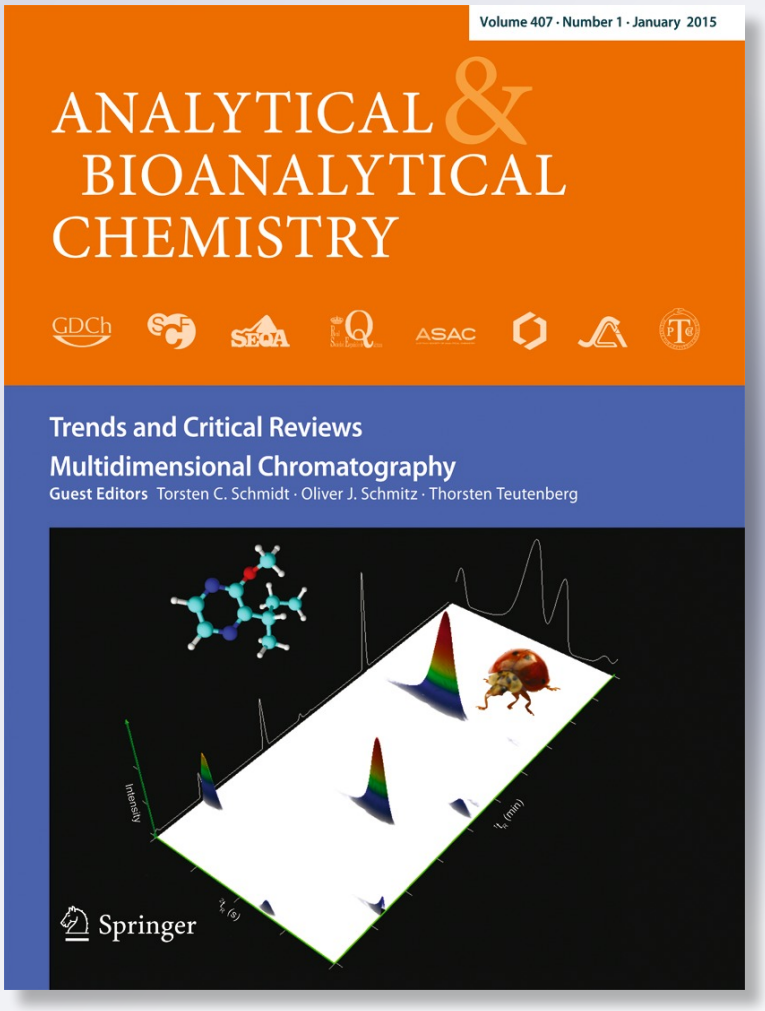

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REVIEW

Column–coupling strategies for multidimensional electrophoretic separation techniques

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Abstract Multidimensional electrophoretic separations represent one of the most common strategies for dealing with the analysis of complex samples. In recent years we have been witnessing the explosive growth of separation techniques for the analysis of complex samples in applications ranging from life sciences to industry. In this sense, electrophoretic separations offer several strategic advantages such as excellent separation efficiency, different methods with a broad range of separation mechanisms, and low liquid consumption generating less waste effluents and lower costs per analysis, among others. Despite their impressive separation efficiency, multidimensional electrophoretic separations present some drawbacks that have delayed their extensive use: the volumes of the columns, and consequently of the injected sample, are significantly smaller compared to other analytical techniques, thus the coupling interfaces between two separations components must be very efficient in terms of providing geometrical precision with low dead volume. Likewise, very sensitive detection systems are required. Additionally, in electrophoretic separation techniques, the surface properties of the columns play a fundamental role for electroosmosis as well as the unwanted adsorption of proteins or other complex biomolecules. In this sense the requirements for an efficient coupling for electrophoretic separation techniques involve several aspects related to microfluidics and physicochemical interactions of the electrolyte solutions and the solid capillary walls. It is interesting to see how these multidimensional electrophoretic separation techniques have been used jointly with different detection techniques, for intermediate detection as well as for final identification and quantification, particularly important in the case of mass spectrometry. In this work we present a critical review about the different strategies for coupling two or more electrophoretic separation techniques and the different intermediate and final detection methods implemented for such separations.

Keywords Multidimensional electrophoretic separations · Column–coupling · Isotachophoresis · Isolectric focusing · Mass spectrometry

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Abbreviations

2DGE	Two-dimensional gel electrophoresis
APTS	8-Aminopyrene-1,3,6-trisulfonic acid
BSA	Bovine serum albumin
C^4D	Capacitively coupled contactless conductivity detection
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CGE	Capillary gel electrophoresis

CSE	Capillary sieving electrophoresis
EE	Electroextraction
EOF	Electroosmotic flow
EPC	Electrochemical preconcentration
ESI	Electrospray ionization
FEP	Fluorinated ethylene propylene
FIA	Flow injection analysis
FQ	3-(2-Furoyl)quinoline-2-carbox-aldehyde
FTICR	Fourier transform ion cyclotron resonance
GC	Gas chromatography
GE	Gel electrophoresis
HILIC	Hydrophilic interaction liquid chromatography
ID	Inner diameter
IEF	Isoelectric focusing
ITP	Isotachophoresis
LC	Liquid chromatography
MEKC	Micellar electrokinetic chromatography
MIPG	Monolithic immobilized pH gradient
MS	Mass spectrometry
NGSE	Non-gel sieving electrophoresis
OD	Outer diameter
PAGE	Polyacrylamide gel electrophoresis
PD	Pepsin digestion
PEEK	Polyetheretherketone
PDMS	Polydimethylsiloxane
PMMA	Poly(methyl methacrylate)
PTFE	Polytetrafluorethylene
RPLC	Reversed phase liquid chromatography
SEC	Size exclusion chromatography
SDS	Sodium dodecyl sulfate
TAMRA	5-Carboxyl tetramethylrhodamine succinimidyl ester
TE	Terminating electrolyte
tITP	Transient isotachophoresis
WBE	Wide bore electrophoresis

Introduction

Multidimensional electrophoretic separations represent one of the most powerful tools for dealing with the complexity of the analytical and bioanalytical problems that the scientific community is currently facing. This complexity arises from the combination of the number of compounds to be analyzed with different physicochemical properties (sometimes thousands), the diverse composition of the biological matrices for the analytes, and the broad range of possible concentrations that can usually span six to ten orders of magnitude [1]. Under these analytical conditions, that are typical in proteomic and metabolomic studies, sample pretreatment is mandatory to eliminate unwanted matrix components (interfering solutes and inorganic salts, among others), to normalize concentrations and enhance separation performance, to improve both selectivity and sensitivity [2]. Historically, two-dimensional electrophoretic separations have been one of the favorite tools for dealing with complex biological samples due to their separation capabilities [3]. An example is 2DGE [4] used extensively in studies on protein expression, peptide mapping, and also in different metabolomics studies [5]. Despite its popularity, its high selectivity and sensitivity, 2DGE as it is performed today involves manually intensive steps: casting of gels, application of samples, running, and staining of gels; all of them are time-consuming tasks that conspire against the reproducibility and quantitative reliability of the method, as well as the possibility of automation. Moreover, the combination of 2DGE with MS, the most effective and promising identification technique, is inefficient [6]. All these problems are also common to most off-line sample pretreatment techniques. In contrast, column–based electrophoretic separations offer impressive separation efficiency, the possibility to easily change separation conditions to account for different physicochemical properties of the molecules, a high compatibility with several detection methods based on electrical, optical (visible and UV range), and electrochemical properties of the analytes, and excellent coupling performance with MS detection [7]. Then, electrophoretic separation techniques offer a unique chance for obtaining high throughput, parallelization automatic analysis of biomolecules, using a complete set of very powerful separation techniques (CE, IEF, ITP, MEKC, CEC, among others) that can be combined in multidimensional methods as well as MS detection, in order to obtain a new generation of analytical tools that are able to satisfy the current analytical needs of the growing proteomics and metabolomics sciences.

Historically, the most common on-column two- and multi-dimensional methods have been chromatographic (both LC as well as GC) with a significantly higher utilization rate compared to electrophoretic methods. It is clear that the coupling of electrophoretic separations is technologically more challenging. These challenges basically ground on two particular characteristics of electrophoretic separations: high electric potentials and low sample volumes. Regarding the high electric potentials, the coupling devices have to be made from electrically isolating materials (hindering e.g. the use of classical LC stainless steel loop valves), but also making it difficult to control unwanted electric fields in those columns where separation is not developing causing inconvenient leakages, dilutions and band broadening. Regarding the low sample volumes, the situation is even more challenging due to the fact that dead volumes, leakages or any kind of failure in the fluidic circuit, as small as some nanoliters, will directly affect the peak shape, detection limits, and separation efficiency of the method. From the fluidic point of view, couplings for

electrophoretic separation require geometric precision in the order of micrometers, making its manufacturing challenging and expensive. Due to these main differences to chromatography, until now there is no standard equipment for coupling column-based electrophoretic separations, in contrast to the plethora of valves, connectors, multiplexers, loops, etc. available for LC and GC.

The most obvious problem that arises when trying to combine column–based electrophoretic methods is the fact that columns (usually fused silica capillaries) are inherently one-dimensional. The most important question is how to implement efficient interfaces to combine the separation performance of individual 1D electrophoretic techniques in a 2D format, keeping the separation performance also in the column–coupled system compared to the one known from inherently two–dimensional method, such as 2DGE [8, 9].

From a practical point of view, 35 years ago, Everaerts et al. [10] proposed a system for the analysis of small anions via the combination of two isotachophoretic separations performed in narrow–bore tubes in order to surmount the shortcomings of 2DGE and take advantage of the capabilities of column–coupling electrophoretic methods. This pioneer work revealed the advantages but also the challenges of implementing 2D separations in column–based methods. Since then, several attempts were made in order to develop coupling systems that enable efficient combination of 1D electrophoretic methods and suitable detection systems. This review presents a critical summary of all these attempts, analyzing their potential and the future of these techniques for solving the current and future analytical questions generated by the growing proteomics and metabolomics research field.

Aims and scope

The present review intends to summarize and discuss different strategies for column–coupling in multidimensional electrophoretic separations and how these methods can be combined with different detection techniques, particularly with MS. Due to the variety and complexity of the technological aspects involved, we have also included some two-dimensional methods that involve one non-electrophoretic separation step (usually included as first dimension) such as LC, FIA, or SPE, among others. The idea behind this broader scope is to provide the reader with crucial information about coupling and sampling strategies also employed in fully electrophoretic multidimensional electrophoretic separations. We have also included some particular implementations of multidimensional electrophoretic separations in microchips in order to discuss common benefits and drawbacks of using the different coupling technologies: column–coupling, microchips or hybrid devices (combination of capillaries and microchips). Complementary information to this review can be found in the literature cited in the previous section: particularly in [2], the reader can find more specific information about coupling of continuous separation techniques to CE, in [11] details on column–coupling electrophoresis in biomedical analysis, and in [12], an excellent summary for multidimensional electrophoretic separations on chips is given.

System performance and sampling strategies

One of the most extensively used concepts to characterize the separation capabilities of an analytical method is the peak capacity (N_p), which represents the maximum number of components that could theoretically be separated on a given column within a given analysis time [13]. All related concepts and definitions about separation performance have been developed for chromatographic separations (the older works for GC and the more recent for LC), and later only adapted to electrophoretic separations [14]. In order to sustain the idea that multidimensional electrophoretic separations can improve analytical performance, we have to consider the way of calculating N_p for a multidimensional electrophoretic separation involving n separation steps. This can be written as follows [15]:

$$N_p = N_1 \prod_{1}^{n-1} \left(N_{j+1} O_{(s_{(j,j+1)})} B_{(j,j+1)} \right) \quad (1)$$

where N_j is the peak capacity of each individual separation technique j, $O_{(s_{(j,j+1)})}$ represents the degree of orthogonality of two consecutively coupled techniques j and $j+1$, which is also a function of the sampling rate between these methods $s_{(j,j+1)}$, and finally, $B_{(j,j+1)}$ is a factor for peak broadening. This factor is intended to quantify the band broadening or dispersion that peaks suffer while they are transferred from the column of the "Method j" to the column of the "Method $j+1$". Ideally, $O_{(s_{(j,j+1)})}$ and $B_{(j,j+1)}$ have values of 1, i.e. full orthogonality and no band broadening due to the coupling. Only then, the peak capacity of a multidimensional method is the product of each individual peak capacity. This demonstrate the large potential of multidimensional separation techniques to face the analytical challenges of proteomics and metabolomics with their complex samples.

The degree of orthogonality is not yet precisely defined in the literature, but it is normally understood as the independence of the employed separation mechanisms, or the underlying physicochemical properties, that are used for each separation, which means that the distribution of analyte signals (selectivity) in one dimension is not correlated with the analyte zone distribution in the other dimension [16].

For example, the combination of SEC or RPLC with CE should provide near–ideal orthogonality because the two methods are based on different physicochemical properties. As a numerical example, Pourhaghighi et al. [15] calculated the orthogonality of CE and off–gel electrophoresis hyphenation, with a theoretical conditional entropy approach, to be 86 %. As a comparison, the highest degree of orthogonality for peptide separation by 2D-LC systems that is reported, refers to the hyphenation of HILIC and RPLC with an identical orthogonality of 86 %. Paradoxically, the orthogonality aspects create an interesting problem in the design of multidimensional separation systems: the higher the orthogonality of the separation mechanisms, the more dissimilar is their operation and the more difficult it is to couple them due to the differences in the characteristics of the required equipment and chemistry [17]. In terms of Eq. 1, this fact can be seen as an inverse, relation between $O_{(s_{(j,j+1)})}$ and $B_{(j,j+1)}$.

As mentioned before, the degree of orthogonality $O_{(s_{(j,j+1)})}$ is a function of the sampling rate $s_{(j,j+1)}$. By sampling rate, we understand the velocity, ratio, or absolute number of sample transfer steps from one separation procedure to the next one including of course, the transfer of the sample peaks between columns. If two sample peaks separated in the first dimension by some distance Δx are transferred to the second dimension, and the two considered methods are completely independent, this distance can be increased, decreased or remain unaltered during the second separation. Of course, this depends on the selectivity of the methods and the relevant physicochemical properties of the two species. If the selectivity of the coupled separation decreases, the sampling rate may be increased in order to sample the two peaks in separate steps [18].

In multidimensional electrophoretic separations, four different strategies regarding sampling are frequently encountered: comprehensive single-step sampling, comprehensive multi-step sampling, heart cut multi-step sampling, and heart cut single-step sampling. *Comprehensive* refers to the transfer to the second dimension of the total sample that was injected at the beginning of the separation while *heart cut* means that only a selected fraction of the separated sample is transferred from the first to the second dimension. *Single-step* refers to those procedures in which the separation in the first dimension is stopped or finished after a single sampling procedure, while *multi-step* involves the restarting (often the continuous operation) of the separation in the first dimension followed by more sampling/restarting steps. Table 1 lists some examples of typical combinations of separation techniques for different sampling strategies.

The influence of the sampling strategy on the degree of orthogonality is beneficial in the case of heart cut single-step sampling allowing the successful coupling of two methods with a low degree of orthogonality, for example CE–CE [19] at different pH values. Of course this situation is not easy to achieve: very precise detection and transfer systems are needed to e.g. (partially) remove excess compounds. Another, favorable situation is the comprehensive multi–step sampling, but in this case the restriction is that the velocity of the second dimension has to be larger than the velocity of the first one. This is the typical situation in LC-CE coupling with their very high compatibility of time scales [20]. All these strategies about sampling involve benefits and drawbacks, and these are characterized in this review.

Table 1 Typical combinations of separation techniques for different sampling strategies

	Single-step	Multi-step
Comprehensive	ITP–ITP, ITP–CE	GC–GC, LC–LC, IEF-CE
Heart cut	CE–CE, IEF–CE	LC–CE, IEF–CE

Finally, for practical analytical purposes the presence of term $B_{(j,j+1)}$ in Eq. 1 implies that the peak capacity is even less than the expected one, because of the additional broadening of component zones during their transfer to the second dimension, particularly if this includes the transfer to a different column [16]. Commonly, these transfer effects involve simple diffusion (which can be minimized by decreasing the transfer time), advective dispersion due to heterogeneous velocity profiles, electrochemical dispersion due to differences in buffer conductivities or surface properties between columns [21], or more complex interactions between solvents of different polarity (Marangoni effect) [22]. All these effects are directly related to the relative dimensions of the peaks, the diameter of the separation columns, and the geometry of the interfaces, particularly the volume/surface ratios [23]. Then, band broadening effects are clearly more significant for smaller columns such as fused silica capillaries or microchannels and methods with intrinsically high N_p such as CE (compared to LC). Due to this, the optimization of interfaces in CE is by far more critical than in LC, generating challenging technological problems that are discussed in the following sections.

Column–coupling strategies

As discussed in the previous section, the coupling strategy between columns has a strong influence on the overall performance of the analytical methods. In this core section of the review we present and discuss different implementations of column–coupling multidimensional electrophoretic separations. As was pointed out, one of the main factors is the sampling strategy, related to the decoupling of flows by mechanical or electrical actuation. The benefits

and drawbacks of the different strategies are discussed. Several column–coupling multidimensional electrophoretic separations, as well as some important approaches including one non-electrophoretic separation technique are discussed. These implementations were specially selected due to their technological importance in the development of more recent or even future multidimensional electrophoretic separations [24].

The section starts first with a historical note about the most popular implementation of column–coupling electrophoretic separations developed by Kaniansky et al. [25], based on the original idea published by Everaerts et al. [10]. It even evolved into commercial devices and enabled the production of a plethora of scientific work in coupled ITP-ITP and ITP-CE separations. The section then continues with a discussion on interfaces based on fused silica capillaries and microcolumns. These types of interfaces are clearly the more abundant in the literature due to the extended use of fused silica capillaries and their high compatibility with various detection methods. They include some commercial structures associated to LC, such as multiport sampling valves, or PEEK MicroTee couplers, but also customized interfaces based on the flow gating principle, dialysis junctions or etched porous tips, among others.

We then discuss a particular type of implementation that is growing especially for methods using MS detection: hybrid systems, which include fused silica capillaries or microcolumns combined with microchips or microfabricated elements. They combine the advantages of standard capillaries, such as their low cost and perfect integration with injection and detection systems with the precision and efficiency of microchips for sample transfer. Finally, some very important microchip separations are discussed due to their influence on defining current and future trends in multidimensional electrophoretic separations and their integration to modern detection systems.

Commercial column–coupling ITP devices

As mentioned, the first work on column–coupling electrophoretic separations was presented by Everaerts et al. in 1979 [10]. Figure 1 presents a scheme of this setup including two separation columns consisting of narrow-bore tubes (0.8 mm and 0.2 mm ID, Numbers 5 and 12 in the figure), connected via a PTFE T-shaped piece (7), PTFE valves for injection (2) and sampling (8, 11, and 18), and semipermeable cellulose–polyacetate membranes (9 and 15) that enable: (i) electrode isolation to avoid the electrolysis products entering the column, (ii) Joule heat dissipation, through the higher volume and thermal transfer coefficient of the compartments, and (iii) EOF suppression due to the fluidic stalling. In this pioneer work, small anions were separated and detected using two conductivity detectors (one at each column end, 6 and 14) and one UV detector (256 nm, 13) located before the second conductivity detector. The total analysis time was 15 min for the ITP-ITP analysis of small acids in orange juice and human urine.

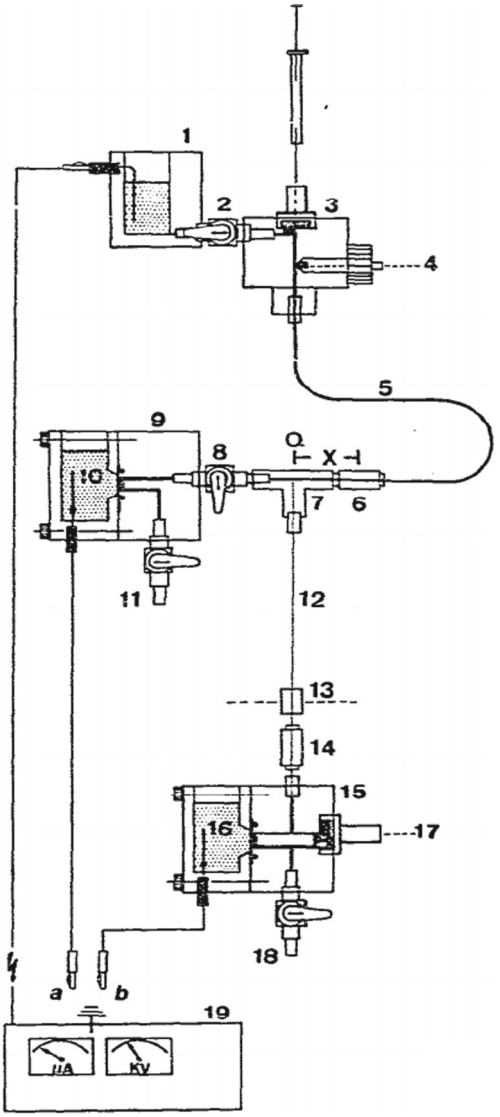

Fig. 1 Device for column–coupling ITP-ITP. 1 = TE compartment; 2, 8, 11, 18 = PTFE-lined valves; 3 = injection block with septum: 4 = drain; 5 = narrow-bore pre-separation tube; 6, 14 = conductivity detector; 7 = T-piece; 9, 15 = counter-electrode compartment with semi-permeable membrane; 10, 16 = counter electrodes; 12 = narrow-bore separation tube; 13 = photometric detector; 17 = septum through which a counter flow of electrolyte can be applied; 19 = current stabilized power supply. Reprinted from [10] with permission

Following a similar concept, in the mid 80's Kaniansky et al. [26] developed a column–coupling commercial device known at the beginning as CS Isotachophoretic Analyzer [27]. This device is produced in Slovakia by the company Villa Labeco since 1992. The first research paper in the literature using this commercial device appeared in 1987, but only used in a single column mode [28]. In

1990, Kanianski et al. reported for the first time an ITP-CE analysis of nitrophenols and 2,4-dinitrophenyl-labelled amino acids [25]. An identical device, the ItaChrom Isotachophoretic System, was produced by the companies Merck (Darmstadt, Germany) from 1997 to 1999, J&M Analytik AG (Aalen, Germany) from 1999 to 2006, and JH-Analytik (Aalen, Germany) since 2006, and mentioned for the first time in literature by Kaniansky et al. in 1997 [29].

In these devices, columns are made of FEP or quartz, with the pre-separation (first) column of 0.8 mm ID, and variable ID and lengths for the analytical (second) column. The coupling is made by a T-shaped plexiglass element [30] including also two standard conductivity detectors (these are contact amperometric detectors, optionally the devices are provided with C^4D detectors) and a UV (200, 220, 254, 280 nm) or UV-Vis photometric detector (190–600 nm) at the end of the analytical column. These isotachophoretic analyzers include dialysis membranes between the electrode compartments and the columns, to suppress the EOF and allow efficient dissipation of Joule heat. The devices also incorporate a connection for an autosampler for automatic analysis. Since 1985 the CS Isotachophoretic Analyzer and the ItaChrom Isotachophoretic System were used in more than 300 reported scientific papers according to the google scholar website. The exhaustive list of all these publications (near 300) using these two commercial devices exceed the scope of this review, it can be found on the cited scholar searches or in [2, 11].

Capillary and microcolumn based implementations

The implementation of column–coupling electrophoretic separations started in the early 90's but still grows today. Table 2 lists the main characteristics of the different strategies for implementing multidimensional electrophoretic separations using microcolumn- and capillary–based technologies mentioned in this section following a chronological order.

During the 90's Jorgenson's group did an extensive work on LC-CE separations [31–35]. They started with a computer controlled 6-port valve [31], but quickly switched to a more efficient flow–gating interface [32] demonstrating the importance of having precise control over the coupling and sampling procedure besides the excellent analytical performance of the separation methods employed (LC-CE, RPLC-CE, SEC-CE). The first version of this interface, as well as a scheme of the full two-dimensional separation setup are shown in Fig. 2.

According to the authors, the flow-gating interface is advantageous compared to the loop-valve system since it eliminates sample collection, storing, and "out-flushing" between each analysis. The interface consisted of two stainless steel plates separated by a Teflon gasket, where the outlet of the chromatography column (SEC microcolumn in [32]) was positioned directly across the inlet of the electrophoresis capillary. Normally, a transverse flow of buffer enters through the top port of the interface for flushing, sweeps through the channel and exits through the bottom port. This flow of CE buffer carries SEC effluent away to waste, preventing transfer of sample to the CE capillary. When an injection is desired, the flush–flow is stopped, allowing the SEC effluent flow to inject sample into the narrow gap separating the SEC microcolumn and CE capillary. When the desired injection is completed, the transverse flow is resumed, terminating the injection process. Figure 3 shows a photograph of this injection procedure, from the SEC column on the left to the CE capillary on the right.

Clearly the flow–gating interface minimizes dead volume (low $B_{(j,j+1)}$ value) compared to the sample loop valve, but also offers the possibility of a flexible and precise control of the injection of the sample to the second dimension. Jorgenson's group and other scientists have used this interface for the successful analysis of various proteins, protein digests and small anions [36]. Jorgenson's group experiments using the flow–gating interface are summarized in Table 2.

Following the idea of using transverse flows, Dovichi's group developed several multidimensional electrophoretic separations using different implementations of this concept. In Fig. 4 the evolution of these interfaces over the years 2002 (Fig. 4a) to 2013 (Fig. 4c) is illustrated. The first interface, shown in Fig. 4a, was constructed by gluing four corners of a microscope slide for aligning the separation capillaries (left and right) and the buffer refilling tubes (top and bottom), assembled between two intact microscope slides and fixated with epoxy glue [37]. This very simple construction was used for the analysis of a complex sample (protein homogenate from *D. radiodurans*) using LIF detection achieving excellent sensitivity in the range of zeptomoles.

Although the setup of the interfaces refers to a similar concept (transverse buffer flow) we must point out some differences between the general strategies from Jorgenson and Dovichi: First of all, Dovichi used electrophoretic separations on both columns, which enabled them to transfer the whole sample plug (in single- or multiple-step mode) from the first to the second column, which is impossible when performing LC-CE due to the differences in eluent flow and separation velocity of the methods. In the case of CE–CE, the transverse flow is used exclusively for refilling the second dimension with BGE and not for eluting sample excess [19, 38].

The evolution of the Dovichi's interface continued, and they produced a set of very interesting interfaces that included enzymatic reactors with immobilized pepsin [39], alkaline phosphatase [40], and trypsin [41]. The interface including the immobilized pepsin reactor is shown in

Table 2 Capillary and microcolumn based interfaces listed in chronological order. For complete acronyms see the abbreviation list at the beginning of the review

Separation methods	Interface	Intermediate detection	Final detection	Sample	Ref.
ITP-ITP	PTFE T-piece	Conductivity	UV (256 nm)	Orange juice and human urine	[10]
ITP-CE	Sampling valve	Conductivity	UV-VIS (405 nm)	Nitrophenol and 2-4 dinitrophenyl labeled amino acids (2 μmol)	[25]
ITP-CE	Direct coupling	UV (234/254 nm)	LIF (488/514 nm)	FITC labeled amino acids (1 ng/ml)	[45]
ITP-CE	PTFE block	Conductivity	UV (214 nm)	Lysozyme, cytochrome c, trypsin, ribonuclease A, $\alpha-$chymotrypsinogen ($\approx$ 1 μmol)	[30]
SEC-CE	Flow–gating interface (Stainless Steel/Teflon)	–	UV (214 nm)	Thyroglobulin, BSA, chicken egg albumin, and myoglobin (0.5 % w/v)	[32]
ITP-CE	Polyethylene T-piece	UV (200 nm)	UV (200 nm)	Neostigmine and propantheline (2.5 nmol)	[46]
RPLC-CE	Stainless steel T-piece	UV (200 nm)	UV (200 nm)	FITC labeled tryptic digest of horse cytochrome c	[33]
EE-ITP-CE	Transparent polyethylene connector	–	ESI-MS	Clenbuterol, salbutamol, terbutaline and fenoterol (2–5 nmol)	[47]
RPLC-CE	Flow–gating interface	UV (200 nm)	ESI-MS	Tryptic digests of ribonuclease B	[34]
ITP-CE	Direct coupling (epoxy glue)	–	UV (200 nm)	Neostigmine bromide and propantheline bromide (2.5 nmol)	[75]
SPE-CE	PTFE tube	–	MS/MS	Proteins from yeast ribosome	[70]
ITP-CEC	Polyethylene T-piece	UV (210 nm)	ESI-MS	Neostigmine, salbutamol and fenoterol (13 nmol)	[48]
ITP-CE	Quartz T-piece	UV-Vis	UV-Vis	Human angiotensins (10 μmol)	[49]
CE-CE	Lexan T-piece	–	LIF (488 nm)	HT29 cell lysate (pmol)	[19]
IEF-tITP-CE	Microdialysis interface	cIEF electric current	UV (214 nm)	Cytochrome c, ribonuclease A, and carbonic anhydrase II digests	[58]
ITP-CE	Quartz T-piece	UV	ESI-MS	Human angiotensins (5 μmol)	[50]
IEF-CGE	Dialysis interface	cIEF electric current	UV (280 nm)	Hemoglobin	[61]
IEF-CE	Dialysis interface	cIEF electric current	UV (280 nm)	Ribonuclease	[60]
IEF-CE	Porous etched interface	cIEF electric current	UV (280 nm)	Myoglobin and hemoglobin (1.0 mg/ml)	[63]
IEF-NGSE	Dialysis interface	cIEF electric current	UV (280 nm)	Rat's lung cancer cell lysate	[62]
CSE-MEKC	Epoxy glued microscope slides assembly	–	LIF-sheath flow cuvette (488 nm)	protein homogenate from *D. radiodurans*	[37]
IEF-RPLC	Microdialysis membrane-based cathodic cell	UV (280 nm)	ESI-MS	Cytochrome c (2 fmol) homogenate from *D. radiodurans*	[52]
IEF-CEC	6-port nanoinjector valve	–	UV (280 nm)	Albumin-depleted human serum	[54]
SEC-SPE-CE	PEEK MicroTee	UV (215 nm)	UV (200 nm)	Enkephalins in CSF	[71]
CE-CE	Tangential connection	–	UV (225 nm)	Mesityl oxide (500 μmol) and m-nitrophenol (50 μmol)	[67]
CE-MEKC	Tangential connection	–	UV (210 nm)	BSA tryptic digest (1.7 mg/ml)	[67]
CE-PD-CE	Micromachined plexiglas	–	ESI-MS	Cytochrome c and myoglobine	[39]
CESE-MEKC	Epoxy glued microscope slides assembly	–	5-port LIF-sheath flow cuvette (473 nm)	Lung cancer cell lysate	[44]
CE-MEKC	Micro-hole	–	Electrochemical	Basic cardiovascular drugs in mouse blood	[66]
CE-CE	Micromachined plexiglas	–	ESI-MS	Insulin chain b oxidized (0.3 mg/mL) and β-casein (1.2 mg/mL)	[41]
EPC-CE	Acrylic cell	Electrochemical	C^4D	Tl, Cu, Pb and Cd (20 nmol)	[68]

Table 2 (continued)

Separation methods	Interface	Intermediate detection	Final detection	Sample	Ref.
(MIPG)IEF-CE	Porous etched interface	cIEF electric current	UV (214 nm)	Milk proteins	[65]
IEF-CEC	6-port micro-injection valve	UV (280 nm)	UV (214 nm)	Human red blood cell lysate	[57]
SPE-CE	Coaxial external tube	–	ESI-MS	APTS-labeled glycans (50 nmol)	[73]
CE-CE	Nicked sleeve	–	LIF-sheath flow cuvette (532 nm)	TAMRA	[43]

Fig. 4b: proteins are separated in the first dimension by CE, digested in the reactor, and the cleaved peptides are transferred to the second capillary, where they are separated by CE. While peptides generated from one digestion are separated in the second capillary, the next protein fraction undergoes digestion in the microreactor [39].

Following a similar construction, a diagonal CE-CE method is used for monitoring the phosphorylation status of a mixture of peptides. Diagonal CE-CE consists in the alignment of the two electropherograms (with and without reaction) on an x-y plot in order to detect spots out of the diagonal which represent the modified (in this case phosphorylated) peptides. This implementation seems to be able to detect any post-translational modification (with different electrophoretic mobilities) as long as an immobilized enzyme (e.g. phosphorylase) is available to cleave the modification under electrophoretic conditions [40]. This setup was also used for studies of post-translational modifications using MS detection [42].

The third enzymatic reactor incorporates a replaceable and more generic enzymatic microreactor for on-line protein digestion with trypsin immobilized on magnetic beads. At the start of each experiment, old beads are flushed to waste and replaced by a fresh plug of beads, which is captured by a pair of magnets at the distal tip of the first capillary. Proteins are separated in the first capillary. A fraction is then parked in the reactor to create peptides. Digested peptides are periodically transferred to the second capillary for separation and MS detection while a fresh protein fraction is simultaneously moved to the reactor for digestion [41].

More recently, a high precision alignment interface was developed. This interface is depicted in Fig. 4c. Here, a nicked sleeve capillary interface was built, using a sleeve capillary which was cut approximately halfway through its inner diameter, resulting in a nick that exposed the inner diameter of the sleeve capillary, while maintaining an adequate length of the supporting capillary under the nick. The sleeve capillary was nicked using a micro-dicing saw and diamond blades. Only transfer efficiency was evaluated for this interface showing impressive results compared to the previous devices [43].

Additionally, a 5-capillary interface was developed for multiplexed LIF sheath-flow cuvette detection. CSE-MEKC expression fingerprints were obtained from homogenates prepared from a lung cancer cell line. The interface was a micromachined polycarbonate block with guide tubes for capillary alignment with a gap of 40 μm between columns of the first and second dimension [44].

The group of van der Greef developed several implementations of two-dimensional electrophoretic separations, particularly ITP-CE. Using commercial equipment at the beginning (Isotachophor; LKB, Bromma, Sweden), they tested different combinations of column diameters and

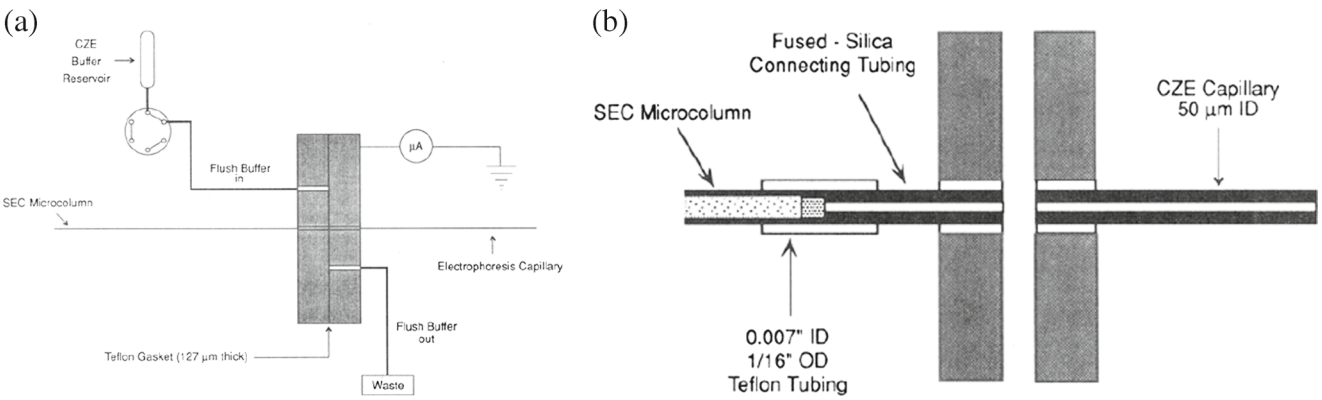

Fig. 2 **a** Full setup for the SEC-CE separation. **b** Constructive details of the flow–gating interface. Reprinted from [32] with permission

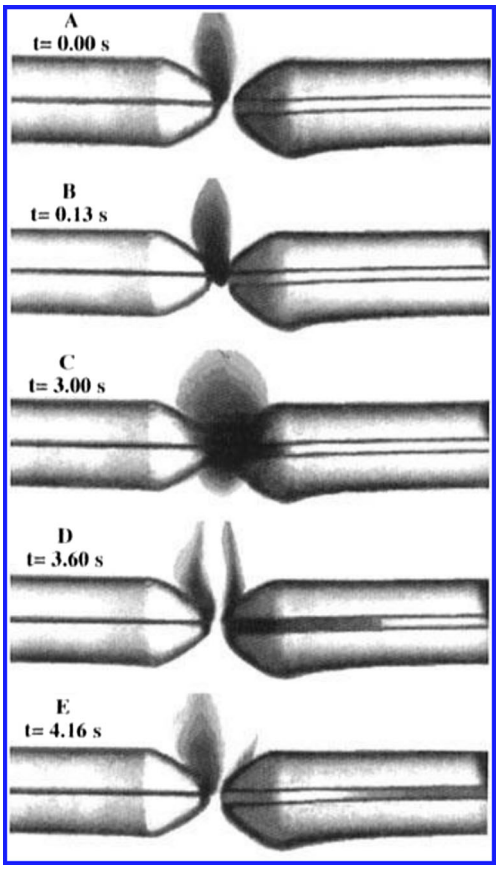

Fig. 3 Photographs of the injection procedure using the flow–gating interface proposed by Jorgenson's group. Injection is performed from the SEC column (*left*) to the CE column (*right*) from 3.00 s to 3.60 s. Reprinted from [35] with permission

materials (PTFE and fused silica) [45]. With this set of experiments they confirmed the postulate that a self stacking technique such as ITP considerably reduces the band broadening (decreasing the $B_{(j,j+1)}$ value) during the sample transfer to the second dimension. Additionally, in order to improve the performance of their method (by reducing the ID of the CE capillary) they developed a T-shaped polyethylene interface for connecting three capillaries [46]. Using this setup, this group has also demonstrated successful developments for EE-ITP-CE [47] and ITP-CEC [48] showing excellent performance for the analysis of important biogenic amines.

Another very elegant implementation of ITP-CE was conducted by Bowerbank et al. [49, 50], who produced the quartz coupling element shown in Fig. 5. The interface was custom-made by Innova-Quartz (Phoenix, AZ, USA), with the capillaries fixed by epoxy glue. When the capillaries broke, the interface was submerged into acetone in order to reuse it. The surface similarity between the fused silica capillaries and the quartz interface enabled the use of commercial coatings for suppressing EOF and reduce adsorption of biomolecules. This interface was used to perform ITP-CE using UV [49] and MS [50] as final detection methods for the analysis of angiotensins.

A particular case of multidimensional separations are those that include IEF. This technique is acquiring more and more popularity in the scientific community due to its excellent properties on separation and preconcentration of proteins and peptides. Mostly IEF is the first dimension due to the fact that the ampholytes used for generating the pH gradient interfere severely with many detection systems, particularly with ESI-MS and for IEF most often performed static. For this reason several groups have implemented combinations of IEF and LC. Due to the high orthogonality between these methods (high $O_{(s_{(j,j+1)})}$ value), the combination of the equipment necessary for the coupling is extremely complex including up to three 10-ports valves and several sample loops, requiring highly complex flow control systems [51–57].

More interesting for us are those 2DCE separations that include IEF. For example, in 2002 Mohan et al. presented a

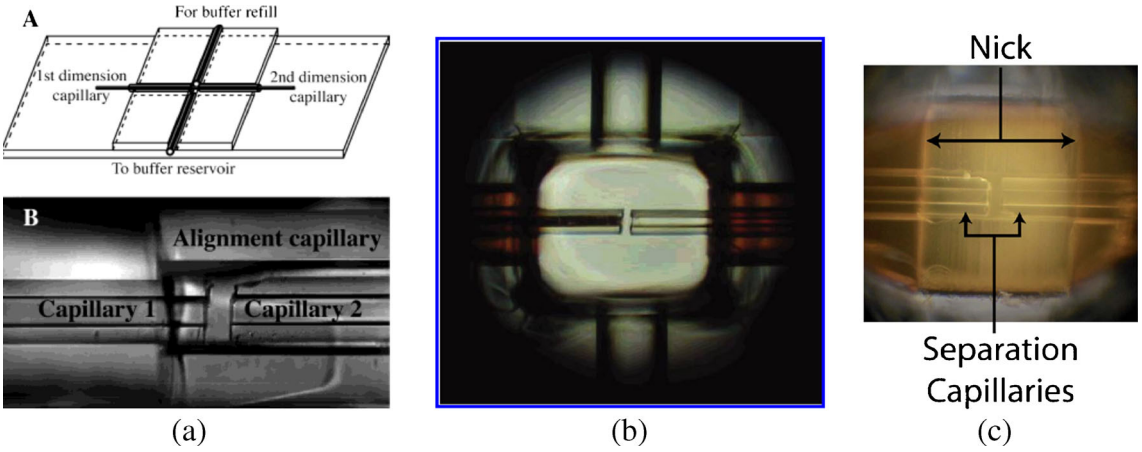

Fig. 4 Three versions of the CE-CE interface developed by Dovichi's group in **a** 2002, **b** 2005, and **c** 2013. Reprinted from [37, 39, 43] with permission

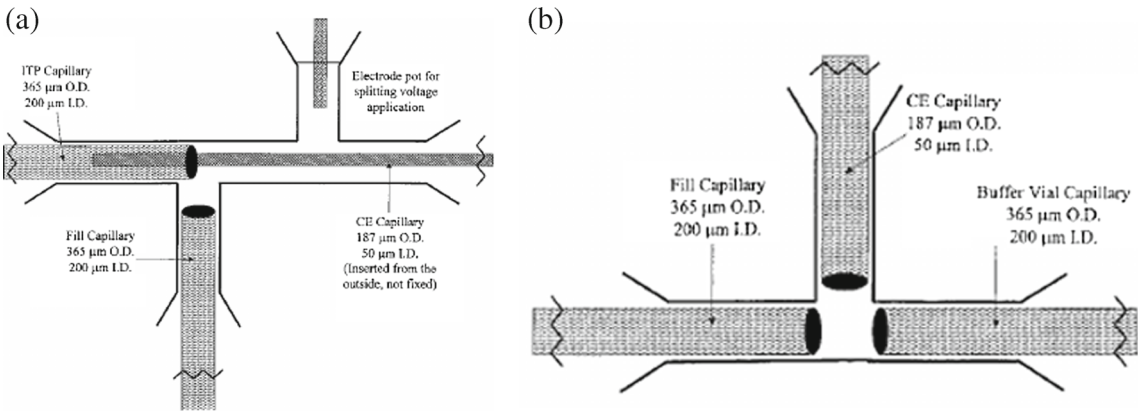

Fig. 5 Quartz interface proposed by Bowerbank et al. with (**a**) and without (**b**) intermediate electrode for voltage splitting. Reprinted from [49] with permission

coaxial arrangement of two capillaries coupled via a dialysis tube for a comprehensive multi-step IEF-tITP-CE analysis of different enzymes. The presented setup and the interface are shown in Fig. 6. The two separation capillaries were butted together inside a 7 mm length polysulfone dialysis tubing (nominal molecular weight cutoff of 10 000 Da) and secured with epoxy glue. The dialysis tube enables the intermediate electric connection and the dilution of the ampholytes in acetic acid prior to the CE separation in order to enhance the analyte detection via UV [58] or MS [59].

Similar implementations of this idea, but using a rigid PMMA reservoir and a hollow fiber were developed by Yang et al. performing IEF–CE of ribonuclease [60] and IEF-CGE of different types of hemoglobin [61]. Especially this study shows the advantages of the two-dimensional method over the independent 1D methods for the separation and detection of the studied proteins demonstrating a clear enhancement of the separation efficiency. In a subsequent work, Liu et al. performed IEF–CGE of protein mixtures excreting from lung cancer cells of rat using the same interface [62].

Another interesting work from this research group is the development of a porous etched interface. This is neither strictly a column–coupling method, because only one capillary is used, but is included here due to its simplicity and efficiency: The interface is fabricated by etching a short segment in the center of a fused silica capillary tube with HF to produce a porous area to enable electric contact between inner and outer regions and selective diffusion of small molecules. This zero dead volume interface was tested and characterized performing IEF-CE experiments with myoglobin and hemoglobin as model samples and using UV detection [63]. This porous etched interface was extensively characterized for its use in multidimensional electrophoretic separations in a more recent work [64]. An application example was presented by Wang et al. who developed an IEF–CE analysis of proteins extracted from milk. The particular feature of this implementation was the monolithic immobilized pH gradient that avoids the use of mobile carrier ampholytes for performing IEF [65]. Although possible, to our knowledge this strategy has not yet been coupled to MS detection.

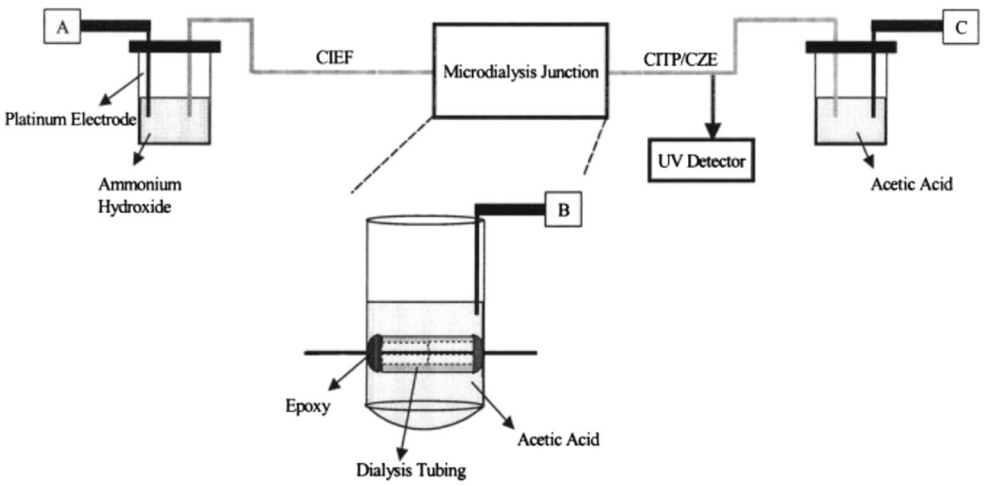

Fig. 6 Scheme for the IEF-tITP-CE setup proposed by Mohan et al. Reprinted from [58] with permission

Similar to the porous etched interface, Zhang et al. developed a microhole interface for performing CE-MEKC separations of cardiovascular drugs. Prior to the CE separation they performed on-line preconcentration procedures such as pH junction and sweeping to reduce the sample zone broadening at the interface. The microhole (30–40 μm) was drilled in the middle part of a PTFE tube of 300 μm ID. Then, the capillaries for the separation were inserted while hot air was blown in order to shrink PTFE tubing ID to the capillaries OD (360 μm). The gap between the capillaries was adjusted to the microhole diameter. An eppendorf tube and a platinum wire were used as reservoir and electrode, respectively. The combination of CE and MEKC in the 2D separation showed clear advantages on separation efficiency compared with the individual methods [66].

Another simple, but very effective interface was developed by Sahlin et al. [67] using tangentially connected capillaries as depicted in Fig. 7: two bi-layer PTFE-FEP tubes were melted jointly at the cross intersection with the help of two tungsten wires: four capillaries (75 μm ID) were coupled to the melted PTFE-FEP tubes to configure a four branches fluidic circuit. A tryptic digest of BSA was analyzed by CE-CE with a comprehensive multi-step procedure, no intermediate detection was used. Although the assay was successful, it is clear that the 3D nature of the fluid and sample flow, and the heterogeneous surface properties generated significant distortions of the peak shape of the analytes detected with UV (225 nm).

An alternative for the enhancement of sensitivity and separation efficiency of electrophoretic separations is the coupling to preconcentration techniques. Due to their current importance in the analysis of samples present in complex matrices and trace analysis, we here include some important studies on column–coupling preconcentration techniques for electrophoretic separations.

Electrochemical preconcentration was successfully coupled with CE using a very complex interface consisting of an electrochemical flow cell with the CE capillary precisely aligned to the working electrode. Despite the laborious and critical alignment process, the method was successfully tested for the analysis of metals. Due to its performance, the authors claimed that this EPC-CE assay is a potential alternative to atomic absorption spectrometry or inductively-coupled plasma techniques for trace analysis of toxic heavy metals [68, 69].

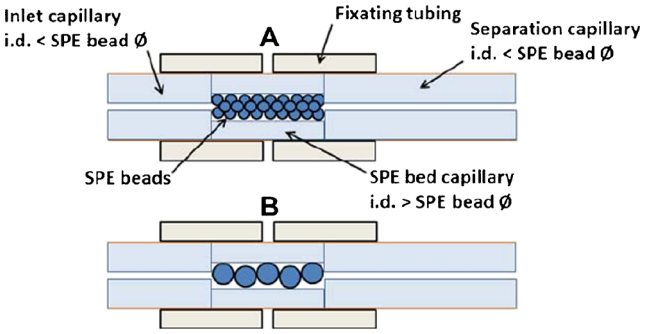

Fig. 8 Packing of two differently sized SPE beads using three capillaries with he same OD. (**A**) Inner diameter of the SPE column is much larger than the SPE particle diameter (**B**) Inner diameter of the SPE column is slightly larger than the SPE particles diameter. Reprinted from [73] with permission

For finishing this section, another strategy that is explosively growing is the on-line coupling of SPE. This (non-electrophoretic) technique enables relatively large sample loadability. The analytes are retained by the solid phase and accompanying compounds, e.g. salts, can be removed. Upon desorption of the analytes from the column with an eluting solvent, the analytes can be concentrated in a small volume plug, resulting in low detection limits. Some examples of coupling SPE with electrophoretic separation techniques include SPE-CE-MS/MS early developed by Tong et al. using a simple PTFE external tube for the packing of SPE material for the analysis of a protein complex from yeast ribosome [70]. Tempels et al. coupled SEC-SPE-CE via a simple commercial PEEK MicroTee with a void volume of 29 nL [71] for the analysis of enkephalins in cerebrospinal fluid (CSF). Puig et al. presented a similar method for determining two cephalosporins (cefoperazone and ceftiofur) in plasma [72]. Jooss et al. presented a really simple but effective strategy for performing SPE–CE in a coupled column by trapping SPE beads in a larger ID capillary between the injection and separation capillary. The three capillaries have the same OD making the coupling easier. This very simple setup is shown in Fig. 8. With this arrangement, and a complex procedure including more than 9 steps, the authors performed the analysis of APTS labeled glycans

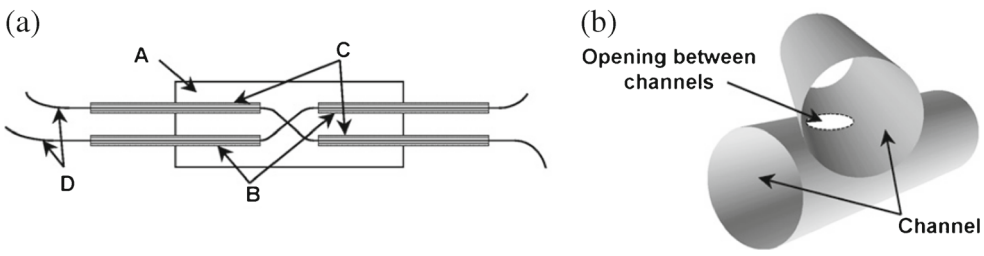

Fig. 7 Interface with tangentially connected capillaries presented by Sahlin et al. Reprinted from [67] with permission

wit MS detection, reaching an enrichment factor of more than 800 [73]. A more complete and detailed review about SPE-CE coupling can be found in [74].

Hybrid systems

Despite the outstanding analytical performance of some of the interfaces that have been discussed in the previous section they still show clear drawbacks due to: (i) their dead volume, maximized when the multiport LC valves were used, (ii) the heterogeneous surface properties of those systems where PTFE or other polymer tubing was used in combination with fused silica capillaries, (iii) and the intricate velocity profiles produced when complex 3D structures are employed as interfaces. The presence of any of these characteristics decreases the efficiency of the separation methods by increasing the broadening of the analyte bands (increasing $B_{(j,j+1)}$ value) that are transferred from the first dimension to the second.

Although these characteristics are present for several interfaces, they are not critical in the overall performance of some previously shown separation methods: particularly, when LC is performed as first dimension, only fractions of the LC sample peaks are transferred (normally a portion near the center of a Gaussian distribution), and the broadening of these fractions is still acceptable due to the statistical properties of the sampled peak. Additionally, this situation clearly illustrates the difference in the characteristics scales of chromatographic and electrophoretic methods: while for LC it is possible to use valves and loops with dead volumes in the order of nanoliters, in electrophoretic methods this is not acceptable. Nevertheless, this difference in sample volumes has its correlation in terms of analysis time, being those much longer for LC.

It is thus mandatory to consider the microfluidic nature of CE, and consequently develop interfaces with much higher precision lowering the dead volume to the range of picoliters. At this point microfabrication configures itself as an alternative for improving the performance of column–coupling multidimensional electrophoretic separations. In the next two sections, we discuss multidimensional separations that were developed using microfabricated elements. We first focus on hybrid systems, i.e. implementations using standard capillaries or microcolumns combined with microfabricated structures. Table 3 lists the main properties of these hybrid systems. The next section covers some pure microchip separations.

The need for developing hybrid systems grounds on the trade-off of improving the interface to the level of the microscale fabrication to minimize the broadening effects during the sample transfer process, and the fact that capillary–based systems can work with standard equipment in terms of pressure and voltage regimes, as well as the integration with commercial equipment such as autosamplers and detection systems. In this sense, the first hybrid developments were created for coupling CE separations performed on chips with MS detection whose capillary-based CE–ESI interface is well established and validated. For example the studies of Figeys et al. [76] and Zang et al. [77, 78] focus on this kind of coupling using glass microchips for the separation and ESI-MS for detection of peptides. In these cases the uniform surface properties of capillaries and

Table 3 Hybrid capillary–microchip based interfaces listed in chronological order. For a complete acronym reference see the abbreviation list at the beginning of the review

Separation methods	Coupling technique	Intermediate detection	Final detection	Sample	Ref.
tITP-CE	Glass microfluidic chip	–	ESI-MS	Angiotensins (10 μg/l)	[23]
tITP-CE	Glass microfluidic chip	–	ESI-MS	Angiotensins (10 μg/l)	[77, 78]
LC-CE	Two-level PDMS structure	UV (210 nm)	ESI-MS	Leucine-enkephaline (300 ng/ml) 9 peptide standard (25 μg/ml)	[84]
LC-CE	Micro flow–gating interface	–	LIF (473 nm)	BSA tryptic digest	[85]
FIA-CE	Cast PDMS interface	UV	ESI-MS	Methionine-enkephalin, neurotensin and substance P	[82]
LC-CE	Two-level PDMS structure	UV (194 nm)	ESI-FTICR-MS	BSA tryptic digest	[83]
LC-CE	Micromachined plexiglas	–	LIF (470/590 nm)	FQ-labelled control serum	[88]
SPE-CE	Two-level double cross PDMS structure	–	ESI-MS	BSA tryptic digest	[93]
IEF-CGE	Triple glass microfluidic chip	–	ESI-MS	β-Lactoglobulin, trypsinogen, ovoalbumin, and BSA (5 μg/ml)	[89]
ITP-CE	Glass microfluidic chip	C^4D	ESI-MS	Human angiotensins (10 nM–100 μmol)	[90]
LC-CE	Glass microfluidic chip	–	ESI-MS	Digested IgG2 containing glycopeptides	[87]

glass microchips enabled the coating of the systems to avoid peptide adsorption.

At the end of the 90's, Harrison's group presented several studies on the optimization of chip–capillary connections, recognizing the problems of dead volume of the connections, the importance of the surface properties, and the difficulties of directly connecting chips with MS [23, 79]. The developed interfaces were tested and compared by performing tITP-CE-ESI-MS analysis of amino acids and CE-ESI-MS/MS of tryptic peptides from *P. sativum* lectin digests.

It is well known that microfabrication in glass is laborious and the sealing of the wafers is problematic [80]. As an alternative, fabrication methods in soft polymers such as PDMS have attracted much attention from the scientific community. Although PDMS chips present several drawbacks for performing electrophoretic separations such as their highly hydrophobic surface, their insufficient heat dissipation, and poor optical properties, many examples of electrophoretic separations have been implemented using this polymer. The main benefits of PDMS are its simple manufacturing process and its excellent capability for sealing interfaces, which configures this material as one of the favorite ones for hybrid implementations [81]. For example, Samskog et al. [82] presented a very simple protocol for the microfabrication of a PDMS interface, using two cross capillaries and a Petri dish for casting the PDMS. Despite its simplicity, the interface revealed several problems related to hydrophobic adsorption, and the electrolysis on the surface of the intermediate electrode. The coupling to MS through a capillary was successful for the detection of methionine-enkephalin, neurotensin and substance P. The same research group used this interface for performing LC-CE-ESI-MS for the analysis of BSA tryptic digests [83] and leucine-enkephaline [84].

An implementation that became extensively used was first presented by Yang et al. in 2003 [85, 86]. The idea was to perform LC in the classical way, and couple the LC column to a microfluidic chip for performing the sampling using a microfabricated interface (cross microchannels in this case), followed by the CE separation as second dimension and finally detect optically [85] or with MS [86]. In these cases, the LC column was fastened to the CE chip simply using PTFE tape.

Following a similar concept, Ramsey's group established a setup for performing mapping of post-translational modifications of complex biotherapeutics [87], e.g. the N-linked glycosylation of a monoclonal antibody by glycopeptide mapping using an LC-microchip(CE-ESI)-MS implementation. In contrast to other studies, the coupling of CE and ESI-MS was performed directly on-chip, including a sheath liquid channel in the glass microchip. Figure 9 shows a scheme of this hybrid implementation.

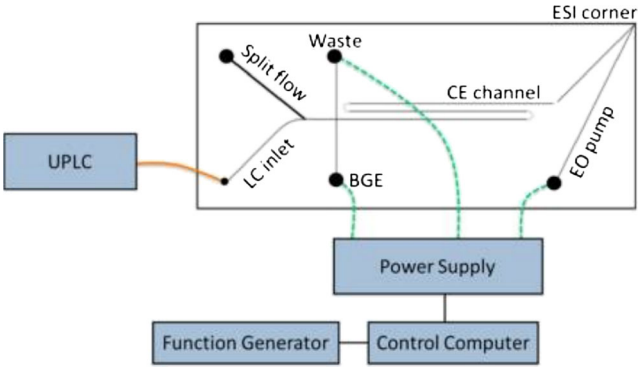

Fig. 9 Scheme of the LC-microchip(CE-ESI)-MS setup presented by Mellors et al. Reprinted from [87] with permission

Analogously, Skinner implemented a gel filtration chromatography interfaced with sub-micellar SDS–CE for on-line multidimensional separations [88]. An alternative interface, where two capillary inlets were moved in continuously flowing sample or BGE streams, was developed demonstrating the potential (and complexity) for multiple array LIF detection. The system was tested by analyzing FQ-fluorescently labeled serum components.

An outstanding hybrid implementation in capillary format was proposed by Lu et al. [89] for performing IEF-CGE with a close emulation of 2DGE. Their implementation consists of an arrangement of three microfluidic chips (see Fig. 10): one for coupling the IEF capillaries (X), one for coupling the multiple CGE capillaries (Z) and one for switching the columns between the two separation modes (Y). Figure 10 shows the arrangement of columns and channels in the IEF mode (Fig. 10a) and in the CGE mode (Fig. 10b). The main drawback of this implementation is

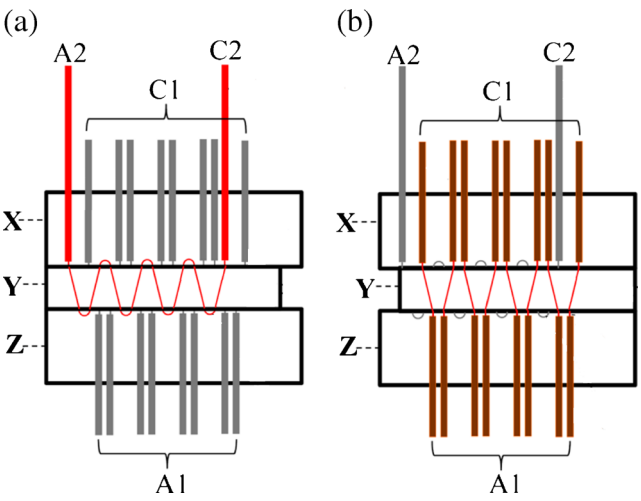

Fig. 10 Scheme of the chips arrangement for the two separation stages: **a** IEF and **b** CGE, performed in the hybrid interface presented by Lu et al. A2-C2: IEF channels; A1-C1: GCE channels. Reprinted from [89] with permission

the critical alignment of the chip Y in order to maximize the transfer efficiency. This was achieved with a micropositioner, but without any automatic closed–loop control, which means that the user has to decide on every measurement which is the right relative position of the chips. This interface was tested with a protein mixture containing β-lactoglobulin, trypsinogen, ovalbumin and BSA, detected via LIF.

Another promising hybrid implementation was recently presented by Kler et al. [90] using glass microchips for coupling commercial fused silica capillaries for implementing ITP-C^4D-CE-ESI-MS. The advantages of this implementation grounds on the use of similar surface properties for the capillary network and the interface, as well as the possibility of using commercial equipment for detection, control of injection, pressure, and voltage regimes. Another interesting fact is the complete flexibility for selecting different lengths and ID's for each capillary in the system enabling, for example, the use of higher ID for ITP to enhance loadability, and smaller ID for CE to increase sensitivity. Detection was performed using standard C^4D capillary heads and commercial CE-ESI-MS interfaces. The coupling of the capillaries with the microchip was achieved through powder-blasted holes in the glass chip with a precise diameter to minimize the space between capillaries and glass wall [91]. Capillaries were fixed with epoxy glue to cover this gap and reduce the dead volume to a few picoliters. This coupling is depicted in Fig. 11a, and Fig. 11b shows a comparison of the performance of the proposed interface, and a commercial PEEK MicroTee, taking an intact capillary as reference. The differences in dead volume and surface properties explained the impressive differences on the performance of the two interfaces for performing a CE separation of three cationic compounds. The chip interface was successfully tested for the separation and detection of four types of human angiotensins, using a comprehensive one-step (back cutting) sampling strategy. The method employed C^4D intermediate detection for the ITP, and MS detection for the final CE separation.

As described in the previous section, the use of SPE as a preconcentration technique is continuously growing in the study of proteins and peptides. This pretreatment technique was also successfully implemented on hybrid systems by Lee et al. [92, 93] using a PDMS chip for coupling the SPE column with a capillary CE separation followed by ESI-MS detection. This setup was successfully tested for the analysis of a complex protein digest (fetuin, α-casein, cytochrome c, myoglobin, transferrin and BSA).

Some important microfluidic chip implementations

Since the use of microfabricated structures for analytical applications was proposed by Manz et al. in the early 90's [94], almost any kind of analytical separation technique has its counterpart on a microfluidic platform. We here consider some of the microfluidic platforms that have been important for the development of two-dimensional electrophoretic separations in general. Their study enables us to understand future trends and the key technologies involved such as detection systems, coupling techniques, etc. A more exhaustive analysis about the implementation of multidimensional electrophoretic separations on chips can be found in several excellent reviews [6, 12, 95–97].

As mentioned in the previous sections, one of the main goals of scientists working on multidimensional electrophoretic separations has always been to transfer classical 2D IEF-PAGE into a column format, ideally with multiplexing. In contrast to the fact that a direct implementation of this method with spatial sampling is impossible using capillaries or microcolumns due to the high number of necessary elements, in a microchip, the simultaneous manufacturing of hundreds of columns in a reduced area is possible. Following this concept, Becker et al. [98] packed 500 columns for performing the second dimension of an IEF-CGE assay. A picture of the developed microchip is shown in Fig. 12. The first separation dimension (Sep. 1) consists of a single channel 16 mm long, 80 μm wide and 37 μm deep; the second separation dimension (Sep. 2) of an array of 500 channels, 5 mm long, 900 nm wide and 36 μm deep. The channels were fabricated using reactive ion etching under maximum anisotropic etching conditions, yielding an aspect ratio of up to 5 for the narrow channels. Despite the great

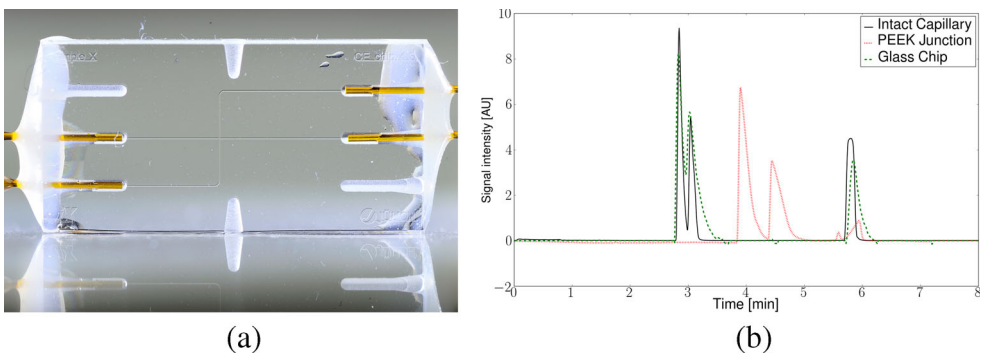

Fig. 11 **a** Hybrid capillary–chip interface proposed by Kler et al. **b** Performance comparison of different coupling devices for the CE separation of three cationic compounds. Reprinted from [90] with permission

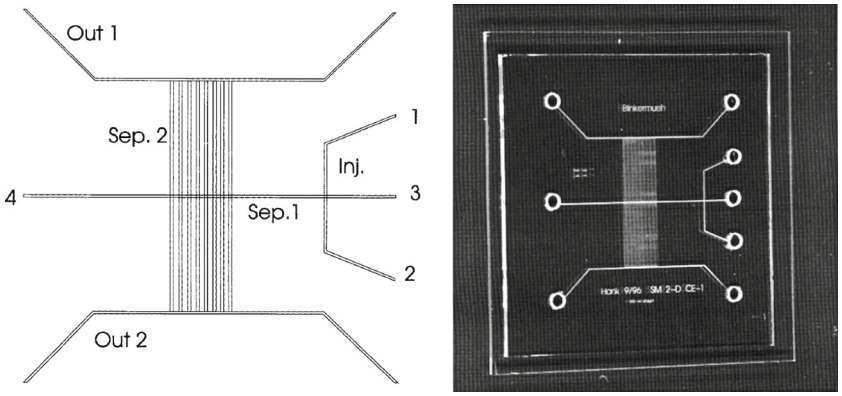

Fig. 12 Scheme and photography of the on-chip implementation of IEF-CE proposed by Becker et al. Reprinted from [98] with permission

technological significance of this chip, there is no literature describing its application.

The use of PDMS and soft lithography techniques was also extensively employed for multidimensional electrophoretic techniques, e.g. by Chen et al. [99]. They presented a complex 3D network of microchannels including vertical structures for performing 2DGE. Here, it was clearly shown, that despite its convenient fabrication costs, PDMS is not a suitable material for working with proteins (suffering selective adsorption) nor with high electric field strengths due to its low heat dissipation capacity.

The wish to implement 2DGE on chips continuously grew, and Emrich et al. [100] presented another version of multichannel on-chip 2DGE. They manufactured a two-layer borosilicate glass microdevice consisting of a single 3.75 cm long channel for IEF, which was sampled in parallel by 20 channels effecting a second-dimension separation by CE. They tested this design performing separations of complex cellular protein mixtures produced by *E. coli*. Detection was performed with a Berkeley rotary confocal scanner with laser excitation at 488 nm. Despite its apparent success, the importance of MS detection for proteomics and metabolomics studies was admitted as well as the difficulties of coupling this detection system to 20 output channels.

Another implementation of IEF-CE was developed by Herr et al. but using a more simple setup, using LIF detection for heart cut sampling into a single second column [101]. In contrast to the three strategies described so far, this implementation inspired several microchip and capillary implementations of IEF-CE and IEF-CE-MS that can be found in the literature of the last 10 years [12].

In terms of ITP-CE, of course the Kanianski group continued their excellent work implementing such techniques on chips [102]. Here, C^4D and MS detection systems were more efficiently coupled, compared to the commercial column–coupling device, and such developments are still a part of the research activities of the group and collaborators including novel CE–CE separations [103].

Other techniques that have been implemented for the fist time on-chip and particularly on hybrid systems were proposed by the Ramsey's group for MEKC-CE [104] and open channel CEC-CE [105]. In this case the authors manufactured the chips out of glass and employed LIF at two detection points.

A clear advantage of microchips is their ability to integrate efficient microelectromechanical structures such as microvalves, as was presented for the first time for multidimensional separations by Wang et al. [106]. In this work IEF-CE and IEF-CGE were successfully implemented. In contrast to their ability to integrate different components, these complex systems require special devices for controlling microvalves and pressure regimes as well as highly skilled operators, and other particular laboratory characteristics restricting their extensive or routine use.

Detection methods

Analytical capabilities of electrophoretic methods are frequently underestimated with the argument of their poor detection limits. The implementation of multidimensional electrophoretic separations are often related to the improvement of the sensitivity by both increasing method loadability (e.g. ITP–CE), and reducing the interference by non-target compounds on the detection systems (e.g. IEF–CE). In the case of multidimensional electrophoretic separations, we can differentiate between intermediate and final detection methods. Intermediate detection methods represent a novelty for multidimensional methods and offer the possibility to construct one of the axes of a 2D (or more dimensional) representation for a comprehensive sampling method. In addition they form a powerful tool to facilitate sample transfer especially in heart-cut procedures thus increasing the overall separation efficiency and also sensitivity of the final detection method. In this section we discuss several intermediate detection methods particularly C^4D in the case of ITP–ITP and ITP–CE, LIF extensively

used in CE-CE, or electric current for IEF–CE. Of course some of these methods are also employed for final detection, depending on the scope of the application and the sampling strategy.

In this section the different intermediate detection methods used in column–coupling multidimensional electrophoretic separations are revised. At the end of the section we especially discuss MS as final detection, and its integration to column–coupling multidimensional electrophoretic separations. Future trends, benefits and drawbacks of implementing and combining detection methods are presented as well.

UV-Vis

Historically the CE community used optical UV or UV-Vis methods present in almost any commercial CE equipment. It is principally well applicable as an intermediate detection technique due to the fact that most analytes, also peptides and proteins are UV active. The major concern can be the detection limit due to the small optical path length defined by the inner diameter of the separation channel. But its adaptation to different geometries is straightforward and thus it is the most common intermediate detection technique for multidimensional electrophoretic separations. In contrast, it requires a special feature for capillaries and channel walls: transparency for the chosen detection wavelength. For this reason windows have to be constructed on the capillaries by removing the polyimide layer considerably decreasing the robustness of the system. In the case of microchips, materials with suitable optical properties have to be employed restricting the applicability of this technique to quartz chips or hybrid systems [107]. This drawback was already mentioned as one of the most problematic limitation of the popular PDMS chips.

LIF

Currently, LIF combines sensitivity and selectivity, especially when derivatization is used. It offers detection limits several orders of magnitude lower than any other technique used in combination with electrophoretic separations. Dovichi's group showed impressive results achieving detection limits in the order of zeptomoles (10^{-21} mol) using the fluorogenic derivatization reagent FQ on lysine to yield a highly fluorescent product. This reagent is non-fluorescent until it reacts with a primary amine in the presence of a nucleophile [19].

In contrast to other techniques, LIF requires complex instrumentation, especially for microchip applications with microscope lenses. It often needs prior labeling steps, although some target molecules show native fluorescence when excited at low wavelengths. In the next years, the impressive technical characteristics of current laser diodes, LEDs, photodiodes, and high resolution CMOS sensors will facilitate cheaper and safer facilities for fluorescence detection. Several studies presented in the previous sections support this idea also for high throughput implementations for 2 [88], 5 [44], and even 60 capillaries in the second dimension [108, 109]. To our knowledge, LIF is currently only used as final detection in multidimensional electrophoretic separations. Nevertheless, this situation is currently changing due to the aforementioned reasons, and the growing use of whole column detection methods for IEF [110].

C^4D

Already in the first column–coupling method reported by Everaerts et al. in 1979, conductivity detection was applied [111]. Conductivity detection was historically implemented for amperometric or conductimetric detection having the working electrodes in direct contact with the solutions. This evoked several drawbacks regarding electrolysis and shielding due to molecule adsorption by induced charges. Alternatively, C^4D offers a comparable sensitivity, avoiding the problems of the immersed electrodes and enabling very convenient arrangements such as capillary heads or imprinted electrodes for on-chip detection. Figure 13 shows the implementation of a C^4D detection system for on-chip intermediate detection of ITP in ITP-C^4D-CE-ESI-MS analysis using a hybrid system. In this case the electrodes are imprinted on the circuit board enabling its positioning at any point of the channel network.

Due to its universal detection principle (every solution has an intrinsic electrical conductivity, which is not the case for UV activity for example), its impressively low costs, simplicity, and flexibility for implementing C^4D on capillaries [112], chips or interfaces [68, 69], it is one of the most promising techniques for intermediate (but also final) detection in multidimensional electrophoretic separations, particularly for low cost or portable systems [113].

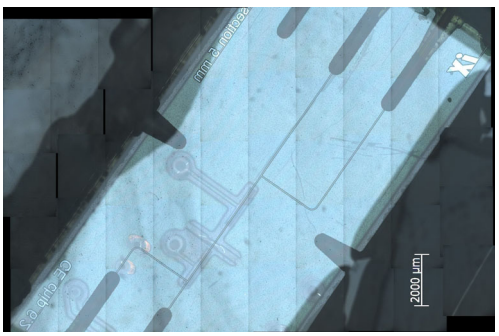

Fig. 13 On-chip C^4D implementation for intermediate detection in ITP-C^4D-CE-ESI-MS analysis on a hybrid system developed at Huhn's group in cooperation with CalvaSens, Aalen, Germany

Electrochemical detection

For this review, only one study was found in literature, where researchers used electrochemical detection for multidimensional electrophoretic separations [66]. Electrochemical detection is a very sensitive technique, but it requires significant efforts to achieve reliable and reproducible results. Despite its relatively low costs, this technique is not extensively used, but for certain applications requiring high sensitivity or monitoring molecular modifications related to redox reactions, electrochemical cells constitute an excellent tool for intermediate detection in multidimensional electrophoretic separations coupled to C^4D or MS.

IEF electric current

IEF is impressively growing as first dimension in column–coupling multidimensional electrophoretic separations. One of the most effective and by far the cheapest way to follow the focusing process during an IEF assay is monitoring the electric current. As far as the carrier ampholytes and the amphoteric samples focus on their isoelectric points, every individual effective electrophoretic mobility decreases, decreasing the overall conductivity of the capillary. As a consequence, the electric current is reduced upon focusing. Once the current is low and/or stable enough according to certain criteria: e.g. a predefined percentage of the initial current [60, 85] or a minimum variation [63, 65] the focusing process can certainly be considered complete. Strictly speaking it is not a detection technique, but it provides such a valuable information about the first dimension of the analysis that it can be considered crucial for IEF-CE assays.

MS coupling

Due to the increasing analytical requirements to obtain more detailed information on biomolecules, MS detection has become the most important detection technique for electrophoretic-based proteomics and metabolomics studies. Initially, in the early 90's the CE-MS coupling was performed via fast atom bombardment [114], but was rapidly replaced by ESI in almost every CE–MS coupling [48].

ESI-MS is extensively used for routine analysis, and commercial interfaces are well established and characterized. For this reason, microchips are often coupled to MS via capillaries as discussed in the section devoted to hybrid systems [77, 78]. Recently the direct coupling of chips was demonstrated offering similar or better performance [87]. Nevertheless, chip interfaces are not yet ready for routine analysis or non-expert operation. Consequently, hybrid systems represent an excellent opportunity to continue using the standard capillary based interfaces combined with the benefits of microchips [76, 79, 90]. For obvious reasons, ESI-MS is not used as intermediate detection in multidimensional electrophoretic separations [34].

A very singular problem appears for coupling multidimensional electrophoretic separations and MS: this end–column method is forcing one of the points of the fluidic/electric network to be at a fixed electric potential (ground potential in the case of Agilent and Bruker devices). This situation clearly interferes with separations performed in columns not directly connected to the MS generating sample leakage, via migration or EOF towards the MS. Strategies to surmount this problem include PDMS valves to isolate the MS potential [106]. Otherwise, complex multiport high voltage sources with closed loop controls would have to be developed.

One of the current challenges is IEF-MS coupling. It is extremely paradox that one of the favorite separation and preconcentration techniques is not compatible with the most favorite detection technique for proteomics and metabolomics analysis. The substantial problems stem from the need for carrier ampholytes in IEF, which coelute with the analytes and suppress their signal intensity. Finally, it is difficult to add reagents, such as urea that enhance solubility of hydrophobic proteins because their presence precludes ESI-MS. Mainly due to this reason, multidimensional electrophoretic separations, particularly IEF-CE-MS, are getting more and more popular. Alternatives to couple IEF to MS are the use of dialysis tubes and membranes to filter out the carrier ampholytes after the IEF and prior to the MS detection [52, 59], the use of monolithic immobilized pH gradients [65] to avoid the carrier ampholytes, or intermediate porous interfaces [63, 64].

It is clear that further effort will focus on the development of robust interfaces for coupling IEF to MS detection, including of course multidimensional electrophoretic separations [42]. More information about recent developments in this area can be found in reviews targeted to MS detection [96, 115, 116].

Conclusions

Column–coupling implementations for multidimensional electrophoretic separations involve several challenges such as the comparatively high dead volumes, the surface heterogeneity, the precise sampling and the intermediate detection techniques. Along this review we have shown different strategies to deal with these problems and how the proposed solutions were integrated with the detection systems.

One of the most common strategies is neglecting all the aforementioned challenges using LC as first dimension, and then perform a multi-step comprehensive sampling into a

second CE dimension. This can be an acceptable strategy if the performance of the LC separation is suitable for the selected sample, but mainly if the total analysis time is not a limitation, as well as the solvent consumption and waste generation are not of major concern.

The strategy most often applied is the use a self-sharpening technique in the first dimension of column–coupling systems that naturally avoids band broadening at the beginning of the second dimension as e.g. for IEF-CE and ITP-CE or ITP-ITP. Since its introduction in 1987 the commercial column–coupling equipment has been used on ITP–ITP and ITP-CE separations in more than 300 publications. If we consider non-commercial strategies for interfaces the most frequent is the use of commercially available components, mostly coming from LC technology e.g. sample loop valves, that are not completely suitable for efficient separations. These elements severely limit the sensitivity and separation efficiency of electrophoretic separations mostly due to the high dead volumes but also due to the surface heterogeneity.

New strategies involve very precise and surface-compatible glass microchips as separation platforms or coupling interfaces with excellent results. Some concerns about Joule heat dissipation, standard equipment for pressure and voltage regimes, valve operation, and packing of chromatographic material still have to be solved for these platforms. Microchips enable a more efficient integration with the electrical and optical detection systems, however, they are still far away from being standardized for routine analysis, despite the recent launching of the Agilent Bionalyzer (Agilent Technologies) and the LabChip GX/GX II (PerkinElmer). As far as microchip-based separations continue growing and its coupling to ESI-MS is getting more robust, it is clear that multidimensional electrophoretic separations will be efficiently implemented in such platforms in the near future.

Meanwhile, the hybrid systems are a trade-off between microfluidic precision, homogeneous surface, and commercially available technology. Based on capillary platforms they enable the use of commercial CE or LC equipment for controlling injection, voltage and pressure regimes and the use of all well established detection techniques: UV/UV-VIS, C^4D, LIF, ESI-MS, among others. In addition, using glass microchip interfaces enables the use of a broad range of coatings developed for controlling EOF and adsorption in capillaries. Moreover, the small dead volumes of microchannels minimize the effects of band broadening due to the sampling process offering an excellent balance between performance and technological availability for the current analytical needs of proteomics and metabolomics.

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