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Advances in Microscale Electro- and Liquid Phase-Based Separation Techniques

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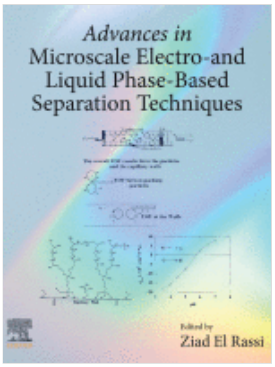

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About the book | Table of contents | Editor(s)

About the book

Key Features

- Summarizes a wide array of microscale separation techniques
- Demonstrates the applicability of microscale separation for the reduction of hazardous waste in numerous industries
- Addresses current and future problems in the field of microscale separation

Description

Advances in Microscale Electro- and Liquid Phase-Based Separation Techniques summarizes Microscale separation techniques characterized by fast, high resolution, and high efficiency with very reduced sample and solvent consumption. The book summarizes the existing body of knowledge, including hyphenated version to mass spectrometry (cLC-MS), capillary electrophoresis (CE), capillary zone electrophoresis (CZE and CZE-MS), micellar electrokinetic capillary chromatography (MEKC), capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF), capillary isotachophoresis (CITP), capillary electrochromatography (CEC), microfluidics, and microchips. It then demonstrates the applicability of these techniques to existing and future problems in the analysis of complex mixtures of biochemicals, pharmaceuticals, environmental, and natural products.

This book will be valuable to separation scientists, applications scientists, and technicians in industrial outlets, including those in pharmaceuticals, biomedicals, biotechnologies, and food and environmental industries. Students and professors who study separation techniques will also benefit greatly from the book's content.

Table of contents

○ *Free access*

Title page, Copyright, Contents, List of contributors, Preface

I - Capillary electrophoresis

Book chapter ○ Full text access

Chapter 1 - Fundamentals of capillary electrophoresis

Bohuslav Gaš

Pa 1

View PDF View chapter View abstract

CHAPTER

22

Fabrication methods for microchip capillary electrophoresis and capillary electrochromatography and their coupling to mass spectrometry

Mariana Bortholazzi Almeida[1,2], *Renata Santos Vieira da Conceição*[1,2], *Matheus Barraca*[1], *and Emanuel Carrilho*[1,2]

[1]Instituto de Química de São Carlos, Universidade de São Paulo, São Carlos, São Paulo, Brasil [2]Instituto Nacional de Ciência e Tecnologia em Bioanalítica - Lauro Kubota, Campinas, São Paulo, Brasil

22.1 Microchip fabrication for capillary electrophoresis

The basic principles of conventional capillary electrophoresis (CE) can be summarized as a method that separates mixtures of compounds using an electric field inside a fused silica capillary tube. One of the most widely used detectors is a UV spectrophotometer; however, electrochemical and mass spectrometry are also widely applied and emerging (Rahman, 2018). The miniaturized form of CE is MCE (microchip capillary electrophoresis). Harrison and colleagues introduced the MCE concept in 1992, and several research groups have worked with it since (Currie et al., 2009; Harrison et al., 1992). MCE is recognized for its advantages in simple microfabrication and the use of low-cost materials, which drive its growing application in various fields, primarily bioassays and environmental applications. Beyond manufacturing, MCE offers several advantages over CE, including the integration of different separation functions onto the chip, reduced consumption of samples and reagents, and faster and more efficient separations. Additionally, sample preparation zones can be integrated into the chip, encompassing washing, incubation, and derivatization steps. These possibilities allow the MCE to be used as a portable device for point-of-care analysis (Coltro et al., 2008; Hassan, 2021; Lobo-Júnior et al., 2020).

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22.1.1 Materials for microchip fabrication

MCEs can be manufactured from various materials, including glass, quartz, polymers, and even ceramics. The choice of material should be based on the viability of the laboratory facility, the desired application, and/or the detection mode. For instance, UV detectors require optically transparent substrates. Additionally, the materials must efficiently dissipate heat, enabling the application of high separation field strengths while minimizing Joule heating (Vandaveer IV et al., 2004). The main characteristics of the most common materials for MCE are summarized in Table 22.1.

For a long time, glass was considered a safe choice of material to fabricate MCE due to its similarity with fused silica capillaries (Coltro et al., 2008; Currie et al., 2009), as well as quartz, which was also widely used during the previous applications of MCE (Vandaveer IV et al., 2004). The use of quartz also has advantages due to its superior optical quality, which allows UV detection (Székely & Guttman, 2005). However, due to the cost associated with these materials, laborious steps, and the need for cleanroom facilities, polymer-based platforms, as well as photolithography, laser ablation, and injection molding techniques, started to be used (Currie et al., 2009).

It is known that electroosmotic flow (EOF) on glass is generated by the charged silanol groups present on the glass surface. Since plastic materials have fewer charged surface groups, the EOF is often lower, and the hydrophobic surface is difficult to wet. However, it is possible to control the surface charge and the EOF through surface modifications, which also help prevent nonspecific interactions between the analyte and the channel wall, allowing for and encouraging the use of plastic materials (Currie et al., 2009). The different types of surface modifications will be addressed in a later section, following the discussion of the materials and fabrication methods used in MCE.

The George Whitesides group first introduced polydimethylsiloxane (PDMS) as an alternative material for microfabrication in 1998, representing an evolutionary step in microchip fabrication (Duffy et al., 1998). The group demonstrated that it was possible to rapidly fabricate microfluidic devices through replica molding, a technique that became known as soft lithography. In addition, it demonstrated the feasibility of performing electrophoretic separations in PDMS microchannels. In this context, PDMS has proven particularly attractive for MCE due to its ease of molding, low cost, optical transparency, and compatibility with fluorescence or UV-Vis detection. Its flexibility and elasticity also enable seamless integration with pneumatic valves and pumps. However, it is essential to note that PDMS has certain limitations, including its inherent hydrophobicity, susceptibility to analyte adsorption, and the tendency of its channels to swell after prolonged exposure to fluids (Duffy et al., 1998; Toh et al., 2009).

PMMA (Polymethyl methacrylate) is one of the most popular polymer substrates for fabricating microfluidic devices. Although it requires specific manufacturing conditions, such as laser cutting and high temperatures and pressures to seal, it has advantageous characteristics, such as being a thermosetting polymer and rigid at room temperature, having optical transparency, low water absorption, low gas permeability, high Young's modulus, low elongation at break, and moderate chemical resistance (Lee et al., 2001; Lobo-Júnior et al., 2020).

Another thermoplastic polymer of interest for MCE fabrication is cyclic olefin copolymer (COC). It offers high optical transparency and low autofluorescence across a wide range of wavelengths. In addition, COC exhibits excellent chemical resistance, including resistance to polar solvents, low water absorption, and high

TABLE 22.1 A concise characteristics comparison of materials used to fabricate microchip capillary electrophoresis.

Material	Mechanical strength[a]	Optical transparency	Electrical insulation[b]	Chemical stability	Fabrication complexity	Surface modification	Estimated cost	References
Glass	High Fragile	High UV-Vis transparent	Excellent	Excellent. Resistant to solvents, acids, and bases (pH < 9)	Complex Requires a cleanroom and etching	Easily modifiable Silanization, coatings	High	Coltro et al. (2008), Currie et al. (2009), Lobo-Júnior et al. (2020)
Quartz	Very high More flexible than glass	Excellent UV-transparent, low autofluorescence	Excellent	Superior Highly resistant to harsh chemicals	Complex Similar to glass	Easily modifiable Similar to glass	Very high	Coltro et al. (2008), Lobo-Júnior et al. (2020)
PDMS (Polydimethylsiloxane)	Low Soft, elastomeric	Good Transparent in the visible range	Excellent	Poor Swells in organic solvents	Easy Rapid prototyping via soft lithography	Surface treatment needed Hydrophobic recovery	Low	Coltro et al. (2008)
PMMA(Polymethyl methacrylate)	Moderate Rigid but brittle	High transparent in the visible range	Good	Moderate Soluble in some solvents	Easier than glass Milling, hot embossing, laser ablation	Moderate Surface modifications possible	Medium	Coltro et al. (2008), Currie et al. (2009), Lobo-Júnior et al. (2020), Tsao et al. (2007)
COC(Cyclic Olefin Copolymer)	Moderate Good toughness	High Low autofluorescence, UV-transparent	Good	High Resistance to most solvents	Moderate Injection molding, hot embossing, laser ablation	Limited Less common functionalization options	Medium	Nunes et al. (2010)
Ceramics	Very high, rigid, and durable	Low to moderate opaque or translucent	Excellent	Excellent Resistance to heat and chemicals	Complex Sintering, machining required	Challenging Requires specialized treatments	High	Henry et al. (1999)
Polyethylene terephthalate- Toner (PET-Toner)	Moderate, flexible, and resistant	Moderate Semitransparent	Moderate	Moderate resistance to aqueous solvents but limited resistance to organic solvents	Very Easy Low-cost fabrication by laser printing and thermal bonding	Limited Difficult to chemically modify	Very Low	Coltro et al. (2008)

[a] *Mechanical strength refers to a material's ability to withstand applied forces without breaking, deforming, or failing.*
[b] *Excellent: high dielectric strength; Good: Low dielectric constant.*

biological inertness (Nunes et al., 2010; Tsao et al., 2007; Wei et al., 2016).

Once some applications of MCE analysis do not require optical detection, it is possible to work with nontransparent substrates and exhibit similar properties to those of glass and fused silica, such as low-temperature cofired ceramic (LTCC) (Henry et al., 1999). The procedure to obtain MCE made by LTCC includes lamination, stamping, laser ablation, or punching tapes. Typically, ceramics are sintered at 1600°C, whereas LTCC foils only require 850°C (Goldbach et al., 2006). The LTCC material has great hermeticity, chemical inertia, and good thermal conductivity (Fercher et al., 2010; Goldbach et al., 2006).

In addition to the polymers previously mentioned, the use of Poly(ethylene terephthalate)-toner (PET-Toner) warrants attention. In the early 2000s, several research groups began to utilize thin-film lamination and constructed devices with individually designed layers (Weigl et al., 2001). Instead of cutting channel designs, another technique was developed using office printers to create microfluidic architecture into ink-toner-coated transparency sheets (Birch et al., 2017; Tan et al., 2001). The channel depth achieved using this methodology could exceed 10 μm, depending on how the layers were defined and laminated.

The PET-Toner system has advantages primarily related to its low cost and ease of fabrication, with no need to use hazardous materials, which also motivates the development of disposable devices. In addition, the toner exhibits negligible solubility in water and operates within a broad temperature range (< 150°C), allowing for the control of lamination to prevent channel blocking (Do Lago et al., 2003). Although the toner can melt during the lamination process, binding the layers together, the PET-toner devices exhibited significant stability and robustness improvements when adhesives were used between layers, such as pressure-sensitive adhesives or heat-sensitive adhesives (Birch et al., 2017; Thompson et al., 2015). It also exhibits some chemical resistance; however, the use of pure organic solvents is not recommended (Do Lago et al., 2003).

22.1.2 Microfabrication techniques

So far, it is evident that MCE has emerged as a powerful, miniaturized analytical technique, offering rapid analysis with minimal reagent consumption. Several substrates have been mentioned for MCE fabrication, but it is essential to note that the performance of these devices heavily depends on the characteristics and fabrication methods of the materials used. Thus various microfabrication techniques have been developed to create precise, reproducible microchannels suitable for electrophoretic separations. The most commonly used techniques will be described below; however, we encourage readers to conduct thorough research on the topic of interest before applying these techniques. Be aware that each method offers specific advantages in terms of resolution, cost, and scalability, depending on the application.

22.1.2.1 Lithography

Lithography is a technique used to transfer the master pattern to a substrate. Depending on the material used, there are two primary methods to obtain microfluidic patterns: photolithography and soft lithography. The primary difference between them is that photolithography is used for solid substrates, such as glass, whereas soft lithography is typically employed for elastomeric polymers, like PDMS, which requires a negative mold (Fig. 22.1) (Gerami et al., 2019).

Photolithography and patterning transfer typically involve a series of laborious steps. A good resolution is achieved when the masks are correctly aligned; however, large-scale production efficiency depends on the number

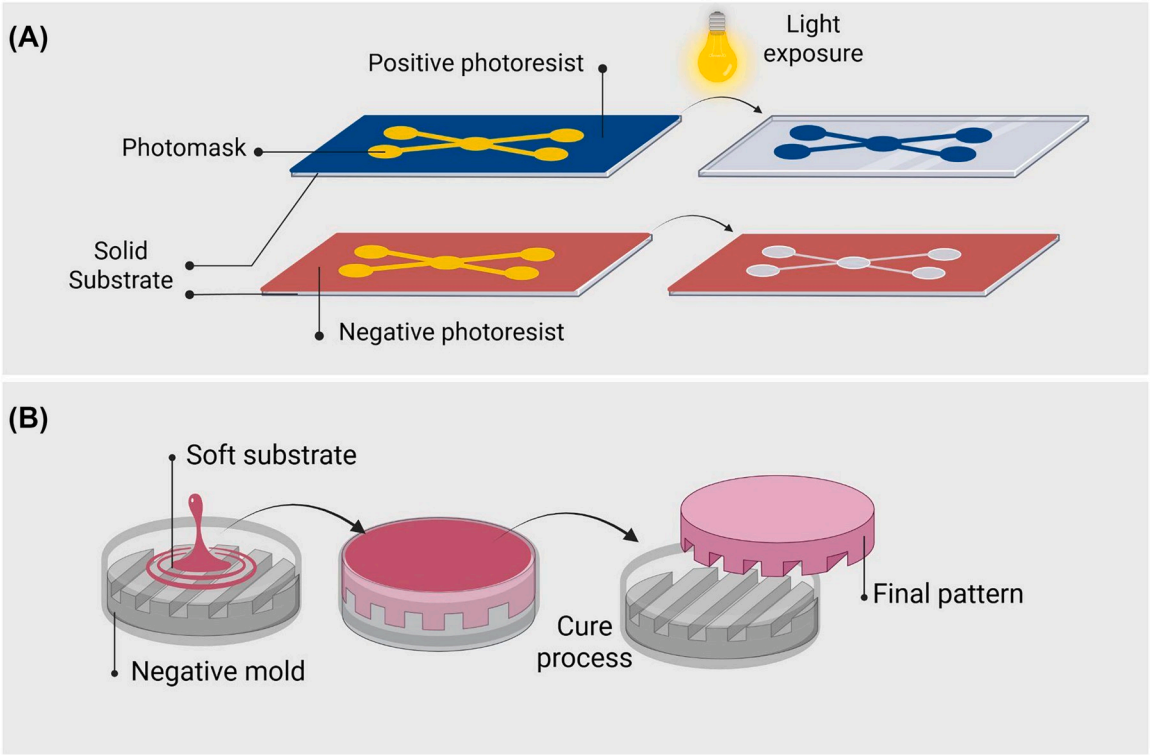

FIGURE 22.1 Comparison of photolithography (A) and soft lithography (B) techniques. *Figure created with BioRender.com.*

of wafers that can be transferred per hour. There are two types of photoresist coatings, positive and negative, which primarily differ in their interaction with light (Fig. 22.1A). For instance, when exposed to light, positive photoresists become more soluble in a developer, allowing the exposed areas to be washed away with a proper solvent. Negative photoresists, on the other hand, become less soluble due to light-induced polymerization and are the areas that remain solid after development. The choice between these types depends on the feature size, adhesion to the substrate, and chemical resistance (Augustin et al., 2006; Madou, 2011).

Photolithographic patterning of silicon wafers is one of the most commonly used standard procedures in microchip fabrication. Silicon offers excellent mechanical properties and chemical inertness, making it a highly suitable material for various applications. Its most significant advantage lies in the flexibility it provides for channel design, enabling the fabrication of various functional units, such as microunit operation blocks, as well as integrated pumping, mixing, and filtering devices (Székely & Guttman, 2005).

As previously mentioned, another common type of lithography is soft lithography. This approach encompasses a series of techniques that use a patterned elastomer as a stamp, mold, or mask to create micropatterns and microstructures (Fig. 22.1B). One of its main advantages is the ability to work with nonplanar

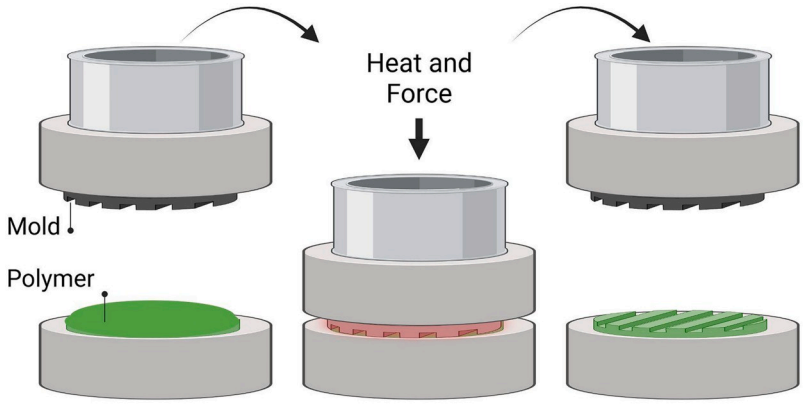

FIGURE 22.2 Schematic representation of the hot embossing method. *Figure created with BioRender.com.*

substrates, unconventional materials, and large surface areas. In summary, soft lithography is a rapid prototyping technique that requires less equipment and is considered the less expensive type of lithography. Today, soft lithography is considered a suite of techniques, including microcontact printing, microtransfer molding, micromolding in capillaries, and microreplica molding. In soft lithography, a master mold must first be fabricated using either conventional lithography or 3D printing. An elastomeric material, such as PDMS, is then cast onto the mold. PDMS is commonly used due to its ease of handling and rapid curing time (approximately 1 hour at 60°C). Additionally, it offers excellent thermal stability, optical transparency, and reusability (over 50 cycles) and is easily modifiable (Gerami et al., 2019; Madou, 2011).

22.1.2.2 Hot embossing

Also known as compression molding or relief imprinting, the hot embossing technique involves heating a polymer substrate above its glass transition temperature (T_g). An embossing master is typically fabricated using photolithography, but silicon can also be utilized as an embossing tool. The master mold is then mounted in an embossing machine, which presses the mold against the softened substrate to transfer the desired pattern (embossing) (Fig. 22.2). Equipped with heating plates and cooling channels, the system operates in cycles, enabling precise temperature control during both the embossing and de-embossing steps. Once the system cools below its glass T_g, the mold is separated from the substrate. The most commonly used polymers in hot embossing have well-established processing conditions in the literature. In summary, this method is considered low-cost because of its scalability and is highly reproducible due to its simplicity (Becker & Heim, 2000; Madou, 2011; Shakeri et al., 2021).

22.1.2.3 Injection molding

Injection molding (IM) is a high-precision, high-throughput manufacturing technique extensively used to fabricate polymer-based microfluidic devices. This process involves injecting a thermoplastic polymer melt into a mold cavity under high and controlled pressure (Fig. 22.3) (Madou, 2011).

Once cooled, the molded part is ejected, resulting in a solid microstructure with micrometer-scale features that replicate the mold design. Polymers commonly used include polymethyl methacrylate (PMMA), polycarbonate,

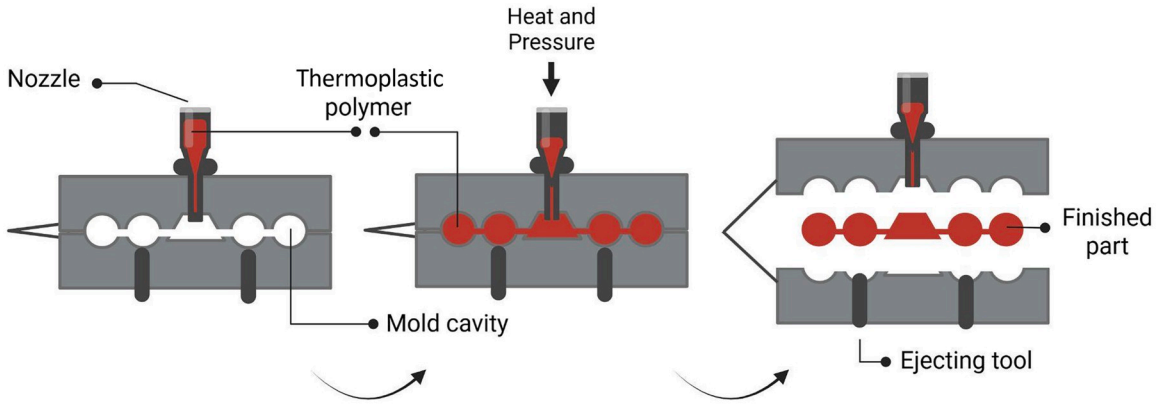

FIGURE 22.3 Injection molding of a thermoplastic polymer representation. *Figure created with BioRender.com.*

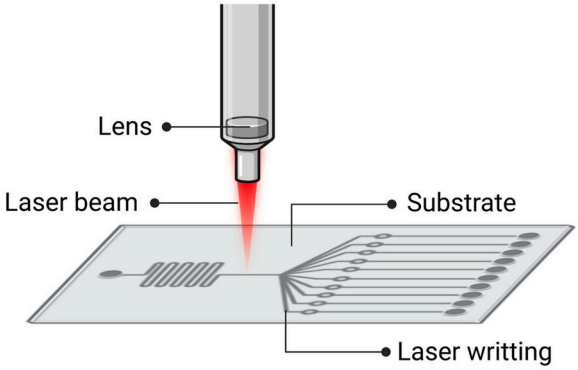

FIGURE 22.4 Laser-induced microchannel structuring. *Figure created with Biorender.com.*

and COC. IM can reproduce microchannels with high fidelity through replication accuracy; however, this process is strongly dependent on mold fabrication and process parameters, such as injection speed, temperature, and mold cooling time. Postprocessing techniques such as thermal or solvent-assisted bonding are used to seal the channels after molding (Attia et al., 2009; Huang et al., 2007; Madou, 2011; Shakeri et al., 2022).

22.1.2.4 *Laser ablation*

Laser ablation is a noncontact, subtractive microfabrication technique in which a focused laser beam removes material from a substrate, allowing for the precise patterning of microstructures, such as channels, reservoirs, and detection zones. It can also be used for mold and mask fabrication (Fig. 22.4). This technique is widely applicable and compatible with a variety of materials, including metals, polymers, ceramics, composites, semiconductors, diamonds, graphite, and glass. However, each material requires a specific type of laser source, as different gases or mixtures operate at distinct wavelengths. The underlying mechanisms vary depending on the material and may include vaporization (for metals, insulators, and ceramics), molecular

disintegration (for polymers), or interface effects such as the exfoliation of thin films. During the process, the laser beam interacts with the material through reflection, absorption, and thermal conduction. Strong absorption at high energy rates typically leads to more effective ablation, although multiple pulses may be necessary. Each material also requires optimization of ablation parameters such as power, beam width, spot size, lasing mode, pulse energy, and pulse duration. In some cases, pretreatment—such as surface coating or oxidation—can improve ablation efficiency and quality (Madou, 2011; Scott & Ali, 2021).

Due to the complexity and variability of laser-material interactions, we strongly recommend that users conduct deeper research to select the appropriate setup for their specific application needs.

22.1.2.5 *Wet and dry etching*

Etching techniques—both wet and dry—are subtractive processes that enable the precise removal of material from solid substrates, particularly glass and silicon, to form microchannels and reservoirs (Fig. 22.5). These processes require the use of a mask made of a resistant material to define the areas to be exposed and etched. Masks can be composed of metallic, ceramic, or polymeric layers or combinations thereof. The etching agents can be either liquid chemicals (wet etching), gases, or plasma (dry etching). Several key parameters must be considered for both approaches, including etch rate, uniformity, throughput (i.e., the number of substrates or wafers processed per cycle), directionality, and selectivity. In practice, combining wet and dry etching methods is often an effective strategy to achieve optimal results. Ideally, the process should ensure the high-fidelity transfer of the mask pattern without distorting critical dimensions (Iliescu et al., 2012; Madou, 2011).

Wet etching uses chemical solutions to dissolve substrate materials, which can generate residues that require proper disposal. In contrast, dry etching is typically performed using reactive ion etching or deep reactive ion etching. In this method, the substrate is etched by reactive gases under vacuum conditions. Dry etching is often preferred because it produces minimal waste, avoids corrosion, reduces undercutting and photoresist feature broadening, and offers excellent control, resulting in cleaner and more precise surfaces. For glass microchips, hydrofluoric acid or buffered oxide etch solutions are commonly used. For silicon patterning, anisotropic wet etching is typically performed using potassium hydroxide or tetramethylammonium hydroxide. In both dry and wet etching processes, the use of an appropriate mask is essential (Madou, 2011; Scott & Ali, 2021).

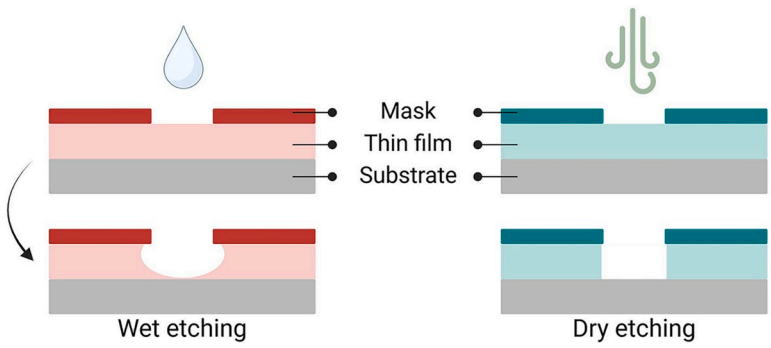

FIGURE 22.5 Visualization of wet and dry etching mechanisms to pattern a thin film. *Figure created with BioRender.com.*

22.1.2.6 Print, cut, and laminate

The Print, Cut, and Laminate (PCL) technique is a low-cost, rapid, and accessible microfabrication method that is increasingly used for developing disposable microfluidic devices. This technique relies on the construction of complex multilayer architectures. The process typically begins with the design of patterns using computer-aided design software, followed by printing onto transparency films and/or adhesive sheets using standard laser or inkjet printers. The toner or ink can serve to define hydrophobic channel walls or act as a sacrificial mask. Next, precise cutting is carried out using mechanical plotters, craft cutters, or laser cutters to form channel geometries, access ports, and external contours (Fig. 22.6) (Do Lago et al., 2003; Kumawat et al., 2022; Thompson et al., 2015).

The PCL method enables rapid prototyping of functional electrophoretic chips with laminated structures that can incorporate integrated reservoirs, sample injection points, and electrophoretic separation channels. Electrical connections for electrophoresis can be established using embedded electrodes or inserted electrode wires, while optical detection can be performed through the transparent film layers. Despite its advantages, the PCL technique has certain limitations, such as the poor chemical resistance of some polymer films (e.g., PET) and reduced pressure tolerance due to the risk of delamination. Additionally, channel reproducibility and dimensional precision are generally lower than those of more advanced techniques, such as injection molding or etching (Do Lago et al., 2003; Kumawat et al., 2022; Thompson et al., 2015).

22.1.3 Surface modification

As mentioned previously, materials used in MCE fabrication often require additional surface treatments to minimize undesirable interactions between analytes (particularly proteins, nucleic acids, and other biomolecules) and the channel walls. These treatments are also essential for controlling surface charge and EOF, especially in devices made from plastic materials (Belder & Ludwig, 2003; Currie et al., 2009; Qian et al., 2023). Analyte adsorption onto channel walls often results in peak broadening and asymmetry due to resistance of mass transfer between the wall surface and the bulk flow, which negatively affects the quality and reliability of analytical results (Geiger et al., 2015).

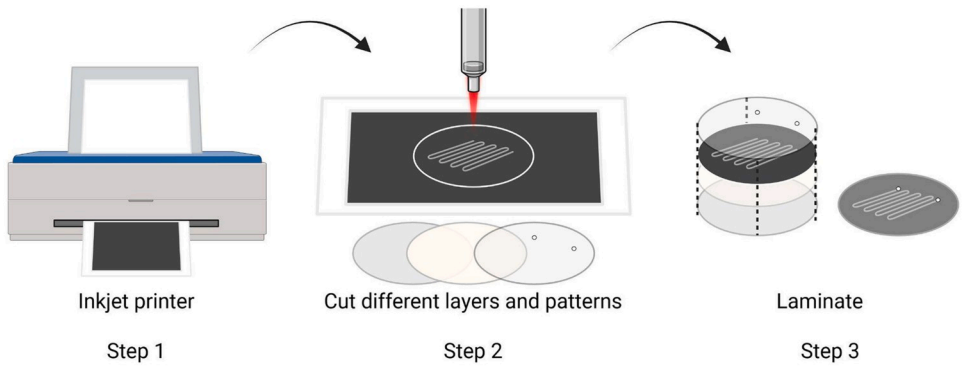

FIGURE 22.6 Representation of Print, Cut, and Laminate process to fabricate microfluidic devices. *Figure created with BioRender.com.*

Given the wide range of materials and surface treatments available, not all will be discussed in detail in this chapter. However, the purpose of surface modification in microchip electrophoresis (MCE) can generally be summarized into two main categories: improving surface properties or functionalizing the surface for specific applications (Jones & Hayes, 2006). It is important to note that surface modification in MCE presents particular challenges due to the complex architecture of microchips, compared to conventional CE, as well as the diversity of substrate materials and chemical reagents involved (Belder & Ludwig, 2003).

Devices manufactured from polymers such as PDMS are typically hydrophobic due to the presence of surface methyl groups (–CH_3), which makes it difficult to load aqueous solutions into the channels (McDonald & Whitesides, 2002; Neves, Afonso, Nobrega, Barbosa, Lima, Ribeiro, 2024). Additionally, the hydrophobic surface tends to interact with nonpolar analytes or molecules containing nonpolar regions, leading to undesired adsorption (Linder et al., 2001).

To address these challenges, several surface treatments have been proposed in the literature to introduce hydroxyl (–OH) or other polar functional groups, thereby rendering the channel surfaces slightly hydrophilic and improving wettability. These treatments include exposure to hydrochloric acid, UV grafting, oxygen plasma, the addition of surfactants, and the incorporation of nanomaterials or polyelectrolyte multilayers (PEMs) (Jones & Hayes, 2006; McDonald & Whitesides, 2002).

Among these, the PEMs approach is particularly noteworthy. This method involves sequentially filling the microchannels with solutions of positively and negatively charged polyelectrolytes, forming electrostatically bonded multilayers on the channel walls. This technique not only ensures reproducible surface coverage but also contributes to the stabilization of EOF (Jones & Hayes, 2006). For a comprehensive overview of PDMS surface modification strategies, the review by Neves and colleagues is highly recommended (Neves, Afonso, Nobrega, Barbosa, Lima, Ribeiro, 2024).

Dynamic coating is also a practical and safe option for surface modification. This approach involves the addition of polymers or surfactants via the running buffer or through rinsing steps, making it an attractive and straightforward solution for polymer-based microchips, which are often intended for disposable use. Another important consideration is that the use of ionic species for surface modification in MCE can increase the conductivity of the buffer. Consequently, this leads to additional Joule heating during separation, resulting in a rise in microchannel temperature. This temperature increase can affect buffer viscosity, chemical equilibria, and pH stability. Collectively, these effects contribute to peak distortion and baseline drift, ultimately compromising the quality and reproducibility of separation (Qian et al., 2023).

In the case of MCE devices fabricated from glass or quartz, surface modification strategies commonly used in conventional CE can often be adapted due to the similarity in substrate materials. For instance, cellulose derivatives are frequently employed as dynamic coatings, particularly in DNA separations, where they serve both as sieving matrices and surface deactivators (Belder & Ludwig, 2003). It is essential to note that dynamic coating must be reapplied before each run, as it is not permanently bonded to the channel surface.

In summary, the choice of surface functionalization strategy in MCE must be carefully aligned with the specific properties of the substrate material, as each material presents distinct challenges related to wettability, surface charge, and analyte interaction. Optimizing surface treatments is, therefore, a critical step

toward enhancing the performance, reproducibility, and applicability of MCE devices in different contexts. Based on this idea, the next section will explore the use of stationary phases in MCE, an approach that further expands the separation capabilities by enabling selective interactions and enhancing the separation.

22.2 Fabrication of capillary electrochromatography and microchip-CEC

The efficiency and reproducibility of CEC (capillary electrochromatography) and MCEC (microchip capillary electrochromatography) are closely linked to the way the stationary phase is incorporated into the system, the type of capillary or microchip material, and the functionalization of the internal surfaces. These aspects have a direct impact on the control of the EOF, the selectivity of the separation, and the robustness of the system. Advances in microfabrication enable a miniaturized, rapid, and potentially disposable approach, making it ideal for clinical, environmental, and field analysis. However, the transition from the traditional CEC to the microchip format presents new challenges in immobilizing the stationary phase, controlling the surface, and selecting materials compatible with microfabrication processes. Therefore, this topic will cover the main aspects involved in the fabrication of CEC systems, including the selection of capillary and column materials, stationary phase immobilization techniques, and surface modification strategies aimed at controlling EOF and enhancing selectivity. MCEC has garnered significant attention for its application potential and the miniaturization of the system. Microchip CE also has the added benefits of low cost, small size, and fast analysis times, which are general goals of many chemical analysis methods. The integration of CEC with microfluidic platforms will be addressed throughout the topics, with an emphasis on the technical and structural adjustments necessary for its effective implementation in miniaturized devices (D'Orazio et al., 2016; Felhofer et al., 2010; Li et al., 2023).

22.2.1 Capillary and column materials

22.2.1.1 Packed versus monolithic columns: structural differences and performance comparison

Packed columns are filled with tiny particles of the stationary phase. These particles, also known as packing materials, can be composed of modified silica, polymers, and chiral phases, among other types of interest. The material can vary in size from 1.5 to 5 μm, and its porosity distribution can also vary, which has an impact on chromatographic efficiency (Angus et al., 2000). This variation in the porosity and size of the material also affects the EOF, resulting in irregular flow with increased resistance due to the presence of interparticle spaces (Colón et al., 2000). Preparing the packed column involves introducing particles into the reservoir and applying high pressure to force them into an empty column, which only has a frit at the outlet end. Once the column is filled, the pressure is gradually released, and the column is disconnected from the packing system so that a frit can be installed at the inlet end, that is, the inlet connector must be modified. The packing material is held in place using retention frits, a porous barrier that secures the material in position. The frits must be mechanically strong enough to contain the packing material, resist the applied pressures, and have high permeability for flow passage (Fanali et al., 2021).

Monolithic columns have a continuous porous structure, such as a monolithic network of silica or polymers, resulting in a continuous separation medium. This structure is commonly

cylindrical with small domains and relatively large channels, which provide high permeability and column efficiency. The EOF passes through the pores of the monolithic material, promoting separation (Fig. 22.7) (Faria et al., 2006). Monolithic phases have stood out in the development of stationary phases, mainly due to the ease of incorporating different functional groups, such as hydrophobic, charged, or specific molecules for selective interactions, already during their synthesis. In addition, one of their main advantages is the possibility of preparation and functionalization in a single process, which simplifies their production and increases their analytical versatility (Allen & El Rassi, 2003).

The choice between a monolithic column and a packed column in CEC depends on several factors, such as the type of sample, the characteristics of the analytes, the desired operating conditions, as well as criteria such as separation efficiency, reproducibility, analysis time, and suitability for the specific application (Fanali et al., 2021).

Packed columns are renowned for their high chromatographic efficiency, primarily due to the large surface area of the stationary phase particles. This characteristic favors analyte-stationary phase interactions, resulting in high resolution and efficient separation of small compounds. However, the presence of interparticle spaces can lead to variations in flow, which in turn impact the dispersion of chromatographic bands (Escalona-Durán et al., 2021; Poole, 2012). The continuous and highly porous structure of monolithic columns enables a more uniform flow of the mobile phase, thereby reducing flow resistance. Although they have a lower surface area compared to packed columns, they compensate for this limitation by providing high flow rates and lower band dispersion, making them especially suitable for analyzing large biomolecules such as proteins (Patel et al., 2022).

Reproducibility is a critical factor in any chromatographic analysis. In packed columns, the packing material can vary, primarily due to the method used to pack the material inside the capillary. Slight differences in particle distribution or failures to retain the stationary phase can affect separation performance. In contrast, monolithic columns have a continuous matrix formed by in situ polymerization, which ensures a more homogeneous structure and reduces variations between different columns, resulting in superior reproducibility compared to packed columns (Tao et al., 2025).

In terms of analysis time, monolithic columns are advantageous, as they enable faster separations due to their highly porous

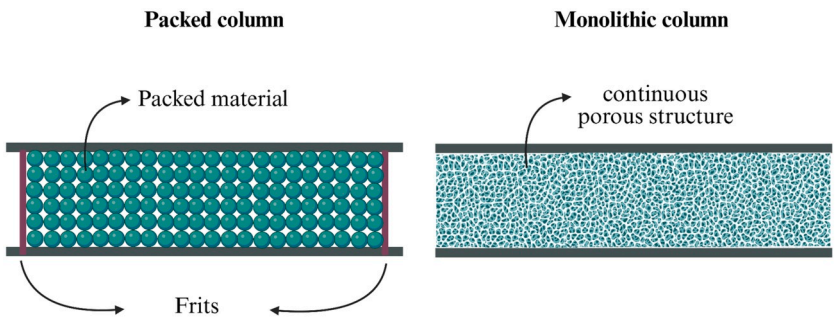

FIGURE 22.7 Schematic illustration of structural differences between a monolithic and packed column. *Figure created with BioRender.com.*

structure, which reduces flow resistance and allows for the use of higher flow rates without compromising separation efficiency (Lima et al., 2023). Packed columns may require longer analysis times, as the presence of discrete particles and retention frits can increase flow resistance. Additionally, due to the need for higher pressures to drive the mobile phase, the total separation time may be longer compared to monolithic columns (Norton & Shamsi, 2008). In general, packed columns are more suitable for analyses that require high resolution, such as the separation of small analytes, drugs, metabolites, and chiral compounds. Due to their high selectivity and number of theoretical plates, they are widely used when seeking accurate separation of compounds with similar chemical properties. Monolithic columns, on the other hand, are more suitable for complex samples such as proteins, peptides, and metabolites in body fluids (Lima et al., 2023).

Mechanical stability and chemical modification are factors that influence the choice of column type. Packed columns are mechanically more stable but may degrade over time due to the movement of the stationary phase particles. Furthermore, their chemical stability can be limited under extreme pH conditions, mainly when based on modified silica (Sun et al., 2019). Monolithic columns, depending on the construction material, may offer greater chemical resistance across a wide pH range, particularly when made from polymer-based materials. However, some polymeric versions may be less mechanically stable than packed columns. Furthermore, they can be functionalized directly during their synthesis, allowing for more efficient incorporation of specific groups, such as hydrophobic, charged, or selective molecules for specific interactions (Lima et al., 2023).

In MCEC platforms, monolithic phases synthesized in situ are often favored over packed particle beds due to their superior mechanical stability, simplified fabrication within microchannels, and compatibility with soft lithography substrates such as PDMS or PMMA. Monolithic columns have the advantage of being able to be patterned directly with ultraviolet radiation, confining them in the columns without the use of packing frits. A fully packed Capillary Electrochromatographic Microchip with Self-Assembly Colloidal Silica Beads was developed by Park et al. (Park et al., 2007) for the detection of fluorescein isothiocyanate (FITC) derivatized amino acids. The microchip was fully populated and did not require any sophisticated chip assembly apparatus. Due to the integrated filter function of the filled spheres, no chip-cleaning process or solution filtration was required. The compact crystalline packing of the colloidal silica in the microchannels served as a stationary phase for sound separation, even with a very short separation column length (Park et al., 2007). Fig. 22.8 shows the structure of the device, the actual size, and scanning microscopy (SEM) of the packing inside the chip.

The separation of amino acid derivatives of fluorescein (FITC) demonstrated efficient analytical performance, even in short separation channels (2 mm in length). The packing reduced the adverse effects of fluid level variations in the reservoirs, allowing stable control of EOF and reproducibility (1.3% RSD in migration time). Despite the good performance, the authors observed problems of uneven packing at the edges and occasional fractures caused by mechanical stress during the sealing process.

Monolithic columns were used by Zhai and collaborators, who developed a glass/PDMS hybrid electrophoretic microchip integrated with a molecularly imprinted solid-phase extraction (SPE) monolithic column (MISPE) coupled to a noncontact conductometric detection system. The stationary phase was synthesized in situ by UV photopolymerization, using auramine O. as a template for the creation of selective recognition sites (Fig. 22.9) (Zhai et al., 2014).

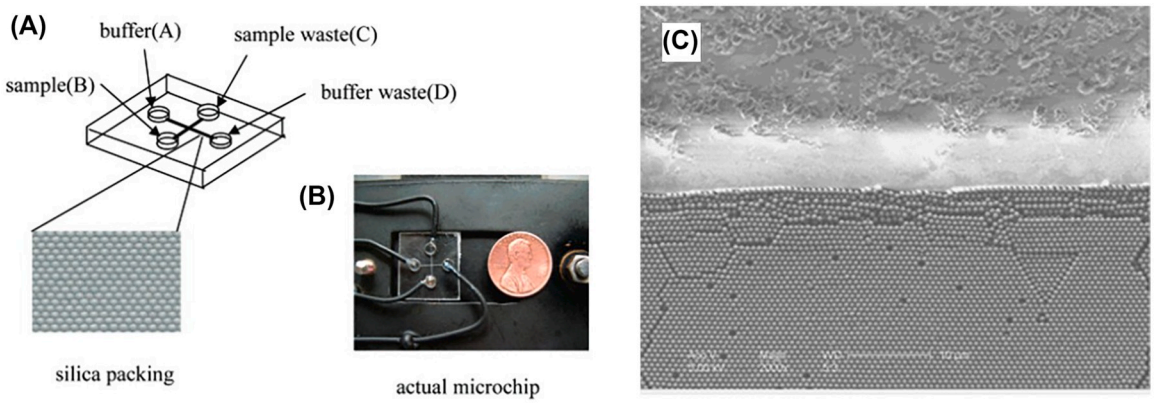

FIGURE 22.8 Schematic of the device structure (A), real device (B), and scanning microscopy (C). *From Park, J., Lee, D., Kim, W., Horiike, S., Nishimoto, T., Se, H.L. & Ahn, C.H. (2007). Fully packed capillary electrochromatographic microchip with self-assembly colloidal silica beads. Analytical Chemistry, 79(8), 3214–3219. https://doi.org/10.1021/ac061714g.*

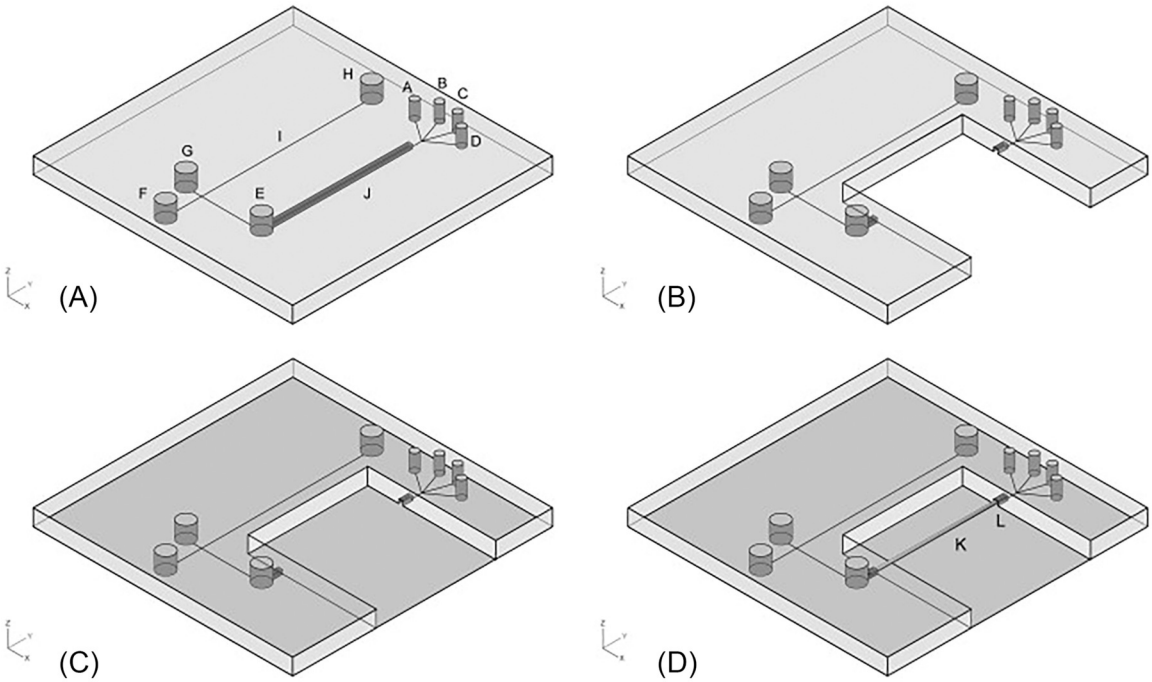

FIGURE 22.9 Schematic of the fabrication of the glass/PDMS microchip: (A) PDMS layer; (B) part of the SPE channel in the PDMS layer has been cut out; (C) PDMS layer sealed with the thin glass.coverslip; (D) monolithic MISPE capillary column attached to the glass/PDMS chip. ABCD: holes, E: sample reservoir, F: buffer reservoir, G: sample waste reservoir, H: buffer waste reservoir, I: separation channel, J: SPE channel, K: MISPE monolithic capillary column, and L: epoxy glue. *From Zhai, Haiyun, Li, Jiangmei, Chen, Zuanguang, Su, Zihao, Liu, Zhenping & Yu, Xiao. (2014). A glass/PDMS electrophoresis microchip embedded with molecular imprinting SPE monolith for contactless conductivity detection. Microchemical Journal, 114, 223–228. https://doi.org/10.1016/j.microc.2014.01.006.*

The device enables the extraction, preconcentration, separation, and detection of analytes to be carried out on a single microfluidic platform. The monolith demonstrated high selectivity and reusability for more than 30 cycles, with an enrichment factor of up to 12 times. The hybrid configuration, featuring PDMS with a glass cover, enhances the sensitivity of conductive detection and enables the modular replacement of columns. Fig. 22.10 shows the electropherograms obtained from the device.

The microchip was used to detect auramine O in shrimp samples, yielding satisfactory results with recoveries ranging from 90% to 92%, good linearity (r=0.9994), and good reproducibility. The authors describe that the homemade MISPE monolithic capillary column can be conveniently integrated into the microchip or replaced from the microchannel. The choice between packed and monolithic columns in CEC and MCEC depends directly on the analytical requirements, the type of application, and the technical limitations associated with miniaturization. Packed columns remain an excellent choice for applications requiring high resolution and separation of low-molecular-mass compounds, as they benefit from their high surface area and wide commercial availability. However, their application in microdevices faces significant challenges related to the complexity of packing, flow control, and mechanical stability, as observed by Park's research group. Despite obtaining good separations on fully packed microchips, they reported problems with fractures and irregular packing (Park et al., 2007; Zhai et al., 2014).

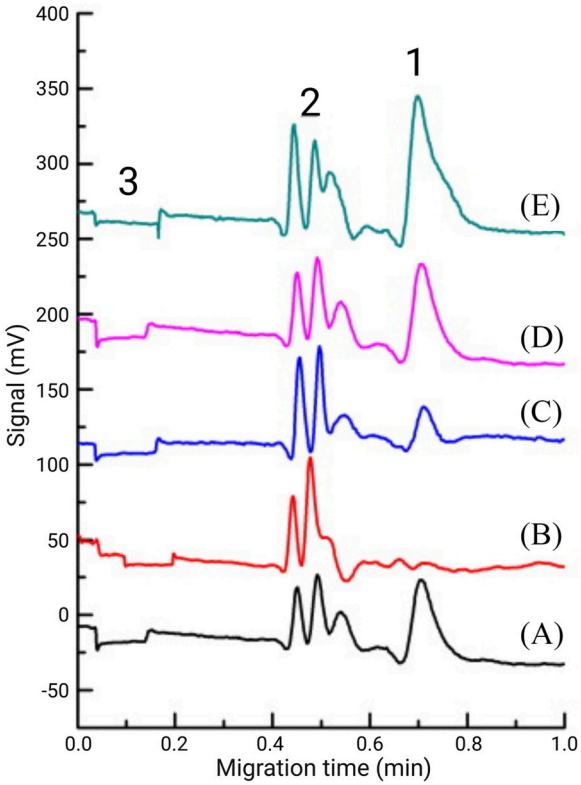

FIGURE 22.10 Electrochromatograms of (A) auramine O standard solution (peak 1, 50 μg mL^{-1}); (B) blank sample solution; (C) sample solution enriched with auramine O standard solution (peak 1, 15 μg mL^{-1}); (D) enriched sample solution prepared with NIP offline and (E) enriched sample solution prepared with MISPE offline. Electrophoretic conditions: buffer, 5.0 mmol L^{-1}; lactic acid, 15% methanol; separation voltage, 2.0 kV (field strength was 400 V cm^{-1}); electrokinetic injection, 5 s at 500 V; SPE elution, methanol-ammonia (8:2, v/v). Peak assignments: (1) auramine O, (2) methanol, and (3) switch mark. *From Zhai, Haiyun, Li, Jiangmei, Chen, Zuanguang, Su, Zihao, Liu, Zhenping & Yu, Xiao. (2014). A glass/PDMS electrophoresis microchip embedded with molecular imprinting SPE monolith for contactless conductivity detection. Microchemicals Journal, 114, 223–228. https://doi.org/10.1016/j.microc.2014.01.006.*

On the other hand, monolithic columns, especially those prepared by in situ polymerization, have become the most practical and effective approach for integration into microfluidic platforms. Their continuous and porous structure offers less resistance to flow, greater reproducibility between devices, and ease of functionalization during synthesis. The work developed by Zhai and colleagues exemplifies this trend by demonstrating the successful application of a molecularly imprinted monolithic column on a PDMS/glass hybrid microchip, which combines extraction, separation, and detection with high selectivity, reusability, and sensitivity (Zhai et al., 2014). Thus, while packed columns are still valued for their high chromatographic efficiency, monolithic columns offer operational and structural advantages that make them more compatible with the requirements of miniaturized devices, reinforcing their central role in the advancement of microscale separation technologies.

22.2.1.2 *Stationary phase selection: silica-based, polymeric, hybrid, and functionalized materials*

Silica-based monolithic columns are composed of a single, continuous rod of porous silica. Similar to conventional packed columns, these capillaries incorporate well-characterized silica particles that provide a high phase ratio and a narrow pore size distribution, resulting in improved retention capacity and enhanced separation efficiency. Notably, the immobilization of microparticles within the monolithic structure enhances the mechanical stability of the separation bed, eliminating the need for retaining frits and thereby reducing complications associated with bubble formation while improving overall column performance (Allen & El Rassi, 2003).

Silica-based materials exhibit physicochemical properties that contribute to their high performance in separation processes, such as high specific surface area, uniform pore size distribution and mechanical stability, as well as the availability of different chemicals that can be used for surface modification and ligand attachment, due to the silanol groups (-SiOH), which can be functionalized with different organic ligands, modulating the polarity, charge and selectivity of the column (Ou et al., 2007). The work of Hemwech and colleagues in 2021 presented a comparative study between coated and uncoated silica in electrochromatography capillaries for the separation of seven phenolic acids within 15 minutes. The uncoated capillary was unable to separate all seven phenolic acids by CEC, demonstrating the effectiveness of the silica coating. The modified capillary exhibited a plate number (N) of $\geq 2.0 \times 10^4$ m^{-1} and a peak resolution (R_s) of ≥ 1.6, indicating high analytical performance (Hemwech et al., 2021).

The review by Ahmed et al. in 2023 discusses the use of narrow open-tube (NOT) columns based on silica, both unmodified and modified fused, for low-volume samples, such as those in metabolomics and single-cell analysis. NOT columns offer significant advantages, including high-resolution separations, short analysis times, and the ability to work with minimal volumes (in the femtoliter range), making them ideal for the highly sensitive detection of biomolecules. The review presents a study that utilized fused silica in capillaries with extremely small internal diameters (e.g., 460 nm), allowing for ultra-low-volume injections with high efficiency. Additionally, the use of silica modified with trimethoxy(octadecyl)silane yielded ultra-high resolution separations with high peak capacities, which could be optimized by controlling the column temperature. Other strategies can also be applied to silica, such as nonporous coatings and control of the mobile phase, which enabled improvements in column loading capacity and detection sensitivity. Thus, the use of silica materials in NOT columns offers benefits such as miniaturization,

high separation efficiency, low sample consumption, and the potential for high-throughput and high-sensitivity applications in biomolecular analysis (Ahmed et al., 2023).

Hybrid materials, which combine the characteristics of silica and polymers (such as sol-gel derivatives or organo-silica compounds), offer a balance between thermal stability, chemical resistance, and functional versatility. The primary feature of hybrid materials is the simultaneous presence of both inorganic and organic components, including silica and polymers or specific ligands (Hu et al., 2020). This combination provides enhanced stability, resistance to organic solvents, greater control over porosity and morphology, and allows for specific functionalizations that increase selectivity. Moreover, hybrid materials exhibit a continuous and homogeneous structure, which facilitates EOF, reduces flow resistance, and improves reproducibility. Many of these hybrid materials can be designed as monolithic columns, offering easier preparation and a lower risk of clogging, a common issue in packed columns. These properties make hybrids an excellent choice for high-performance applications such as complex analyses by capillary electrochromatography coupled with mass spectrometry (CEC-MS), enantioseparations, and biomolecule separations. They are also ideal for miniaturized systems or microfluidic platforms due to their robustness and chemical flexibility (Lei et al., 2012; Qu et al., 2016).

Finally, functionalized materials are designed with specific groups to enhance selectivity in targeted separations. This category includes phases with chiral ligands, affinity groups, or selective interactions with metal ions, pharmaceuticals, or biomarkers. The chemical stability of these materials depends on the base matrix used and the quality of the functionalization. One example of functionalized materials is the study by Sun et al. (2021). The authors developed a vinyl-functionalized spherical covalent organic framework (COF-V) and synthesized it at room temperature, employing it as a stationary phase for CEC-MS analysis targeting antiepileptic drugs, triazine herbicides, and active ingredients from traditional Chinese medicine. The COF-V-based column demonstrated strong EOF, high separation efficiency, and excellent loading capacity. The maximum column efficiency exceeded 1.4×10^5 plates m^{-1} for methylbenzene. Furthermore, the COF-V-modified column outperformed an open-tubular column in terms of repeatability and stability (Sun et al., 2021).

In MCEC platforms, the choice of the stationary phase is a critical factor, directly influencing separation efficiency, system selectivity, and compatibility with the microfluidic design. Due to volume restrictions, the miniaturization of channels, and the need for integration with processes such as preconcentration and detection, the stationary phases used in MCEC must have high stability, specific selectivity, and ease of manufacture in situ. Organic monoliths, typically based on polymethacrylate or polystyrene, are widely used due to their straightforward synthesis via in situ polymerization, which enables the phase to be directly molded into microchannels. These materials can be easily functionalized with hydrophobic, ionic, or bioactive groups during synthesis, promoting selective interactions with the analytes. In addition, the continuous and porous structure of the monoliths enables uniform EOF and low resistance to flow, even under high electrical voltages, which is essential for ensuring efficiency in small-volume systems (Gunasena & El Rassi, 2012; Štulík et al., 2006).

Hybrid phases, which combine a polymeric matrix with inorganic components (such as silica or metal oxides), have gained prominence in MCEC because they offer a combination of chemical resistance, mechanical stability, and targeted functionalization. The presence of an inorganic component, such as silica, increases surface area and thermal stability, while the

organic phase ensures synthetic flexibility and compatibility with microchannels made of different materials, including PDMS, glass, or PMMA. This approach is beneficial when seeking specific selectivity for polar analytes or biomolecules while maintaining a robust structure even in integrated and reusable systems (Hu et al., 2020; Lin et al., 2012).

The incorporation of nanomaterials, such as metal nanoparticles, carbon nanotubes, metal oxides, or functionalized graphene, into the stationary phase represents an emerging trend in microextraction by packed sorbents in MCEC. These materials offer a high surface area, a high density of active sites, and a selective affinity for specific classes of compounds, including drugs, proteins, or environmental contaminants. In addition, they enable faster analytical responses and, in some cases, can be integrated into the detection system as built-in electrochemical or optical sensors. However, their use requires strict control of the dispersion and anchoring of the nanomaterials in the stationary phase matrix to maintain stability during multiple cycles of use (Jozanović et al., 2023).

One example is the study made by Goswami in 2009, which uses nanotubes as the stationary phase. The article reports on a new approach to developing a microfluidic electrochromatographic chip device using vertically aligned and patterned carbon nanotubes as the stationary phase material. The methods used in the study include the patterned growth of nanotubes at a specific location in the channel, utilizing both a solid-phase Fe-Al catalyst and a vapor-deposited ferrocene catalyst. The device is tested using reverse-phase capillary electrochromatographic separations and solid-phase extraction (SPE) of a glycosylated protein (Goswami et al., 2009). Fig. 22.11 shows the fabrication of the device.

The results of the study include the successful development of a microfluidic electrochromatographic chip device using patterned vertically aligned carbon nanotubes as the stationary phase material. The device is capable of performing reversed-phase capillary electrochromatographic separations and SPE of a glycosylated protein (Goswami et al., 2009).

The stationary phases most commonly used in MCEC are those that combine high selectivity, good reproducibility, ease of in situ synthesis, and compatibility with the materials used in microdevices. Modified polymer monoliths are the predominant choice due to their ease of manufacture and functional flexibility. At the same time, hybrid phases and nanomaterials represent advanced solutions for specific applications, such as the selective detection of biomarkers, pollutants, or trace compounds in complex samples.

22.2.2 Stationary phase immobilization techniques

The efficiency of separation processes in chromatographic systems depends on how the stationary phase is immobilized on the support. The stationary phase is the component responsible for selective interactions with the analytes, and its stability, distribution, and functionalization directly impact the sensitivity, selectivity, and reproducibility of analytical systems. Various approaches have been developed for immobilizing this phase, mainly particle packing, in situ polymerization, and the synthesis of monolithic phases (Faria et al., 2006; Li et al., 2023). Each of these techniques has specific advantages and limitations that influence EOF and the choice of solvents, depending on the desired application, especially in contexts where miniaturization, speed, and sensitivity are crucial. Immobilization strategies can be classified based on the nature of the structure used, the synthesis method, and the type of chemical bond formed between the functional phase and the support. Understanding these techniques is crucial for the development of advanced and customized analytical platforms (Jandera, 2011; Poole, 2012).

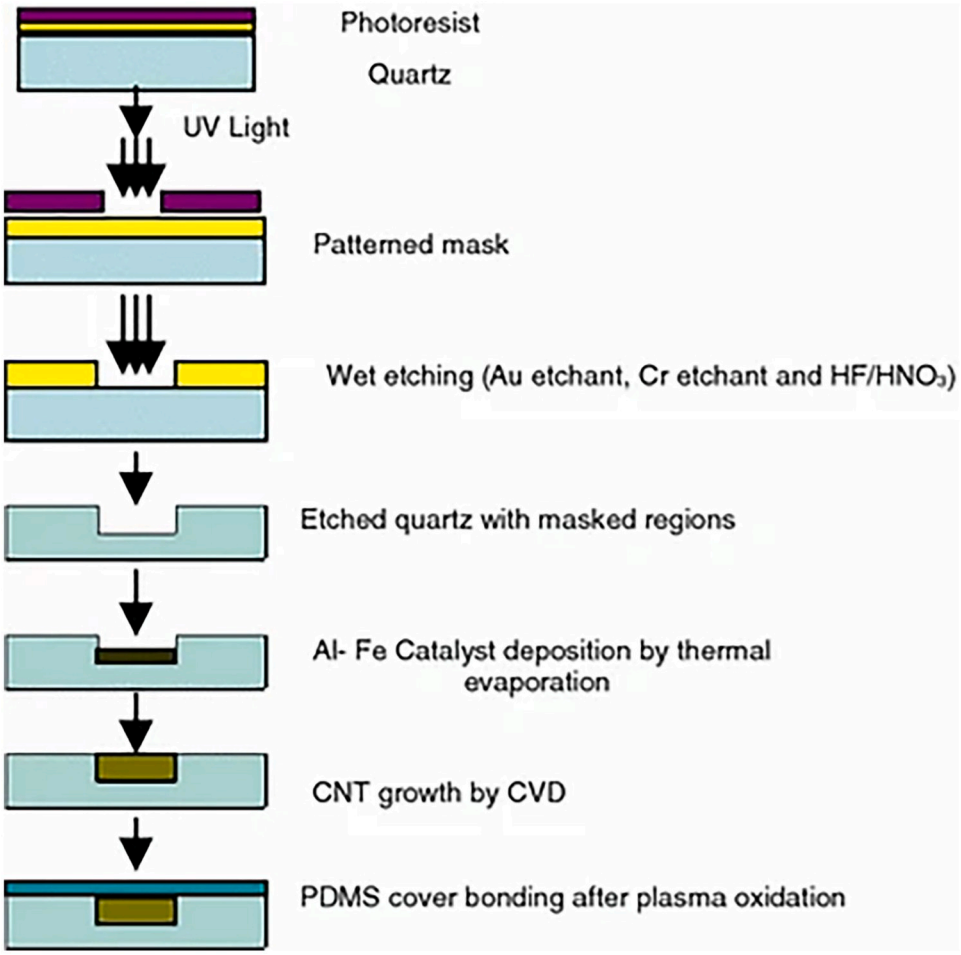

FIGURE 22.11 Carbon nanotube microfluidic device fabrication using thermally deposited Fe–Al/et**hylene growth process**. *From Goswami, S., Bajwa, N., Asuri, P., Ci, L., Ajayan, P.M. & Cramer, S.M. (2009). Aligned carbon nanotube stationary phases for electrochromatographic chip separations. Chromatographia, 69(5–6), 473–480. https://doi.org/10.1365/s10337-008-0948-0.*

22.2.2.1 Particle packing versus in situ polymerization: advantages and challenges

The choice of stationary phase immobilization technique is a determining factor in the performance of chromatographic systems and miniaturized analytical devices. Among the most widely used approaches, particle packing and in situ polymerization have specific characteristics that directly influence separation efficiency, reproducibility, matrix stability, and integration with modern analytical platforms, such as microfluidic systems. The particle packing technique is widely established, with a high degree of standardization, and allows the use of commercial particles with well-defined properties, favoring the reproducibility of results. However, its application in microdevices can be limited by the complexity of

microscale packing and the risk of channel formation (Maloney & Colón, 2002). Despite the technical difficulties associated with uniform packing in small-diameter capillaries, when appropriately done, particle packing provides columns with excellent analytical performance, making it a preferred choice in applications requiring high sensitivity and resolution.

Numerous studies have been conducted using packing particles, with one notable example being the work of Hernández-Mesa and colleagues in 2015. The authors utilized C18 silica beds as the stationary phase for determining 5-nitroimidazole (5-NDZ) residues in milk. The stationary phase was selected due to the high selectivity for 5-NDZ separation, and the fabrication is represented in Fig. 22.12 (Hernández-Mesa et al., 2015).

Packing was carried out at 420 bar to achieve the best results, and frit formation was optimized through sintering. The authors obtained an efficient stationary phase with a limit of detection value of less than 12 μg/L. Another interesting study is the fabrication of packed columns modified with polyethyleneimine (PEI) metal oxides for CEC and capillary liquid chromatography (Wiedmer et al., 2011). The stationary phases were manufactured using a nanocasting process, which enabled the production of mesoporous particles of SiO_2, ZrO_2, SnO_2, and Mn_2O_3 with controlled morphology and porosity. Subsequently, these particles were surface-modified with PEI, imparting unique characteristics, as well as hydrophobic properties and the ability to interact with analytes electrostatically.

In the column packing stage, two primary methods were tested: slurry packing by pressure and electrokinetic packing. The slurry packing method, also known as suspension packing under pressure, is the most common method for packing chromatographic columns, including capillaries. This method has proved to be the most efficient, especially with PEI-modified silica particles (SiO_2-PEI), allowing the preparation of stable columns up to 23 cm in packed length. On the other hand, packing with modified metal oxides had limitations, such as clogging of the column, formation of bubbles, and instability of the electric current, rendering it less effective or even unfeasible in some cases, as seen with Mn_2O_3-PEI and SnO_2-PEI. The results obtained demonstrated that the modification with PEI was successful, resulting in materials with a high density of amino groups and hybrid interaction behavior, which combines hydrophobic and electrostatic interactions. The column packed with SiO_2-PEI exhibited excellent performance in chromatographic separations, characterized by good stability over a wide pH range, a stable electric current, and a separation efficiency of up to 47,000 theoretical plates (Wiedmer et al., 2011).

The primary advantage of using particle packing in CEC and MCEC is the ability to utilize widely available and well-characterized commercial stationary phases, such as silica functionalized with C18, phenyl, and amino groups, among others. Additionally, the use of packed particles provides greater retention and control over the migration time of analytes, making it suitable for applications in the analysis of drugs, biomolecules, and metabolites in complex matrices. However, packing particles into capillary channels or microchannels requires highly precise procedures, which can lead to bubble formation, preferential channels, or partial obstructions that compromise EOF and analytical reproducibility. The presence of particles significantly increases the hydraulic resistance of the system, potentially requiring

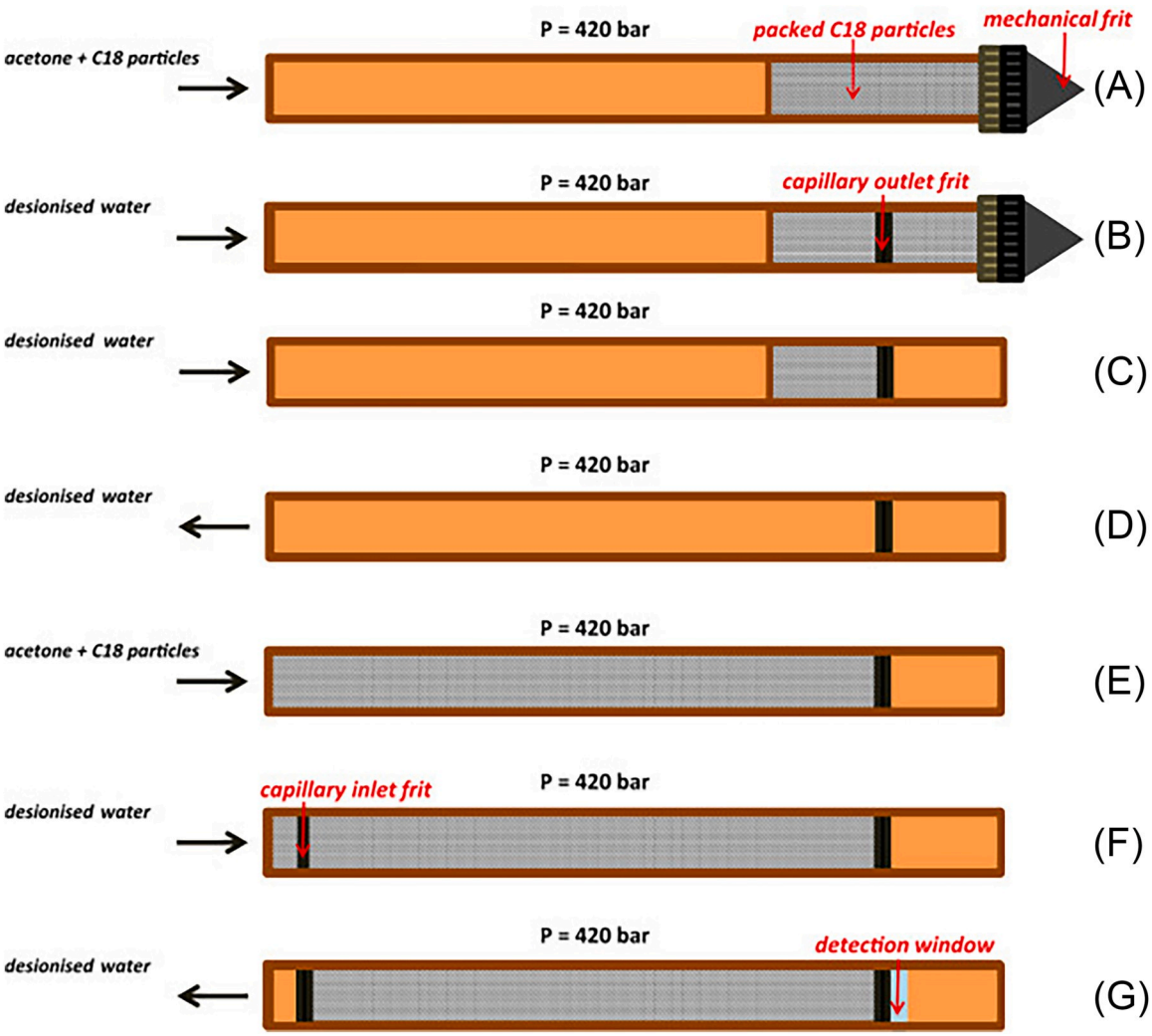

FIGURE 22.12 Capillary packing procedure scheme. (A) The capillary is partially filled with C18 silica particles. (B) Deionized water is passed through the packed capillary, and the outlet frit is positioned 10 cm away from the mechanical retainer. C18 particles are sintered, and consequently, the frit is made by heating a nichrome ribbon. Deionized water is passed through the capillary when frit formation is carried out. (C) The mechanic retainer is removed. (D) The capillary is emptied. (E) The Capillary is fully packed at high pressure. (F) Deionized water is passed through packed capillaries. Afterward, the inlet frit is sintered, considering the desired packed capillary length, as an outlet frit is made. (G) The excess of the stationary phase at the capillary inlet is removed. The detection window is completed, and the capillary ends are cut to the desired capillary dimensions. *From Hernández-Mesa, M., Lara, F.J., Cruces-Blanco, C. & García-Campaña, A.M. (2015). Determination of 5-nitroimidazole residues in milk by capillary electrochromatography with packed C18 silica beds. Talanta, 144, 542–550. https://doi.org/10.1016/j.talanta.2015.06.049.*

higher electrical voltages or additional pressures to maintain flow, which can lead to operational instability or device degradation (Breadmore et al., 2019; Park et al., 2007).

On the other hand, in situ polymerization occurs directly inside the capillary through the polymerization of monomers. The advantages of using this type of stationary phase include the simplicity of the process due to the uniformity within the capillary itself, which promotes excellent reproducibility and avoids common packing flaws, such as empty channels or uneven particle distribution. Additionally, the resulting polymeric monoliths exhibit high permeability due to their interconnected porous structure, allowing for efficient mobile phase flow with minimal back pressure. The technique also offers chemical flexibility, allowing the incorporation of various functional groups by adjusting monomers and porogens, which enables the design of selective stationary phases (Xu et al., 2023).

An example of in situ polymerization is the work done by Fu and collaborators in 2023. The authors used an in situ immobilization of covalent organic frameworks as a stationary phase for CEC. The developed material, called COF TZ-BPTA, was synthesized in situ and immobilized directly on the inner wall of capillary columns at room temperature. After that, the COF was synthesized directly within the capillary, resulting in a uniform coverage of spherical particles, as demonstrated by scanning electron microscopy (SEM) images. The column modified with COF TZ-BPTA demonstrated excellent chromatographic performance. Efficiencies higher than 2.8×10^5 plates m^{-1} were obtained, standing out from other COF-based columns reported in the literature. The system was able to efficiently separate a wide range of analytes, including alkylbenzenes, polycyclic aromatic hydrocarbons, parabens, sulfonamides, amino acids, herbicides, and antiepileptic drugs (Fu et al., 2023).

In situ polymerization has been highlighted as a robust approach for fabricating stationary phases in MCEC systems, primarily due to the limitations associated with traditional methods of packing particles into microchannels with dimensions of less than 100 μm (Nischang et al., 2010). Polymerization has several advantages, as particle packing in narrow microchannels often results in uneven distribution and void formation, which compromises separation efficiency and reproducibility. In contrast, in situ polymerization enables the formation of continuous monoliths directly within the microchannels, resulting in a homogeneous and cohesive structure that enhances the mechanical integrity of the system (Zou et al., 2002).

In situ polymerization eliminates the need for frits or additional supports to hold the stationary phase, simplifying the manufacturing process and facilitating integration with microfluidic devices. This procedure is particularly beneficial in MCEC applications, where miniaturization and the integration of multiple functions on a single chip are desirable. The technique enables the incorporation of various functional groups during monolith synthesis, allowing for customization of the stationary phase for specific applications, such as separations based on hydrophobic, ionic, or affinity interactions. In situ polymerized monoliths exhibit high porosity and pore connectivity, resulting in lower flow resistance and improved separation efficiency, even at high flow rates. This flow rate is crucial for fast, high-throughput analysis in MCEC systems (Levkin et al., 2008).

Furthermore, the technique represents an effective solution to the challenges associated with manufacturing stationary phases in MCEC, offering significant advantages in terms of homogeneity, mechanical integrity, versatility, and efficiency. Its ability to produce continuous, functionalized monoliths directly within microchannels makes it a preferred technique for developing advanced microfluidic devices.

22.2.2.2 Monolithic phase synthesis: sol-gel chemistry, photopolymerization, and thermal polymerization

Sol-gel is a wet chemistry technique used for the synthesis of solid nanomaterials from small molecules, as well as for the production of glassy materials and oxides. It is, therefore, widely used for the preparation of monolithic inorganic phases, especially silica-based, in microfluidic devices with vitreous substrates such as glass or quartz, and has stood out as a versatile and efficient approach for the synthesis of monolithic stationary phases, particularly inorganic ones, for CEC applications (Ur Rahman, 2021).

Sol-gel chemistry enables the formation of three-dimensional networks of metal oxides, primarily silica, through the hydrolysis and condensation of precursors such as tetraethylorthosilicate (TEOS), which operates at relatively low temperatures and is compatible with vitreous substrates like glass and quartz (Constantin & Freitag, 2000). A key advantage is the strong chemical bond between the stationary phase and the capillary walls, which improves mechanical stability and reduces leaching. Columns coated with β-cyclodextrin sol-gel showed improved enantioseparation of compounds such as chlorphenamine and zopiclone, with excellent repeatability between steps (RSD < 0.89%) and between columns (RSD < 2.9%) (Jiang et al., 2019). Encapsulation of biomacromolecules is also possible. BSA and ovomucoid were immobilized on sol-gel silica to create chiral monoliths, achieving up to 72,000 plates/m and good repeatability in the separation of enantiomers (Kato et al., 2002).

A sol-gel method was employed to fabricate a PMMA electrophoresis microchip featuring a hydrophilic channel wall, as developed by Chen et al. (Chen et al., 2007). The main conclusions of the study were that the sol-gel modified PMMA microchip had a smaller contact angle with water (≈ 27.4°) compared to pure PMMA (≈ 66.3°), and the EOF increased from 2.13×10^{-4} cm^2 V^{-1} s^{-1} for the native PMMA channel to 4.86×10^{-4} cm^2 V^{-1} s^{-1} for the modified channel. In addition, the uric acid separation efficiency increased to 74,882.3 m^{-1} compared to 14,730.5 m^{-1} for the native PMMA microchips. After removing the excess TEOS, the channel was filled with an acid solution for a period of 3 hours. The channel was washed with water and pretreated in an oven to obtain a sol-gel-modified PMMA microchip. The analytical performance of the sol-gel-modified PMMA microchip was demonstrated for the electrophoretic separation of various purines, accompanied by amperometric detection. The result of this simple modification is a significant improvement in the performance of PMMA for MCE and microfluidic applications (Chen et al., 2007).

Sun et al. (Sun et al., 2004) have developed an electrochemical detector based on sol-gel-derived carbon composite material for CE microchips. The main conclusions of the study include the successful manufacture of a rigid, easy-to-manufacture detector and the development of a modified copper particle detector for carbohydrate detection—an electrochemical detector based on a sol-gel-derived carbon composite material for CE microchips. An on-chip disk electrode based on sol-gel-derived carbon composite material can be manufactured reproducibly. Experimental procedures, including the fabrication of this detector, the configuration of the outlet of the separation channel and the electrode edge, and the performance characteristics of this new electrochemical detector, were investigated (Sun et al., 2004).

In situ photopolymerization is a widely used technique for preparing monolithic stationary phases for CEC and microdevice applications, owing to its speed, simplicity, and ability to be performed directly within capillaries or microchannels. This approach enables the formation of robust porous structures with good adhesion

to the internal wall of the support. The efficiency of photopolymerization depends on several experimental factors. The pretreatment of the inner capillary wall is critical for obtaining a structurally homogeneous stationary phase. The introduction of methacrylate groups through prior silanization yields more uniform monoliths, as demonstrated by SEM, compared to the direct addition of the silanizing agent into the polymerization mixture. Furthermore, UV irradiation energy is a key parameter: below $3\,J/cm^2$, a suitable monolith is not formed; above $12\,J/cm^2$, the polymer structure is destroyed. Within this range, columns showed good reproducibility (RSD < 10%) in terms of plate height, retention factor, and electroosmotic mobility (Augustin et al., 2006).

Regarding the polymer formulation, the choice of monomer and crosslinking agent directly impacts chromatographic performance. Monoliths prepared with benzyl methacrylate and bisphenol A dimethacrylate, using porogenic solvents such as cyclohexanol and 1-decanol, resulted in materials with good permeability, mechanical stability, and improved selectivity for aromatic analytes compared to systems based on EDMA (Ou et al., 2010).

This technique has also proven promising for analytical miniaturization. In glass microfluidic devices, acrylate-based monoliths were photopolymerized within the channels using masked UV exposure, enabling the precise design of injection, separation, and detection zones. These microchips achieved the separation of six peptides in just 45 seconds, with efficiencies of up to 600,000 plates/m. Device regeneration was made possible by thermal incineration of the polymer (Throckmorton et al., 2002).

Different photopolymerizable systems, including photopolymerized sol-gel approaches, were also investigated. In this context, the choice of photoinitiator (such as Irgacure 819, 1700, or 1800, or benzoin methyl ether) and the presence of additives, including sodium dodecyl sulfate (SDS), significantly influence the morphology and chromatographic behavior of the columns. All resulting phases exhibited reversed-phase behavior, and efficiencies of up to 74,470 plates per column were achieved for neutral compounds, such as thiourea (Gong et al., 2008). Additionally, photopolymerization has been applied to the preparation of molecularly imprinted polymers (MIPs). Using 4-hydroxybenzoic acid (4-HBA) as a template, monoliths with specific recognition sites were prepared, enabling baseline separation of hydroxybenzoic acid isomers in under 8 minutes—an effect not observed with nonimprinted polymers. These MIPs also demonstrated the ability to separate neutral compounds under pressure-driven CEC conditions (Zhang et al., 2007).

Yamamoto et al. developed an online MCE-mediated preconcentration method for cationic compounds using cationic polyacrylamide gels fabricated by in situ photopolymerization in 2018. The main key findings of the study include the development of a simple and efficient method for fabricating a cationic sample preconcentrator and the achievement of preconcentration factors exceeding 10^4-fold. This approach is based on a simple photochemical copolymerization method for fabricating a permselective preconcentrator. The intersection of the PMMA microchip was filled with a gel solution comprising acrylamide, N,N-methylene-bis-acrylamide, (3-acrylamidopropyl)trimethylammonium, and riboflavin, which functioned as a photocatalytic initiator. In situ polymerization near the cross-section of the sample outlet channel was performed by pinpoint irradiation with a 488 nm second-harmonic generation laser beam, which served as the light source for fluorimetric detection. The utility of the cationic preconcentrator gel was demonstrated by analyzing rhodamine derivatives, oligosaccharides labeled with rhodamine 110, and cytochrome C labeled with fluorescein isothiocyanate (Yamamoto et al., 2018).

The same author, a few years later, also studied, in the same line, an in situ photopolymerization of functionalized polyacrylamide-based preconcentrators for highly sensitive specific detection of various analytes by MCEC. Some of the key findings of the study include the development of a new method for fabricating an ion preconcentrator on a commercial cross-channel PMMA chip based on the photopolymerization of ionic acrylamide. The study also found that the preconcentrator efficiently captured oppositely charged ionic analytes in front of the nanofilter by applying hundreds of volts for a few minutes (Yamamoto, 2021).

Thermal polymerization is considered a bottom-up approach in which smaller units self-assemble to form larger and more structurally complex systems through intermolecular interactions, such as Van der Waals forces, hydrogen bonds, and/or electrostatic adsorption. This strategy has been effectively employed in the synthesis of polymeric monolithic stationary phases within confined microdevices, enabling high control over structure formation and its precise location within the device.

Recent studies have demonstrated the fabrication of thermally initiated monoliths in 3D-printed titanium devices under controlled temperature conditions. By maintaining distinct hot (70°C) and cold (4°C–10°C) regions during the reaction, it was possible to obtain clear interfaces between the filled and empty regions, ensuring a homogeneous monolithic structure well anchored to the channel walls. This thermal confinement enabled the localized formation of monoliths with excellent reproducibility. The produced monoliths exhibited high porosity (~ 60%) and good permeability (~ 4×10^{-15} m^2), essential characteristics for high chromatographic performance (Passamonti et al., 2020). Their application in reversed-phase liquid chromatography resulted in the effective separation of intact proteins, demonstrating the potential of thermal polymerization as a robust and versatile technique for developing stationary phases integrated into miniaturized analytical systems.

In addition to the aforementioned strategies, other innovative approaches have made significant contributions to the advancement of monolithic phase synthesis for CEC. Among these is the in situ synthesis of porous polymer monoliths (PPM) on PDMS/glass microchips integrated with microvalves. Through the precise control provided by the PDMS microvalves, it was possible to perform the grafting of the microchannel surface and the localized photopolymerization of the poly(methacrylic acid-co-ethylene glycol dimethacrylate) monolith (Kang et al., 2010). The optimization of parameters, such as polymerization time, PDMS elastic properties, UV intensity, and grafting conditions, demonstrated that PPM can be successfully synthesized on the PDMS microchip, resulting in a homogeneous structure and excellent mechanical properties. This technology allowed the microextraction, separation, and online detection of dopamine with a high enrichment factor (up to 80 times), demonstrating the potential for integrating monoliths into high-throughput and reduced-complexity analytical systems.

Another prominent strategy is the fabrication of capillary monolithic columns functionalized with chiral nanostructured materials, such as chiral molecularly imprinted polymers (CMIPs) and chiral metal-organic frameworks as stationary phases. Miao et al. (Miao et al., 2025) developed a column for the efficient enantioseparation of the herbicide dichlorprop, a compound with distinct biological and toxicological properties. The innovation lies in the use of a novel functional monomer, allyl-β-d-pyrone galactoside (PG), with the capacity for "chiral prerecognition" of the racemic template. This ability enabled the use of the racemic compound as a template for the synthesis of CMIPs, thereby dispensing with the need for conventional single-enantiomer templates, reducing costs, and simplifying the process. The column showed

excellent selectivity and efficiency, and the enantioseparation mechanism was elucidated through molecular docking simulations and static adsorption studies (Miao et al., 2025).

These approaches demonstrate the potential of integrating microdevices, novel functional monomers, and nanostructured materials to expand the limits of monolithic phase synthesis, offering greater selectivity, sensitivity, and versatility in the separation of complex compounds in real samples.

22.2.3 Optimization of capillary surface properties

Modifying the internal surface properties of capillaries and microchannels is a crucial step in ensuring stable and reproducible performance in CEC systems, particularly when integrated with MCEC. Controlling the EOF through selective functionalization of surfaces is a strategy to enhance separation efficiency, analytical selectivity, and compatibility with various sample matrices. Two main approaches are employed to stabilize the EOF: dynamic coatings, which use polymers or surfactants added to the mobile phase, and permanent covalent modifications, such as silanization or plasma activation, which offer greater durability and consistency. The choice of technique depends on the type of substrate, the time of use, and the complexity of the sample (Fernández-Abedul et al., 2013; Rocco et al., 2013).

22.2.3.1 EOF control: dynamic and covalent coatings

EOF is the primary mechanism for propelling the mobile phase in CEC and MCEC systems. This flow is generated by the movement of cations in the electrical diffusion layer near the inner surface of the channels under the application of an electric field. In microchips, precise control of the EOF is more important due to the miniaturization of the channels and the absence of pressurized pumps (Fernández-Abedul et al., 2013). Slight variations in the channel surface can cause significant fluctuations in the flow velocity, compromising the reproducibility and efficiency of the separations. To better stabilize the EOF, surface modification techniques are employed, which adjust the charge density of the channel wall or suppress the formation of adsorption zones. Among the most widely used approaches are dynamic coatings, in which substances such as soluble polymers or surfactants are added to the mobile phase. These coatings form a temporary layer on the surface of the channel, reducing interaction with the analytes and smoothing out fluctuations in the flow. Polymers such as polyethylene glycol and polyacrylamide, as well as surfactants like SDS, are typical examples (Rocco et al., 2013).

In contrast, covalent coatings offer a more durable solution. Using techniques such as plasma activation, photopolymerization, or silanization, functional groups are chemically attached to the channel wall. This approach creates a stable layer that is less susceptible to chemical variations and repeated use. In addition to increasing the stability of the EOF, covalent coatings also enable subsequent chemical modifications, allowing, for example, the addition of affinity groups or specific hydrophobic properties. The silanization method utilizes silica or glass-based substrates, where silane reagents, such as 3-aminopropyltriethoxysilane or octadecylsilane, react with the silanol groups to form Si-O-Si bonds. This approach enables the surface to be modified with functional groups, such as amino, carboxyl, alkyl, or epoxide, thereby adjusting both the charge and hydrophobicity of the channel (Shakeri et al., 2021).

UV-grafting is the process in which a surface is exposed to UV radiation in the presence of vinyl monomers and photoinitiators, promoting the formation of a covalently bonded polymeric

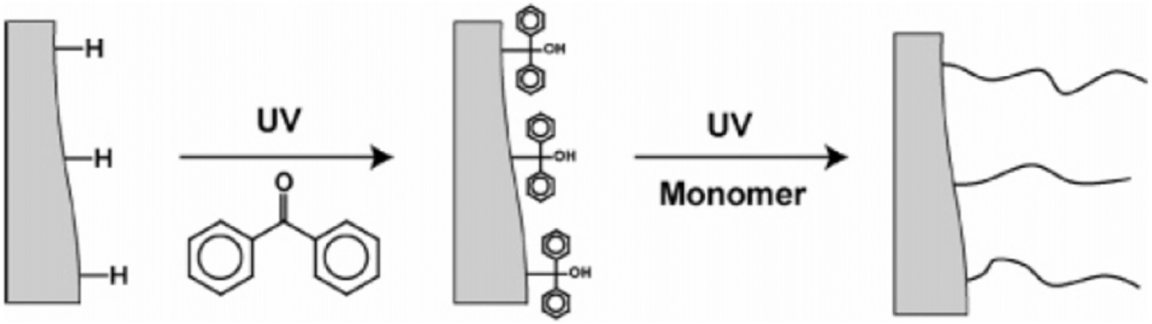

FIGURE 22.13 Sequential two-step graphitization performed on a previously formed monolithic column, using benzophenone as a photoinitiator. *From Stachowiak, T.B., Svec, F. & Fréchet, J.M.J. (2006). Patternable protein resistant surfaces for multifunctional microfluidic devices via surface hydrophilization of porous polymer monoliths using photografting. Chemistry of Materials, 18(25), 5950–5957. https://doi.org/10.1021/cm0617034.*

layer. This technique is particularly advantageous for polymeric substrates, such as PDMS or PMMA, where traditional silanization is limited. Fig. 22.13 involves the immobilization of the initiator on the internal surface of the monolith using photoinduced lysis, as described by Stachowiak et al. (2006), which leaves a free radical bound to the surface. When a polymeric material is exposed to UV radiation, its electrons are excited, resulting in the removal of hydrogen atoms from the polymer surface. If there are no monomers present, the radicals generated on the surface bind to another type of radical (semipinacol radical) formed by the activation of benzophenone by UV light. However, when monomers are present and a second exposure to UV radiation occurs, benzophenone releases the radicals from the surface, allowing them to initiate the formation of polymer chains (grafting) directly onto the material. This process enhances grafting efficiency by preventing excessive monomer dispersion in the solution, thereby directing chain growth toward the channel surface. Additionally, this technique reduces the viscosity of unreacted residues, facilitating their subsequent removal from the column (Stachowiak et al., 2006).

Plasma activation (oxidative or functional) is used to generate reactive groups (such as –OH or –COOH) on the surfaces of polymers, which subsequently serve as anchor points for covalent coupling reactions with other functional groups or polymers. Plasma activation involves exposing the channel surface to an ionized gas (plasma) generated under low pressure in a reactive chamber through an electrical discharge. The ions, electrons, and free radicals present in the plasma interact with the surface, breaking chemical bonds and introducing new functional groups, such as hydroxyls, carboxyls, and amines. The composition of the plasma defines the functional groups that will be inserted. For example, oxygen plasma introduces hydroxyl and carbonyl groups, increasing hydrophobicity and making the surface more reactive. Ammonia plasma (NH_3) inserts amine groups, which are functional for coupling reactions with aldehydes or esters. In microchips, covalent coatings are preferred because of their mechanical strength and stability under applied electrical stresses (Currivan & Jandera, 2014; Keppeler & Hüsing, 2011; Shakeri et al., 2021).

Another powerful strategy involves the use of "click chemistry," a set of highly specific,

efficient, and biocompatible coupling reactions that can be performed in aqueous solvents. An emerging technique for in situ postpolymerization modification of monolithic columns has been developed from a technique originally used in organic synthesis. This strategy is advantageous when seeking to immobilize functional groups in restricted regions of the channel or when it is necessary to preserve the biological activity of sensitive ligands (Keppeler & Hüsing, 2011). Combined, these functionalization strategies increase the analytical potential of MCEC devices, enabling more selective separations with greater chemical control and suitability for highly complex analyses. Therefore the choice between a dynamic or covalent coating depends on the type of application, the required durability of the system, and the composition of the matrix. In reusable devices or more demanding analyses, covalent coating is usually preferred due to its consistency over time.

22.2.3.2 *Functionalization strategies for improved separation efficiency and selectivity*

Functionalization strategies are crucial for optimizing the performance of CEC and MCEC systems. These modifications significantly enhance separation efficiency, minimize nonspecific adsorption, and improve chemical selectivity—critical factors for complex separations and high-sensitivity analyses. In miniaturized platforms, these strategies are even more important, as they contribute to flow control, integration of multiple analytical functions, and device reproducibility. Several studies reported in the literature describe the potential of functionalization strategies aimed at improving separation efficiency and selectivity.

Fu and colleagues developed in 2023 a synthesis of carbon dot-based covalent organic nanomaterials (CONs) as a stationary phase for open-tubular CEC. The functionalization strategy involves integrating CONs derived from carbon dots (CDs) into the sol-gel matrix to enhance separation performance. In a recent study, CONs synthesized via Schiff-base reaction between CDs and 1,3,5-tris(4-aminophenyl)benzene (TAPB) were used as a stationary phase in OT-CEC. The resulting CON CDs-TAPB structure provided abundant interaction sites and improved analyte accessibility, leading to high separation efficiency—up to 1.6×10^5 plates m^{-1}—and an excellent loading capacity for hydrophobic analytes, such as methylbenzene. This approach highlights the potential of organic-inorganic hybrid materials to tailor surface chemistry and porosity, thereby enhancing selectivity and resolution in microseparation platforms (Fu et al., 2023).

Functionalization strategies play a key role in enhancing chromatographic performance. In the case of the poly(VBS-co-TAT-co-AHM) monolithic column, the incorporation of sodium p-styrene sulfonate (VBS) as a functional monomer provided multiple interaction modes—π–π stacking, hydrophilic, and ion exchange—enabling efficient separation of diverse analytes under RPLC and HILIC conditions. The sulfonate groups introduced by VBS also contributed to the generation of a stable and controllable EOF, which is essential in CEC for maintaining resolution, reducing analysis time, and improving peak symmetry. The in situ polymerization ensured uniform distribution and stability of functional groups, while the cross-linkers AHM and TAT helped optimize porosity and mechanical strength. This multifunctional design yielded high separation efficiency (up to 1.7×10^5 plates/m), excellent selectivity, and reproducibility, highlighting the impact of rational surface functionalization and EOF control in modern stationary phase development (Li et al., 2023).

Selectivity and separation performance in CEC monolithic columns are also a challenge. In the study developed by Dai et al. in 2023, sulfonic acid-modified covalent organic polymer (COP-SO_3H) was synthesized via postsynthetic functionalization of a vinyl-functionalized precursor

(TAPT-DVA-COP). The introduction of $-SO_3H$ groups significantly improved the interaction profile of the stationary phase, enabling selective separation of a broad range of analytes, including monosubstituted benzenes, alkylbenzenes, hydroxybenzoates, nucleobases, and biogenic amines. The enhanced performance is attributed to the synergistic effect between the sulfonic acid groups and the aromatic framework of the COP, resulting in a rich combination of noncovalent interactions, including ion exchange, π–π stacking, hydrogen bonding, and hydrophobic forces. This multifunctional surface chemistry yielded increased selectivity, good reproducibility, and strong application potential for complex sample matrices, including pharmaceutical and environmental samples (Dai et al., 2023).

22.3 Coupling with mass spectrometry

22.3.1 Interfacing strategies for MCE-MS

The primary challenge in coupling microfluidic chips with MS is transferring the solutes present in the microchannels as ions to the analyzers without compromising the separation techniques. A review by Naghdi et al. (2023) summarizes the key concepts and technological advances in MCE-mass spectrometry (MCE–MS) coupling from 2016 to 2021. The authors point out that while establishing electrical contact at the sample and buffer inlets is relatively straightforward, doing so at the outlet where ionization occurs is significantly more challenging. Electrical contact is essential for achieving efficient molecular separation and ionization. Although electrode-free methods offer operational simplicity, they often compromise flexibility and precision due to system constraints, such as the inability to adjust separation and ionization potentials independently (Naghdi et al., 2023).

The direct (online) coupling of microfluidic chips with mass spectrometry is typically achieved through electrospray ionization (ESI), making MCE-ESI-MS the most widely adopted technique for this type of analysis. In the electrospray process, fused silica capillary emitters serve as needles that facilitate the ionization of samples. These capillaries are integrated into the microchips, enabling ionization to occur before analysis by mass spectrometry (Feng et al., 2015). Fig. 22.14 illustrates a schematic of the connection between a microfluidic chip and ESI for drug analysis in cells (Jie et al., 2017).

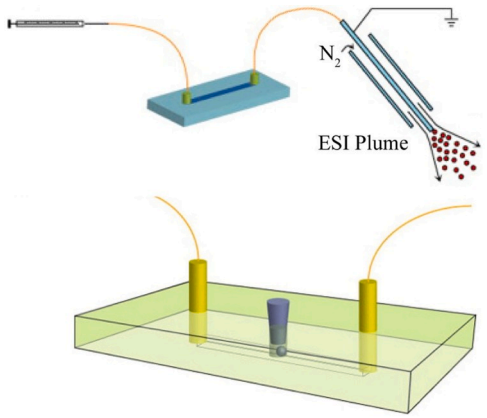

FIGURE 22.14 Microchip coupling with mass spectrometry in ESI-MS mode using capillaries for spray formation. *From Jie, M., Mao, S., Li, H. & Lin, J.M. (2017). Multi-channel microfluidic chip-mass spectrometry platform for cell analysis. Chinese Chemical Letters, 28(8), 1625–1630. https://doi.org/10.1016/j.cclet.2017.05.024.*

Microfluidic devices can also be coupled with matrix-assisted laser desorption/ionization (MALDI), resulting in the MCE–MALDI–MS method—an indirect, offline coupling approach. In this configuration, the microchip itself can function as a sampling plate. As the solvent evaporates, the analytes remain on the chip's surface, which is then directly inserted into the MALDI ionization source. However, unlike in CEC–MS coupling, MCE is rarely combined with MALDI–MS. One of the key advantages of microfluidic chips—their high separation speed—is diminished in the offline coupling, where maintaining rapid separations becomes challenging during fraction collection (Naghdi et al., 2023).

A coupling approach that eliminates the use of sheath liquid—known as the sheathless method—has also been developed. Traditionally, a solvent mixture (sheath liquid) is employed to assist in electrospray formation and facilitate ion transfer to the mass spectrometer. Moini (2007) designed a capillary with a porous tip that allows electrolytes to pass through while avoiding sample dilution by the sheath fluid. The ion-conductive glass membrane enables an efficient electrical connection without compromising analyte concentration. This innovation marked a significant advancement in the sheathless coupling, enhancing ion transfer efficiency and resulting in higher sensitivity (Moini, 2007). Fig. 22.15 presents a schematic illustrating the coupling using a porous tip for ion transfer, originating from a nanoLC operating under two flow conditions.

A study by Huang et al. (2011) evaluated three different interfaces for directly coupling a micellar electrokinetic chromatography (MEKC) microchip to ESI-tandem mass spectrometry (ESI-MS). The authors observed that few studies focus on MEKC microchip-MS-MS coupling, as most applications still rely on optical detection methods such as UV/Vis spectroscopy, fluorescence spectroscopy, or electrochemical detection. MEKC-ESI-MS can employ both conventional sheath liquid interfaces and low-sheath-flow configurations. Additionally, a dual-junction interface (liquid junction/low-flow) may be used to prevent the MEKC surfactant from entering the ESI-MS system. In this study, ammonium dodecyl sulfate (ADS) was used as a surfactant to reduce ion suppression effects (Huang et al., 2011).

In summary, coupling MCE with mass spectrometry has advanced significantly, offering a range of interface options tailored to different analytical demands. While MCE-ESI-MS remains the most commonly used method due to its practicality and continuous operation, other strategies, such as MCE-MALDI-MS and sheathless setups, provide valuable alternatives. Each configuration brings its strengths and limitations, particularly in terms of sensitivity and system complexity. As these technologies continue to mature, they are likely to enhance further the speed, resolution, and versatility of microfluidic-MS analyses across various scientific fields.

22.3.2 Interfacing strategies for CEC-MS

Regarding the coupling of CEC with mass spectrometry, (Klampfl, 2004) conducted a review evaluating various interface designs that enable this hyphenation. As with MCE-MS, the main challenge lies in efficiently transferring analytes from the capillary column to the MS inlet. Additionally, CEC-MS techniques require that the capillary's electrical circuit be grounded appropriately to ensure stable operation (Klampfl, 2004).

The first interface used for CEC–MS coupling was continuous flow fast atom bombardment; however, this technique exhibited high chemical noise and difficulties in maintaining a stable electrical current. Consequently, it was eventually replaced by atmospheric pressure ionization interfaces, with ESI and atmospheric

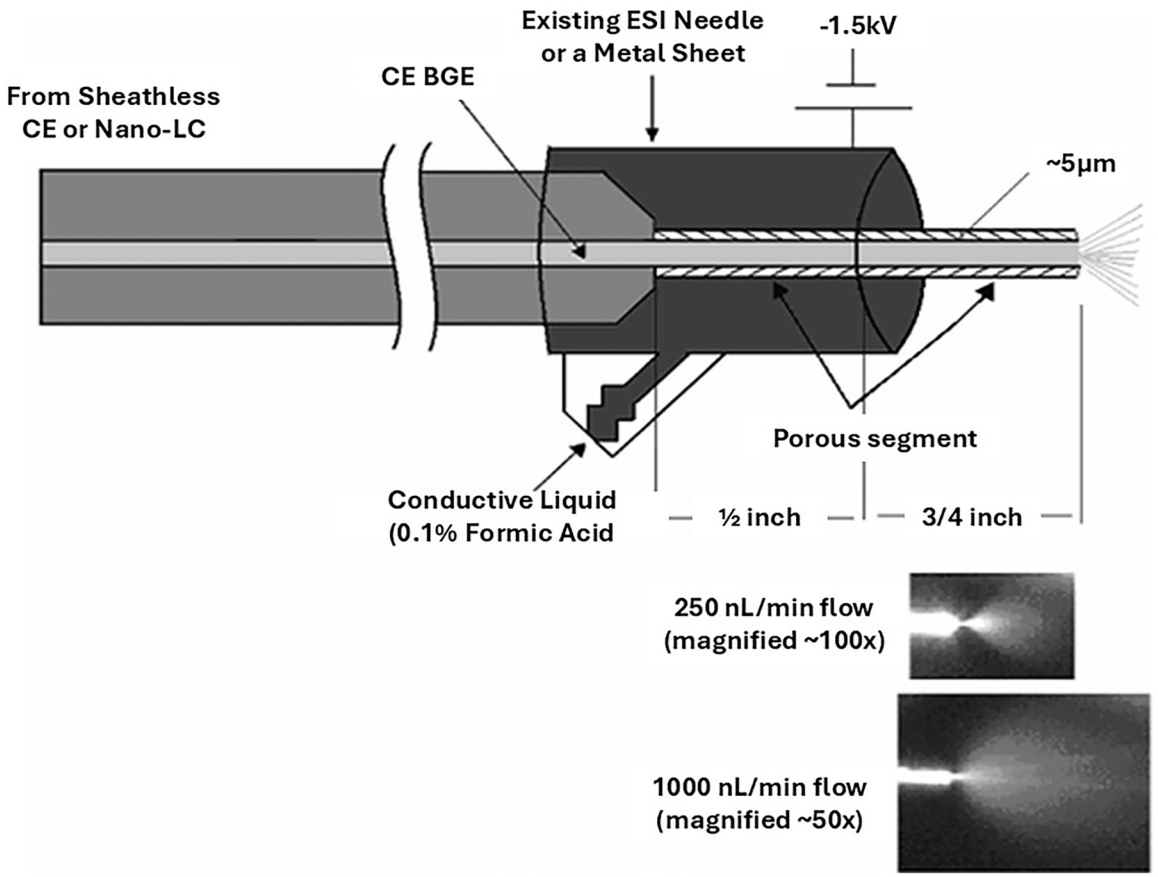

FIGURE 22.15 Illustration of a CE and nLC porous tip assembly generating the ESI plume from an nLC, operating at 250 nL/min (magnified by ~100×) and 1 µL/min (magnified by ~50×). *From Moini, Mehdi. (2007). Simplifying CE–MS Operation. 2. Interfacing Low-Flow Separation Techniques to Mass Spectrometry Using a Porous Tip. Analytical Chemistry, 79(11), 4241–4246. https://doi.org/10.1021/ac0704560.*

pressure chemical ionization being the most prominent techniques. In these systems, the capillary column itself can function as the electrospray emitter, or, if necessary, a transfer capillary may be placed between the column and the ESI needle. One of the ongoing challenges in this type of coupling is the development of customized interfaces using shorter columns to reduce analysis time without compromising separation efficiency (Klampfl, 2004).

D'Orazio and Fanali developed in 2010 a lab-built liquid junction interface for online CEC-MS coupling, machining a PMMA (polymethylmethacrylate) block. When combined with nanospray, this setup initially led to bubble formation, which was mitigated by using packed capillaries and applying external pressure to both vials during analysis. However, this additional pressure could ultimately compromise separation efficiency in the capillary, negating the method's benefits.

Since the group had previously tested the liquid junction interface for CE-MS coupling, the same approach was adapted for CEC without external pressure, proving to be highly effective. This modification enabled a bubble-free operation, stable current, and high sensitivity. Ongoing research continues to focus on optimizing capillary connections to improve further interface performance (D'Orazio & Fanali, 2010). Fig. 22.16 shows a schematic of CEC-MS coupling for a separation experiment involving pesticides and enantiomers.

In a more recent study, Wang et al. employed a CEC-MS coupling for the analysis of chiral amino acids. Since chiral selectors are nonvolatile, they pose a risk of contaminating the MS ion source. To address this issue, the selectors (cyclodextrins) were immobilized on the separation column as a chiral stationary phase, enabling the resolution of enantiomers before they entered the MS. Although the technique shows promise, it still faces technical challenges and specific limitations that hinder its application in online separations, particularly those involving enantiomers. For effective coupling, background electrolytes

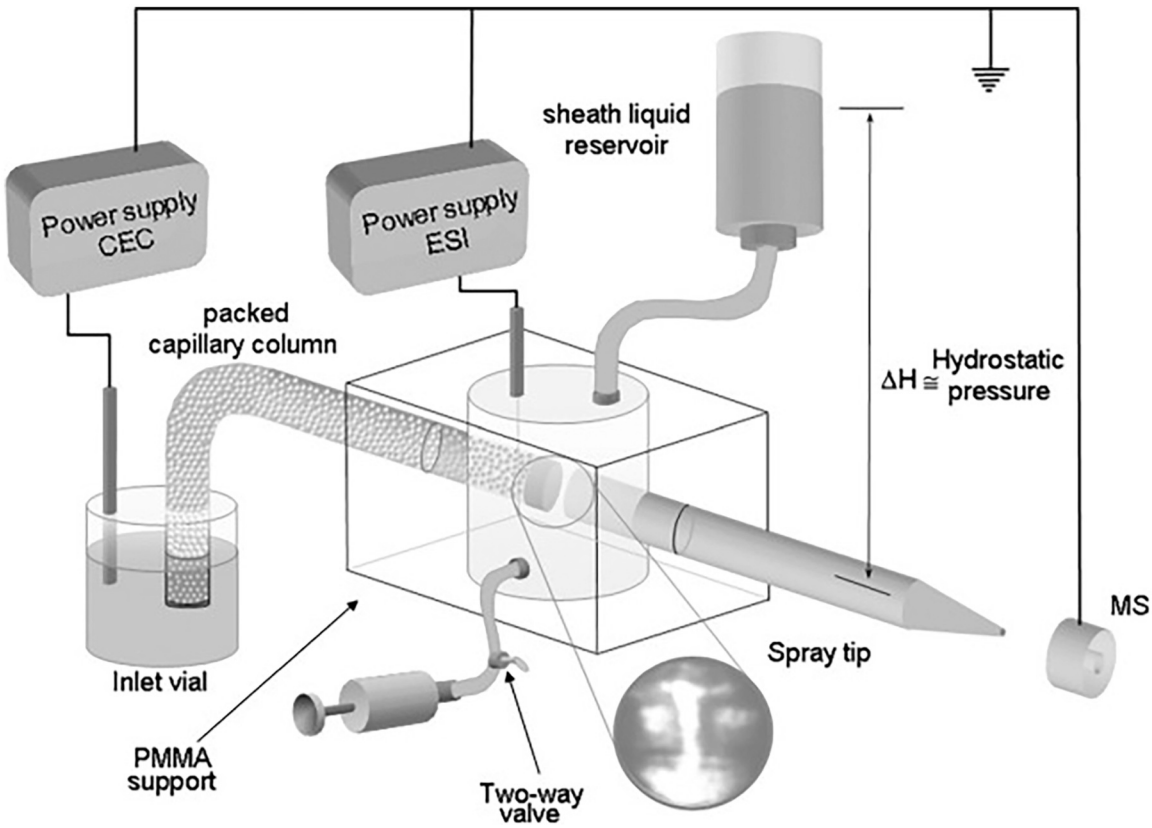

FIGURE 22.16 Scheme of the CEC-MS instrument with a liquid-junction ESI interface (no scale drawing). The distance between the packed capillary and the tip was about 100 µm. *From D'Orazio, Giovanni & Fanali, Salvatore. (2010). Coupling capillary electrochromatography with mass spectrometry by using a liquid-junction nano-spray interface. Journal of Chromatography A, 1217(25), 4079–4086. https://doi.org/10.1016/j.chroma.2009.11.004.*

must be volatile and of low ionic strength, and the interface must be carefully designed to ensure consistent and controlled sample introduction into the mass spectrometer (Wang et al., 2022).

CEC-MS continues to demonstrate tremendous potential as an analytical technique, particularly in applications that require high-resolution and chiral separations. Recent improvements in interface design, combined with the use of immobilized chiral selectors, have enhanced performance and sensitivity. Still, the technique faces important limitations, especially for online analysis, where issues such as electrical stability and electrolyte compatibility must be carefully addressed. Even so, steady progress in overcoming these challenges suggests that CEC-MS will play an increasingly relevant role in future analytical strategies.

22.3.3 Overcoming technical challenges

One of the challenges in coupling CE techniques with ESI mass spectrometry is the requirement for a capillary column longer than 65 cm, which can result in relatively longer separation times. Performing electrophoresis on microchips can overcome this issue, as the miniaturized chip can be integrated directly into the ionization source, and the separation channels can be customized in length to suit the specific application (Zhong et al., 2014).

One notable limitation of the technique is that its high-speed separation speed tends to generate narrow, short-lived peaks. In a study by Black et al. in 2015, a CE microchip coupled with hydrogen-deuterium exchange mass spectrometry via ESI was used for the analysis of intact proteins and protein complexes. When compared to an LC-MS method, the latter showed better performance in terms of protein coverage. This reduced coverage with MCE-ESI was likely due to a combination of technical constraints, including the high separation speed, lower sensitivity compared to LC-MS, minimal injection volumes, and the absence of a preconcentration step. The results reflect the common limitations of early-stage technologies, indicating that while the technique has strong potential, further improvements are still necessary to overcome technical hurdles and enhance overall performance (Black et al., 2015).

It can be said that the introduction of microchips was primarily driven by the small sample volumes used in CE-MS techniques, leading to a coupling system based on a fluidic microchip that integrates both the CE capillary and the nanoESI emitter, making it essential for any analysis based on this technique. Moreover, there is no flexibility to adjust the capillary parameters within the microchip, such as diameter and length, which limits the possibility of performing more customized experiments (Gahoual et al., 2019).

22.3.4 Applications and future directions

In the context of current applications of microfluidics coupled with mass spectrometry, bioanalytical research remains a primary area of advancement, particularly at the single-cell level. This field of research enables the conduct of studies such as quantifying intracellular metal elements using a spiral chip coupled to ICP-MS, performing real-time quantification with internal standards, directly analyzing cell membranes with ESI-QTOF-MS, distinguishing between human tumor subtypes, and quickly separating isobaric compounds (Berlanda et al., 2021).

Still, in cellular analysis and proteomics, a device developed by Holland-Moritz enables the screening of droplets based on specific mass spectrometry signals. It works by splitting the droplets and analyzing the product ions with ESI-MS, while the rest are held back for later separation using dielectrophoresis (Holland-Moritz et al., 2020).

For the analysis of complex biological samples, the most commonly chosen platform is

two-dimensional separation, as it offers higher peak capacity and resolution power. As an orthogonal alternative to liquid chromatography, MCE can be an ideal choice for separation since its high separation speed improves the sampling rate of the first dimension (Zhong et al., 2014). In a review conducted by Štěpánová and Kašička (2023), studies are presented that demonstrate how MCE-MS coupling can be a valuable and effective tool for proteomic and peptidomic studies, as well as for clinical applications and biological and biochemical research. Its miniaturized dimensions make the technique particularly attractive for protein analysis in single cells. Further advancements in MCE-MS methods are anticipated in the future to expand their applications in this type of analysis (Štěpánová & Kašička, 2023).

Sah et al. used an MCE-Orbitrap-based method to monitor ovarian cancer progression. The method was validated and applied to determine 40 metabolites, showing good reproducibility and sensitivity for the target analytes while reducing sample consumption, solvent use, and analysis time. They analyzed amino acids, amino acid derivatives, and nucleosides. The validation demonstrated that the MCE-MS method can be applied in metabolomics without significant issues. In the future, more experiments could be conducted by coupling the electrophoresis system with faster mass spectrometry instruments, such as time-of-flight mass spectrometry (Sah et al., 2022).

When it comes to CEC-MS coupling, methods using this technique often receive considerable attention because they can be highly efficient and provide rapid analyses. Mass spectrometry is highly advantageous because it provides more detailed information about the molecular structure being analyzed. When combined with electrochromatography, which integrates the features of both CE and HPLC, it becomes a powerful separation and detection technique with high sensitivity and selectivity, particularly for biological analysis. In the future, we can expect improvements in the robustness of CEC-MS methods, better coupling interfaces, and further miniaturization (Luedtke & Unger, 1999).

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