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# Advances in steady-state continuous-flow purification by small-scale free-flow electrophoresis

Fletcher J. Agostino, Sergey N. Krylov *

*Centre for Research on Biomolecular Interactions, York University, Toronto, Canada*

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ABSTRACT

This year marks the 150th anniversary of the first continuous-flow chemistry (CFC) technique, developed by Ernest Solvay, which revolutionized industrial level synthesis. CFC is defined by multi-stage processes in which mixing and reacting of chemicals occur without interruption. A significant challenge of CFC lies in post-reaction purification. Free-flow electrophoresis (FFE) could be integrated with CFC. FFE separates chemicals by an electric field that is directed orthogonally to a pressure driven hydrodynamic flow. Although there are problems with FFE, both macro-scale and small-scale FFE are feasible for CFC integration, and realizing long-term steady-state continuous-flow purification can have significant benefits. In this review, we discuss (i) the progress of CFC, (ii) existing continuous-flow purification techniques, (iii) small-scale FFE limitations associated with steady-state continuous-flow purification, and (iv) advances in FFE performance.



**Contents**



*Abbreviations:* ATPS, Aqueous two-phase system; CE, Capillary electrophoresis; CFC, Continuous-flow chemistry; FFE, Free-flow electrophoresis; IEF, Isoelectric focusing; MEKC, Micellar electrokinetic chromatography; μFFE, micro-FFE; mFFE, Milli-FFE; NOFFE, Non-orthogonal FFE; OEFFE, Open-electrolyte FFE; SacC, Sacrificial channel; SMB, Simulated moving bed.

* Corresponding author. Tel.: +1 416 736 2100 x22345; Fax: +1 416 736 5936.
*E-mail address:* skrylov@yorku.ca (S.N. Krylov).

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## 1. Introduction

### 1.1. Large-scale continuous-flow synthesis

In continuous-flow chemistry (CFC), chemical reactions are carried out in a fluid stream in a steady-state fashion. CFC takes its roots from successful attempts to streamline nineteenth-century industrial-scale production [1]. In 1864, Ernest Solvay developed a revolutionary technique that continuously produced sodium carbonate by employing the ammonia-soda process. This process, using continuous-flow synthesis, allowed for interconnection of multiple feedback loops, which maximized reaction efficiency and minimized reagent waste. Largely due to this dramatic improvement, the production of sodium carbonate increased over 10-fold by the turn of the twentieth century [2]. The Solvay process, with some modifications, is still in use today.

The benefits of CFC for large-scale production were obvious from the beginning. Large-scale production can be achieved without the need for large-volume reactors, which are difficult to control and often unsafe. The amount of waste generated can be dramatically decreased, while the intermediate products or unreacted starting material can be recycled. The build-up of large amounts of hazardous chemicals can be avoided and hazardous materials can be managed in a streamlined, safe manner. These benefits facilitated decreased production cost and increased production scale. Appreciation of these benefits has led to the use of CFC in many modern large-scale industrial applications. To date, the most abundantly produced chemicals are the raw chemicals that form the majority of starting reagents and solvents in chemical reactions. Raw chemicals are required in large amounts and this demand can be met because they are typically produced by CFC processes, thus benefitting from high efficiency and low cost. Interestingly, CFC chemical plants serendipitously adopted at least two of the principles of Green Chemistry formulated at the turn of the twenty-first century: prevention and atom economy [3]. The greener nature of CFC can offer important insight into new synthesis strategies and optimization of pre-existing protocols. Additional green advantages were realized when small-scale continuous-flow synthesis was introduced.

### 1.2. Small-scale continuous-flow synthesis

While produced in large abundance, raw chemicals represent only a minor fraction of the variety of all synthesized chemicals. The vast majority of chemicals are fine chemicals produced at small or very small scale, with an emphasis on quality and purity. An ultimate example is the synthesis of highly-diverse combinatorial libraries containing billions of different structures with as few as 1000 copies of every structure [4]. Until recently, batch production of fine chemicals was the only available option. Fine chemical synthesis strategies changed over the past decade when small-scale continuous-flow synthesis was developed using capillary and channel micro-reactors. The advantages of small-scale continuous-flow synthesis, over a batch approach, include:

1 greater control over the precision of reaction conditions (i.e., temperature, pressure, quality of mixing) [5,6];
2 ability to use high temperature and pressure [7];
3 suitability for in-line monitoring of reaction efficiency [8];
4 automation capabilities [9,10];
5 safer handling of hazardous reactions [11]; and,
6 simple scale-up strategies for larger product quantities [12,13].

The efficiency, the versatility and the safety associated with micro-reactors are possible due to rapid mass- and heat-transport processes. The success of small-scale continuous-flow synthesis is validated by the availability of commercial equipment, which has been used for a variety of chemical reactions [14–16]. Overall, the multitude of advantages associated with small-scale continuous-flow synthesis has been acknowledged by both the fine-chemicals and pharmaceutical industries, which are beginning to adopt continuous-flow methodologies.

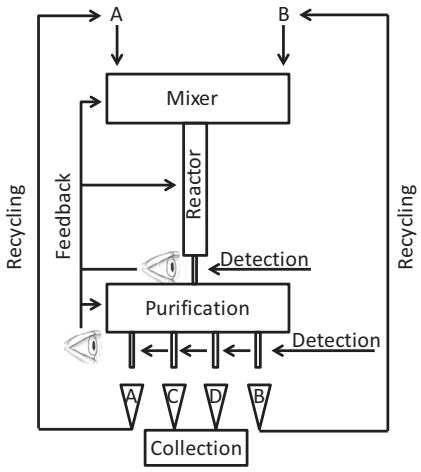


**Fig. 1.** A fully integrated and interconnected design for continuous-flow chemistry (CFC).

To exploit the benefits of small-scale CFC fully, it would be ideal to combine chemical synthesis in micro-reactors with continuous-flow purification and continuous-flow analysis. Such a combination would allow feedback for automated process control and improved recycling of unused materials for highly economical operation (Fig. 1). However, continuous-flow synthesis is typically followed by discontinuous purification. The main reason for the use of discontinuous purification is that a limited number of options for continuous-flow purification are available. Here, we discuss all continuous-flow purification techniques that are viable in-line options for continuous-flow synthesis.

## 2. Continuous-flow purification strategies for continuous-flow synthesis

We define continuous-flow strategies as those that process an undivided sample stream that is propagated by an unperturbed flow. The integration of continuous-flow purification to continuous-flow synthesis must satisfy the following criteria:

- a purification device should resolve target analytes from impurities, by-products, and unused reagents, with minimal contamination;
- the purification device should also be compatible with the solvent used in the flow reactor; and, finally,
- integrating continuous-flow synthesis and purification should not interfere with the steady-state performance of the entire CFC design.

Until recently, difficulties in satisfying these criteria precluded integration of continuous-flow purification into CFC designs.

For the sake of our definition of continuous-flow strategies and the scope of this review, we cannot consider simulated moving bed (SMB) chromatography as a purely continuous-flow technique. Automation and robotic handling achieve continuous processing of discrete volumes, so the original fluid stream is divided into smaller volumes. To date, there exist only four continuous-flow purification platforms that can be considered as potentially viable with CFC

– magnetophoresis, liquid-liquid extraction (LLE), fractionation, and electrophoresis. We review them in this section.

### 2.1. Magnetophoresis

Particles, which are naturally magnetic or functionalized with a magnetic tag, can be separated from a sample in the presence of a magnetic field. Larger magnetic particles (1 μm and greater) are more susceptible to the force applied by a magnetic field. Since very few particles exhibit magnetic properties, efficient separation can be achieved with minimal contamination. Free-flow magnetophoresis separates particle analytes from a stream of sample, which is driven continuously through a magnetic field and typically orientated orthogonally to the flow. Magnetophoresis, in free-flow form, has been applied to the separation of cancer cells from normal cells [17,18], DNA purification [19], water purification [20,21], and nanoparticle (NP) purification [22].

Aside from being an efficient, precise means of separation, steady-state performance is easily achieved in magnetophoresis, which is an attractive advantage for continuous-flow purification. One caveat is that magnetophoresis in small channels is prone to clogging. The removal or the prevention of a clog would require the magnetic field to be weakened or turned off. If this occurred in an integrated CFC design, magnetophoresis would force the entire process to a halt. Furthermore, since very few analytes can be resolved naturally by magnetophoresis, all other analytes would have to be labelled with different magnetic tags in order to achieve separation. In CFC, such labeling would follow a flow reactor and must be extremely selective, making a tandem continuous-flow synthesis and purification set-up highly impractical.

### 2.2. Liquid-liquid extraction

LLE is a purification technique that is based on the separation of non-miscible liquids. Conceivably, target solutes can also be extracted and isolated based on their solubility within a specific liquid phase [23–25]. Extraction efficiency depends on the difference in polarity of each liquid phase and the solubility of the target solute within each phase. Membranes are unnecessary but, in some cases, can help to maximize extraction efficiency. Classically performed in batch, LLE procedures have been developed for continuous processing.

A proof-of-principle continuous-flow extraction unit, with an integrated membrane, was developed by Kralj et al. [26]. In their work, two non-miscible liquids were continuously combined in a slug-flow microreactor and then separated by an in-line extraction unit. The principles of extraction include that the extraction unit is divided in two halves by a porous Teflon membrane, allowing only the organic phase to pass to the opposite side of the membrane (Fig. 2); and, organic-phase transfer is dependent on the hydrophobicity of the membrane and the density and the size of the pores.

Having smaller pore size diameters (~0.5 μm) is crucial in managing the efficiency of phase transfer. The small dimension of the pores allows high capillary pressures to force the transfer of the organic phase exclusively from the original mixture.

In a successful application, the LLE unit was used for continuous preparation of aliskiren, a common pharmaceutical. The combined production and purification of aliskiren was achieved for 100 h at a rate of 40 g/h [27]. The only disadvantage with this specific process was that, over time, the integrity of the Teflon membrane deteriorated by contamination or structural damage.

Recently, Campos et al. described a membrane-less electro-extraction unit, which separates compounds based on differences in their electrophoretic mobility and solvent affinity. Here, two physical properties are exploited to achieve efficient extraction using an aqueous two-phase system (ATPS) [28]. Being a relatively new technique, ATPS is not as commonly used as aqueous-organic LLE. However, ATPS can be applied to a versatile library of analytes with potentially higher extraction efficiency [29].

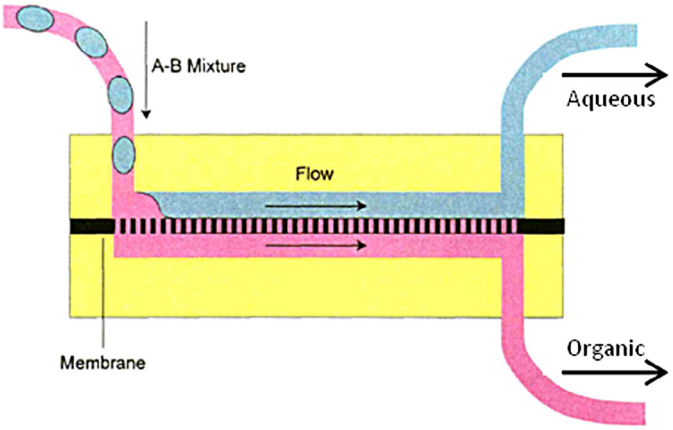


**Fig. 2.** Continuous liquid-liquid extraction. A segmented flow of an aqueous solution (**A**) dispersed in an organic phase (**B**). The organic phase wets the hydrophobic membrane and is driven through the membrane pores by the imposed pressure difference leaving the aqueous solution behind in the top portion of the device. {Adapted from [26] with permission from The Royal Society of Chemistry}.

Regardless of the manner of liquid extraction, contamination can be difficult to avoid due to incomplete extraction during the purification process. Extraction becomes even more challenging when purifying multiple targets, particularly if they exhibit different solubilities. Such a scenario could require significant optimization and multiple tandem extraction units that process different solvents, depending on the target solute. In one case, optimizing the extraction of a single solute required six identical LLE units in series [30]. There is continuing effort towards optimizing stable mechanisms of LLE for future industrial applications, and a recent review highlighted the current research [31].

### 2.3. Free-flow fractionation

Originally introduced in 1966 [32], free-flow fractionation (FFF) was considered to be a separation technique with a broad range of potential applications [33]. An FFF device propagates analytes by a pressurized flow through a narrow channel, without the need for a stationary phase, and separation of the analytes is achieved by a field force that is directed perpendicular to the flow. Such fields would exploit a unique physical property of the analytes in order to achieve separation. Field-fractionation modes include flow, sedimentation, thermal, and electrical [34].

Typical analytes are macromolecules; however, FFF resolution is theoretically possible for analytes with sizes of 0.001–100 μm [35]. More impressive is the capacity for FFF to process high-molecular-weight analytes ($10^9$ Da), where such species would be difficult to separate using size-exclusion chromatography. FFF is also convenient in that it uses mild separation conditions, thus allowing the analysis of fragile species. Furthermore, multiple detection schemes (multi-angle light scattering, mass spectrometry, UV detection, and refractive index) have already been integrated on-line and off-line for characterization [34]. Despite the broad range of applications, FFF has a niche that satisfies the size sorting and characterization of macromolecules. Separation of simple molecules, especially multiple analytes, is still best done by chromatography and electrophoresis.

### 2.4. Free-flow electrophoresis

Free-flow electrophoresis (FFE) continuously separates target molecules from a sample that is exposed to an electric field (Fig. 3). The

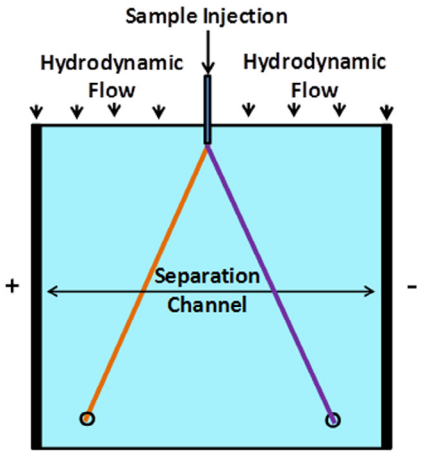


**Fig. 3.** Free-flow electrophoresis (FFE). A continuous hydrodynamic electrolyte flow carries a sample through a wide separation channel. The sample is separated by an electric field into its individual components. The migration of each component depends on its respective electrophoretic mobility.

electric field is directed orthogonally to a pressure-driven hydrodynamic electrolyte flow, which carries the sample. FFE was originally used to purify large volumes of complex biological samples continuously. Furthermore, in this application, the components of an FFE device were reusable for different samples. These advantages demonstrated that FFE was a viable high-throughput preparative platform, at least for the application mentioned.

FFE devices can be classified into four categories based on their scale – macroscale [36,37], midscale [38], milliscale (mFFE) [39–41], and microscale (μFFE) [42,43]. The scale categories are mainly defined by the volumes of the separation channels but also by acceptable ranges of flow rates and electric field strengths (Table 1). The lower range of flow rates employed by macro, mid and mFFE devices are comparable because of the similarity of their separation-channel heights. With the exception of field-step FFE, employing flow rates that are too high would cause rapid dispersion of sample in a narrow channel and poor separation performance. However, field-step FFE allows the rapid separation of analytes and focuses the charged species that approach the boundaries of the separation channel (due to conductivity differences of the media), thereby minimizing band broadening of analytes at high sample flow rates [44]. In general, sample flow-rate conditions used in FFE devices depend on the type of sample that is being separated (i.e. large analytes, like cells, require higher flow rates), and background electrolyte flow rate. It is worth mentioning that the maximum electric field strength is defined not by the gross size of the device or the separation channel, but mainly by the dimension of the separation-channel height and the resultant ability to dissipate heat efficiently through the top and bottom plates of the device. Notably, the midscale and macroscale FFE devices employed higher electric field strengths than mFFE due to an efficient cooling-liquid strategy (discussed in the next subsection), which mitigated Joule heating. We chose the optimal separation conditions from each scale where an FFE device was used as a preparative technique and, thus, potentially capable of high-throughput CFC.

**Table 1**
Defining parameters of various free-flow electrophoresis (FFE) scales used as a preparative technique. Unless referenced otherwise, the parameters were extracted from the literature source listed under "Device"

| Device | Maximum volume of separation channel (mL) | Range of sample flow rate (μL/min) | Maximum electric field strength (V/cm) | Aspect (width to height) ratio | Surface area to volume ratio ($mm^{-1}$) |
|---|---|---|---|---|---|
| Macro-scale FFE[36] | 35.7 | 6–97 | 250 [37] | 143 | 1.4 |
| Midscale FFE [38] | 1.89 | 5.4–18.6 | 140 | 85 | 2.9 |
| mFFE [39] | 1.32 | 4–600 [40] | 100 [41] | 300 | 5 |
| μFFE [42] | 0.025 | 0.08–4 | 708 [43] | 400 | 20 |

To date, only μFFE has been successfully integrated into a CFC design. Recently, an in-line μFFE device was used to purify three amino acids that were labelled continuously in a micro-reactor [45]. In general, however, μFFE flow rates are too small for high-throughput preparative technology and do not reflect flow rates that are typically used in continuous-flow micro-reactors. The flow rates used in larger-scale FFE are compatible with the flow rates in most continuous-flow micro-reactors, making these purification scales better suited to CFC design [39].

Macroscale FFE, in particular, could be a good candidate for CFC integration. Aside from compatible flow rates, macroscale FFE is a rugged, reliable technique, as is evident in commercial devices (e.g., Octopus), which have existed since before the twenty-first century [46]. Such devices have high-throughput capabilities, high separation power, and well-established steady-state purification strategies. However, a major caveat with respect to macroscale FFE integration to CFC is that macroscale FFE dimensions are large (separation channel volume in Table 1). As a result, these devices consume large volumes of sample and reagents, and the residence time of samples can also be large. The essence of CFC, especially continuous-flow synthesis, is to minimize waste and maximize efficiency, which can be achieved through small-scale processing. The miniaturization of FFE has certain attractive attributes, such as small volumes, efficient heat and mass transfer, and facile on-line detection schemes, thus making midscale and mFFE more practical for CFC integration. However, midscale and mFFE are not nearly as reliable purification techniques as their larger counterpart.

Scaling FFE from the macroscale down to smaller dimensions incorporates a significant limitation – the need to address, *de novo*, all issues of instability. While macroscale FFE performance has solved the problems that prevent steady-state purification, its strategies are not easily transferrable. In this section of the review, we focused on recent advances that help maintain steady-state continuous-flow purification in small-scale FFE. In addition to steady-state purification, we will discuss two other important limitations that need to be addressed before considering the integration of FFE into CFC. The first limitation is the inadequate resolving power of FFE [with the exceptions of isoelectric focusing (IEF)] when compared to existing discontinuous purification techniques. The other limitation is the inability of standard modes of electrophoresis to separate many of the potential target molecules, such as pharmaceuticals, which are either neutral or are difficult to dissolve in aqueous solutions. Steady-state purification, FFE resolving power and potential FFE target molecules are reviewed in the next sections.

## 3. Limitations of FFE purification

### 3.1. Steady-state continuous-flow purification

While being the driving force of separation in electrophoresis, an electric field creates a number of problems that prevent the use of small-scale (milli-, mid-, and micro-scale) FFE for steady-state continuous-flow purification. The electric field affects steady-state purification by three associated mechanisms – Joule heating, bubble generation, and $H^+/OH^-$ transport that forms pH gradients. To date, the regeneration of a steady state typically requires frequent shutting down of a device, which is not a viable option for integrated CFC. In the next sub-sections, we discuss in detail each of the three negative effects of electric fields.

*3.1.1. Joule heating*

Joule heating occurs when a voltage is applied across a conductive media. The amount of heat released is related to the voltage, *V*, electric current, *I*, and the resistance of the respective media, *R*, in the following ways: $Q = V^2/R = I^2R$. In FFE, the heat is generated within the electrolyte, thus increasing the electrolyte temperature and possibly leading to boiling. Recently, Dutta estimated, through computation, the effects of Joule heating on the quality of separation [47]. In general, Joule heating and its consequences can easily destroy steady-state purification in FFE.

One of the consequences of Joule heating is the generation of temperature gradients due to uneven heating of the buffer. Temperature gradients affect the rates of convection and diffusion, leading to significant band broadening, and can also affect analyte stability and electromigration. The effect of temperature on electrophoretic mobility is not trivial and depends on a variety of factors, which can have unique effects on different ions [48]. Temperature affects electrophoretic mobility by modifying buffer properties, such as viscosity, pH and ionic strength. Joule heating can be reduced by simply decreasing the strength of the electric field, but this would also reduce the separation power. Alternatively, other strategies can limit Joule heating without compromising separation power, including:

- maximizing the aspect ratio of the separation-channel geometry;
- introducing active cooling; and,
- using low-conductivity buffers.

In FFE devices, the conductive media is contained within a high aspect-ratio separation channel. The height of the separation channel is at least an order of magnitude smaller than the width or the length. Such geometry provides a large surface area-to-volume ratio, which allows rapid heat dissipation and reduces Joule heating. μFFE and mFFE incorporate separation channels with the highest aspect ratios. In Table 1, we present the aspect ratios of different FFE device platforms. Larger aspect ratios do not necessarily correlate with smaller surface area-to-volume ratios. In particular, macro FFE has a separation channel with a larger aspect ratio than midscale FFE because its width is significantly larger, but the narrower depth of a midscale separation channel provides a surface area-to-volume ratio that is twice as large as that of macro FFE. In general, heat is dissipated most rapidly in μFFE, whose surface area-to-volume ratio is the greatest.

Active cooling was a major strategy to circumvent excessive Joule heating in the first FFE devices. Hannig et al. introduced a cooling liquid that circulated on the top and bottom surfaces of a macroscale FFE device to help remove heat [36]. Most commercial FFE devices use such cooling schemes and can maintain consistent temperatures over long periods of time. A similar cooling set-up was also incorporated in μFFE devices [49]. More recently, μFFE devices employed thermoelectric cooling plates, which can remove heat even faster [50].

Arguably, the most successful implementation of thermoelectric cooling for steady-state purification was developed last year by Cao et al. [51]. They designed a mid-scale FFE device with a ceramic bottom surface, a material with high thermal conduction. Joule heat efficiently passed through the ceramic and was absorbed by thermoelectric coolers attached to air-cooled heat sinks. Temperature sensors were also incorporated into the ceramic plate to ensure that the coolers maintained a strict temperature. At an electric field strength of 67 V/cm, the temperature was held with a precision of 6°C. This mid-FFE device was used for over an 8-h period (a great improvement from 5 min without cooling) after which the generation of bubbles became significant.

The selection of a buffer with low conductivity can greatly minimize the amount of Joule heating, thus allowing higher electric field strengths to be used. In the first μFFE device, Raymond et al. investigated the influence of multiple buffers, with different conductivities, on Joule heating. They found that Joule heating could be mitigated if a buffer with higher conductivity was washed over the electrodes and a lower conductivity buffer was introduced exclusively in the separation channel [52]. Lowering buffer concentrations can also reduce Joule heating, but at a risk of forming pH gradients, a topic that we discuss later in this review. From all of these strategies, there is no obvious choice for circumventing Joule heating. Rather, a combination of active cooling, low-conductivity buffers and high aspect-ratio channels need to be used to address the Joule heating issue in steady-state FFE purification.

*3.1.2. Electrolysis*

*3.1.2.1. Bubbles.* In FFE, the energy of an electric field drives the decomposition of water into molecular hydrogen and oxygen:

$$2H_2O_{(l)} \rightarrow 2H_{2(g)} + O_{2(g)} \quad (1)$$

Both gases can form bubbles near the electrodes and, if not removed efficiently and rapidly, can disrupt steady-state purification by FFE. One consequence of bubble accumulation is electric field disruption [52]. Bubbles that adhere to the electrodes can behave as individual insulators, thereby increasing ohmic resistance and cause Joule heating, thus decreasing separation power [53]. Furthermore, bubbles can easily interfere with the original trajectories of the sample components. In a closed FFE device, normal performance can be regenerated by halting FFE operation and evacuating the bubbles. In other words, achieving steady-state continuous-flow purification requires continuous and efficient bubble removal from the FFE device.

One of the first efforts to prevent bubble accumulation was to use palladium, in place of platinum, as an electrode material. Palladium acted as a catalyst to combine $O_2$ and $H_2$ back into water instead of producing bubbles [54]. However, the maximum electric field that was used without generating bubbles was only 17 V/cm. Such a low field is insufficient for efficient FFE purification, so it is necessary to minimize the rate of bubble formation without decreasing the electric field strength.

Kohleyer et al. developed a μFFE device that introduced a redox-active species, quinhydrone, at the electrode channels [55]. Quinhydrone is a molecular complex consisting of hydroquinone and p-benzoquinone, which acted as reducing and oxidizing agents, respectively. Quinhydrone reacted at the electrode faster than water, causing less electrolysis of water to occur. Thus, the formation of bubbles occurred at a slower rate, minimizing their accumulation. However, the effectiveness of quinhydrone was limited to electric field strengths lower than 150 V/cm. Higher electric field strengths would increase the production of gases to a quantity at which the electrolysis of quinhydrone could not outcompete bubble accumulation. Increasing the concentration of quinhydrone is not a viable option, as there would be solubility issues. Quinhydrone also suffers from stability issues, making its use difficult in long-term steady-state FFE. Furthermore, Kohleyer et al. claimed that there were significant challenges with controlling pH within the separation channel, and reported a drop in voltage efficiency where the separation voltage was 20–30% of the total applied voltage due to rapid concentration polarization. As a result, this FFE device operated for a maximum of 15 min.

FFE operational times can be increased by employing physical barriers, such as membranes, to isolate bubbles from the separation channel. Macroscale FFE devices successfully incorporated membranes to prevent fluid exchange between the separation channel and the electrodes [56,57]. However, small-scale FFE devices cannot benefit equally from membranes. A major limitation with membranes is that electric field strengths are severely reduced due to the electrical resistance of the membrane, thus requiring higher

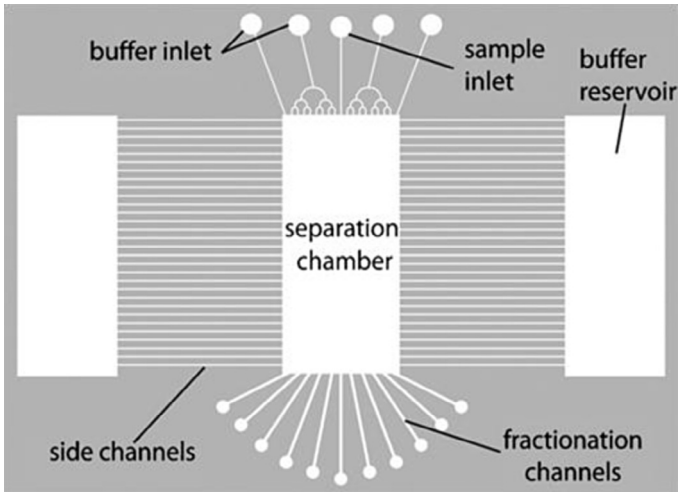


**Fig. 4.** Free-flow electrophoresis (FFE) device with side-channel geometry and buffer reservoirs. The side channels limit bubble interference in the separation channel. {Reproduced from [58] with permission from Elsevier}.

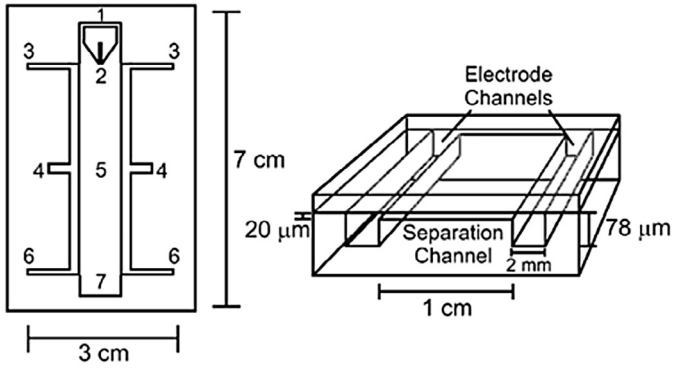


**Fig. 5.** Deep electrode channels in a micro-free-flow electrophoresis (µFFE) device. The high flow rates in the deep channels rapidly remove bubbles and can prevent bubble interference without complex geometric modifications or the use of membranes. {Adapted with permission from [61], ©2006, American Chemical Society}.

voltages to be used. Furthermore, electric field distortions can occur because bubbles are still able to accumulate around the electrodes. In general, the use of membranes is not a perfectly practical solution to achieve steady-state purification, as they are delicate and must be regularly replaced.

The isolation of bubbles in µFFE devices can also be achieved by placing the electrodes into large reservoirs, which are connected to the separation channel by thin side channels (Fig. 4), which are sufficiently thin to prevent bubble transfer but still allow an electrical current to pass [58–60]. However, the electric current can experience great resistance, and a major disadvantage with the use of side channels is that exceptionally high voltages (of the order of kV) need to be applied to realize useful electric field strengths in the separation channel. Such a system is impractical for use in larger FFE platforms. Ideally, a strategy used for solving the bubble problem should be transferable to all FFE scales.

One of the most simple, effective, and transferable strategies developed is to remove the bubbles from a FFE device at their point of formation. Fonslow et al. fabricated a µFFE device that incorporated deep electrode channels in which bubbles were removed rapidly (Fig. 5) [61]. According to the Hagan-Poisuelle equation, deeper channels support higher flow rates, $q$:

$$q = \frac{\Delta P H^3 W}{12 \eta L} \quad (2)$$

where $P$ is the pressure, $\eta$ is the viscosity, and $H$, $W$, and $L$ are the height, width, and length of the channel, respectively. It is clear, from Equation (2), that the height of a channel has the greatest influence on the flow rate. Optimizing the electrode-channel depth can allow sufficiently high flow rates to wash away bubbles rapidly immediately after their formation. In the best case scenario using deep electrode channels, a µFFE device was operated for a maximum of 2 h. This time was achieved by combining deep electrode channels with a detergent in the buffer to minimize the size and the surface tension of the bubbles [62]. More recently, however, a µFFE device was fabricated with an integrated nanoporous hydrophobic membrane as a top cover layer, thereby removing gas bubbles and allowing stable separation conditions over 3 h [63]. Alternatively, another highly effective method of removing bubbles was to incorporate tall channels, called partitioning bars, above the electrodes [64]. As bubbles formed, they became trapped within these bars, preventing them from entering the separation channel, and were flushed out by hydrodynamic flow (Fig. 6).

Another strategy was inspired by the encouraging results obtained with deep electrode channels and partitioning bars. Our team has developed a prototype, in which the electrolyte, directly above the electrodes, was open to the atmosphere [40]. Unlike the conventional closed FFE system, bubbles could now easily escape into the atmosphere. To prevent any bubbles from entering the separation channel, the electrodes in the FFE prototype were positioned

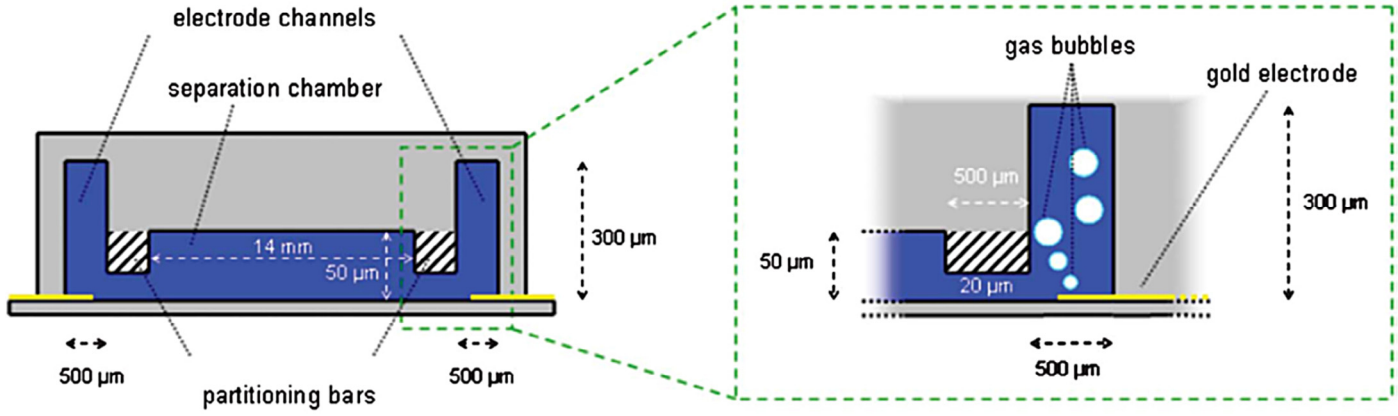


**Fig. 6.** Chip layout and cross-sectional diagram of the separation chamber, the electrode channels and the partitioning bars showing the principle of bubble retention. {Reproduced from [64] with permission from The Royal Society of Chemistry}.

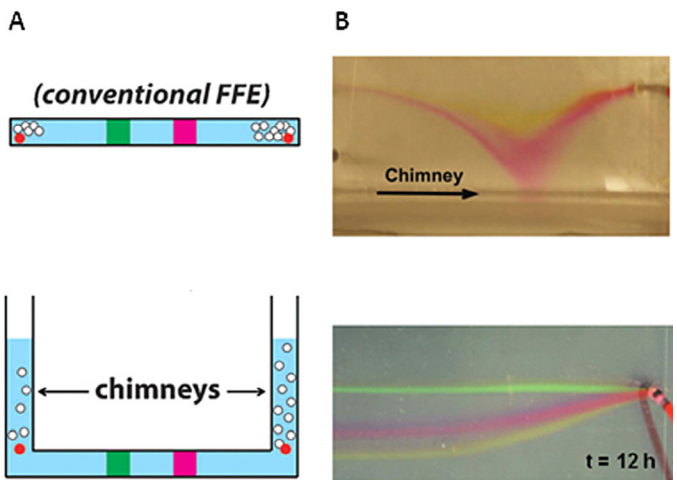


**Fig. 7. Panel A** shows the conversion of a conventional free-flow electrophoresis (FFE) device into an open-electrolyte FFE (OEFFE) device. Generated bubbles (o) are allowed to escape into the atmosphere because of the location of the electrodes within tall chimneys open to the atmosphere. **Panel B** demonstrates reduction of the effect of chimneys on the hydrodynamic flow achieved by the inclusion of sacrificial channels (SacCs) (not shown). As a result, steady-state continuous-flow purification was achieved over 12 h with OEFFE. {Adapted from [40] with permission from Wiley}.

in a plane situated higher than the top plate of the separation channel. As bubbles formed on the electrodes, they rose, due to buoyancy forces, and escaped from the FFE device through tall structures called chimneys (Fig. 7A). The chimneys were needed to balance the pressure inside the separation channel with that of the electrolyte column in the chimneys. However, a significant disadvantage of the chimneys was that they affected flow uniformity in the separation channel. Fluid exchange between the separation channel and the chimneys influenced analyte trajectories and decreased separation quality.

To circumvent flow non-uniformity, a new set of fluidic channels, termed sacrificial channels (SACs) were invented. Due to the increased depth of a SacC, it has a much larger volumetric flow rate than the separation channel and, as a result, the flow within it is less likely to diverge into the chimneys. Implementing multiple SacCs further attenuated the fluid exchange between each successive SacC, thus re-establishing flow uniformity (Fig. 7B).

A mFFE device, with optimized SacC geometry, was fabricated after thorough computer modelling with COMSOL [40]. This new platform, named open-electrolyte FFE (OEFFE), was the first successful implementation of milli-scale steady-state continuous-flow purification by FFE over a 12-h period. By comparison, a macroscale FFE device, with membranes, was operational for a 15-h period [65]. Furthermore, combining OEFFE with efficient temperature-control strategies can potentially allow steady-state continuous flow purification over an unlimited period of time.

*3.1.2.2. pH gradients.* In addition to gases, the electrolysis of water also produces ions:

$$2H_2O_{(l)} \rightarrow O_{2(g)} + 4H^+_{(aq)} + 4e^- \quad (3)$$

$$2H_2O_{(l)} + 2e^- \rightarrow H_{2(g)} + 2OH^-_{(aq)} \quad (4)$$

$H^+$ and $OH^-$ migration are responsible for the formation of local pH and conductivity gradients. Local pH changes can adversely affect analyte electrophoretic migration, optical properties (rendering it undetectable), reactivity, and structural configuration [55]. Changes in local conductivity can cause temperature fluctuations and distort electric field strengths. Since the electrophoretic mobilities of $H^+$ and $OH^-$ are, in general, greater than those of larger ions, both ions can migrate rapidly across the separation channel and cause drifts in pH over large areas of the separation channel. The extent of $H^+$ and $OH^-$ migration depends on the buffering capacity of the electrolyte, the electric field strength, and the hydrodynamic flow rate. However, ideal conditions for reducing pH gradients are not favorable for FFE separation conditions. High buffer concentrations will increase the buffering capacity, but can lead to increased Joule heating. Applying low voltages will decrease the rate of electrolysis and electrophoretic velocities of $H^+$ and $OH^-$, and high hydrodynamic flow rates will reduce the lateral migration velocity of $H^+$ and $OH^-$; however, such conditions will also decrease separation power. A balance between pH gradient formation and separation power therefore needs to be established.

However, exploiting pH gradients can substantially increase separation power for unique samples using IEF-FFE [66]. Gradients are established by the use of ampholytes, but have also been formed in ampholyte-free media [67], and that is attractive for CFC integration. IEF-FFE forms a defined pH gradient across the width of the separation channel after a voltage is applied. Analyte streams migrate into the region at which the analyte pI value is equal to the pH. The pH regions in IEF-FFE are typically distinct and spatially narrow, thus allowing the analyte to focus. An established pH gradient will remain stable as long as a steady-state is maintained, but could easily be shifted or disturbed by the same limitations associated with FFE mentioned above. A spatial shift of the pH gradient, in IEF-FFE, was reduced by employing ion-exchange membranes [57]. Commercial FFE devices use stabilization media to control pH [68]. In IEF-FFE, such media are, typically, a strong acid and a strong base that flow next to the anode and the cathode, respectively, and are used to help stabilize a pH gradient. However, the major limitation with IEF-FFE is that only amphoteric analytes can exclusively benefit from focusing.

Non-amphoteric analytes should be separated by conventional FFE (or zone FFE) in a region of uniform pH. Again, commercial devices use stabilization media, which have similar electrophoretic mobility or pKa to the separation buffer, to maintain pH uniformity [69]. However, uniform pH is challenging to achieve in small-scale FFE.

A recent publication addressed the lack of stable pH regions in both IEF-FFE and zone FFE using a microscale device [70]. A switchable pH actuator was integrated upstream to a separation channel to ensure uniform pH in zone FFE or to ensure a stable pH gradient in IEF-FFE. When affected by a voltage bias, the actuator was activated, so that $H^+$ and $OH^-$ ions migrated across ion-exchange membranes and generated precisely-defined pH gradients well before the separation channel. In the absence of voltage bias, the actuator remained inactive, allowing a buffer with a constant pH to travel downstream into the separation channel (Fig. 8). The use of these bipolar membranes and actuators can help stabilize a uniform pH, and pH gradients, in μFFE devices. Due to the delicate, complex nature of the membranes, switchable pH actuators are not easily transferrable to larger FFE devices. However, a geometric modification is a transferable strategy that could be used to reduce pH gradients.

We recently published an example of a transferrable modification that effectively reduces pH gradients and limits their influences on separation power [41]. We used deep SacCs to reduce pH gradients in an mFFE device. The electrolytic ions were washed away rapidly before migrating into the separation channel, due to increased flow rates within the SacCs. Additionally, similar to the OEFFE design, SacCs were optimized in order to maintain flow uniformity within the separation channel. In this mFFE device, a uniform pH profile can be maintained over a broad range of flow rates and electric field strengths (Fig. 9).

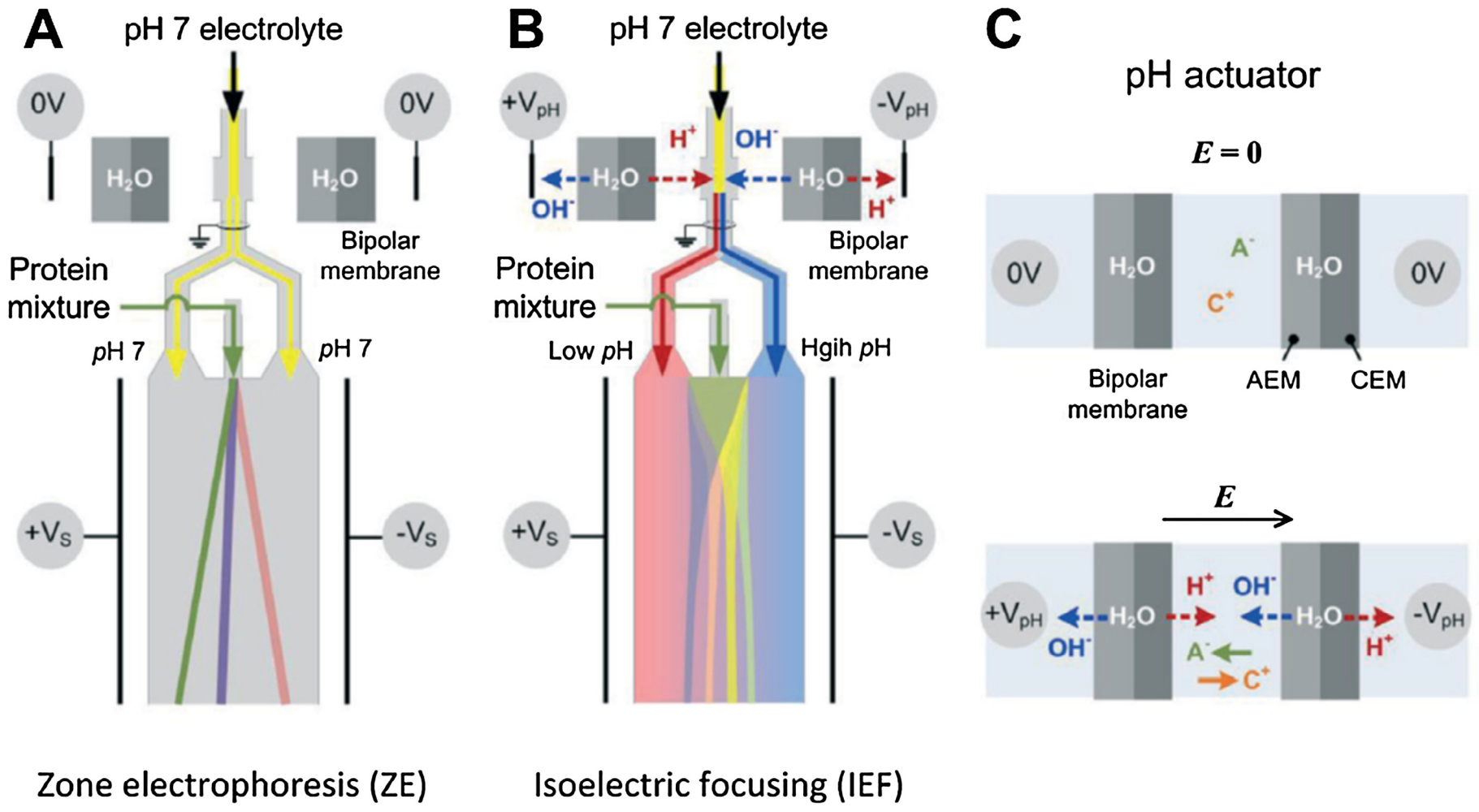


**Fig. 8.** The switchable pH actuation provides reconfigurability of the platform for dual-mode operation; it produces a constant neutral pH medium for zone electrophoresis (**A**) or a pH gradient for isoelectric focusing (**B**). (**C**) The function of the pH actuator is based on an electric field-enhanced water-dissociation phenomenon in the integrated bipolar membranes upon voltage bias. The process dopes electrolyte microflows with excess $H^+/OH^-$ ions and redistributes the existing cations ($C^+$) and anions ($A^-$) in the solution, resulting in a two-layer laminar flow containing a low-pH flow in one half and a high-pH flow in the other half. A symmetric voltage arrangement was employed for both upstream pH actuation ($\pm V_{pH}$) and downstream electrophoresis ($\pm V_S$) to avoid electrical crosstalk. {Adapted from [70] with permission from The Royal Society of Chemistry}.

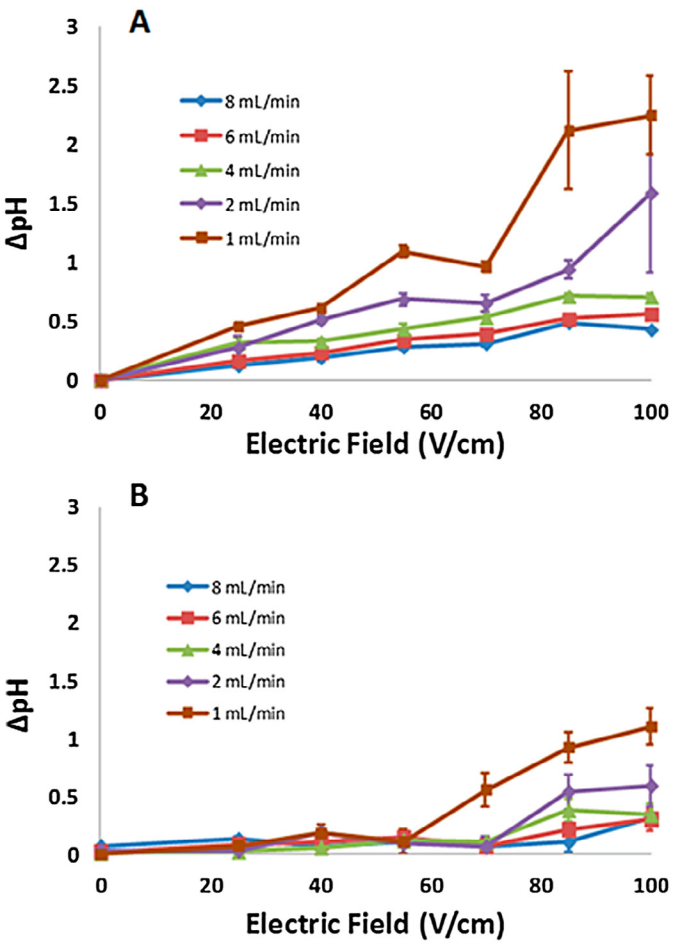


**Fig. 9.** ΔpH between the anode outlet and cathode outlet of a milli-free-flow electrophoresis (mFFE) device without (**A**) and with (**B**) sacrificial channels (SacCs). $H^+$ and $OH^-$ migrate across the separation channel and change the pH, thus affecting the separation conditions. This effect worsens at higher electric field strengths and lower flow rates. SacCs provide a region of faster flow that can evacuate $H^+$ and $OH^-$ more efficiently and limit the change in pH. mFFE devices with SacCs can decrease ΔpH further than devices without SacCs and maintain a uniform pH over a broader range of separation conditions. Error bars represent the standard deviation of three separate pH measurements at each outlet. {Reprinted with permission from [41], ©2014, American Chemical Society}.

### 3.2. FFE separation efficiency

FFE, with the exception of IEF-FFE (as described in the previous sub-section), lacks the separation power of other non-gel electrophoretic techniques. For example, capillary electrophoresis (CE) uses electric field strengths that are significantly higher than those in macro, midscale, and mFFE. Simply increasing the electric field strengths in FFE cannot match the separation power of CE because of the instabilities described above. Furthermore, it would be impractical to have a separation channel as long as the capillary because continuous-flow purification introduces additional sources of hydrodynamic band broadening. Addressing FFE separation efficiency therefore requires other strategies that do not depend on increasing the electric field strength or the channel length.

Another strategy to reduce band-broadening effects, named interval FFE, was introduced by Bauer and Weber in 1998 [71]. In this platform, a sample was injected into a device and passed through the separation channel in the absence of an electric field. Once the sample spanned the entire length of the separation channel, the hydrodynamic flow was turned off and a voltage was applied. The true benefits of interval FFE were investigated in a quantitative study in 2012. It was demonstrated that FFE separation power could be increased by reducing band broadening caused by the simultaneous effects of electrophoresis and hydrodynamic flow [72]. While interval FFE is an effective way to increase separation power, the platform becomes a combination of discontinuous flow and discontinuous separation. Thus, it is difficult to consider interval FFE as complementary to continuous-flow synthesis.

Our team has demonstrated from first principles that improved resolution could be achieved by re-orientating the electric field in a direction non-orthogonal to the hydrodynamic flow (Fig. 10) [73]. Following this work, non-orthogonal FFE (NOFFE) designs were suggested [74]. NOFFE theoretically proved that two analytes, whose electrophoretic mobilities differ by less than 0.5%, can be resolved. However, having an electrode non-parallel to the hydrodynamic flow makes injection and collection of target analytes challenging, so making a practical NOFFE device will require a great deal of study and optimization.

In general, FFE separation efficiency needs to be improved. The ultimate challenge will be to find a strategy that will allow the separation of analytes with similar electrophoretic mobilities. It will be imperative for FFE to address difficult separations in order to complement continuous-flow processing, and continuous-flow synthesis in particular [75,76].

### 3.3. Samples

The final FFE limitation is that its purification capabilities are restricted mainly to charged analytes in aqueous media. However, it would be ideal to separate analytes that are neutral or have limited solubility in water. In this sub-section, we explore the niche of biological analytes that first defined FFE success and the potential to separate unorthodox analytes by unconventional electrophoretic techniques in the FFE format.

#### 3.3.1. Biological samples

The original purpose of FFE was purification of biological samples. Early [77–80] and modern [81–83] original research papers and reviews [84–87] describe a large amount of work devoted to the separation of cells, vesicles, organelles and bacteria.

The purification of biological samples was natural for FFE as most biomolecules are diverse in size and charge. Protein separation, in particular, was incredibly successful using IEF-FFE. μFFE devices have also been employed extensively to purify biological materials using higher electric field strengths. However, due to short-term steady-state conditions and μFFE dimensions, separation is limited to small sample volumes and the sample components are restricted in size to avoid clogging. As a result, rather than being used exclusively as a preparative technique, μFFE found an important purpose as an analytical tool to measure equilibrium constants of biomolecular interactions [88]. μFFE was also used to separate and to select DNA aptamers for a target molecule [89]. Furthermore, such devices were successful because on-line detection could be easily integrated, thus allowing continuous-flow analysis and real-time separation monitoring.

#### 3.3.2. Inorganic samples

To date, both metals and NPs have been purified using FFE devices. Metals and NPs have the potential to precipitate or to form agglomerates that would cause clogging in smaller-scale FFE devices, so only macroscale FFE has been used for inorganic purification. In one instance, isotachophoresis FFE was used to separate platinum-group elements from liquid waste [90]. Macroscale FFE has also been used as a size-fractionation technique for a variety of NPs [91,92]. NPs can be sorted by size, resulting in fractions with low polydispersity. Size separation is essential, as NP functions are often defined by its size and shape [93].

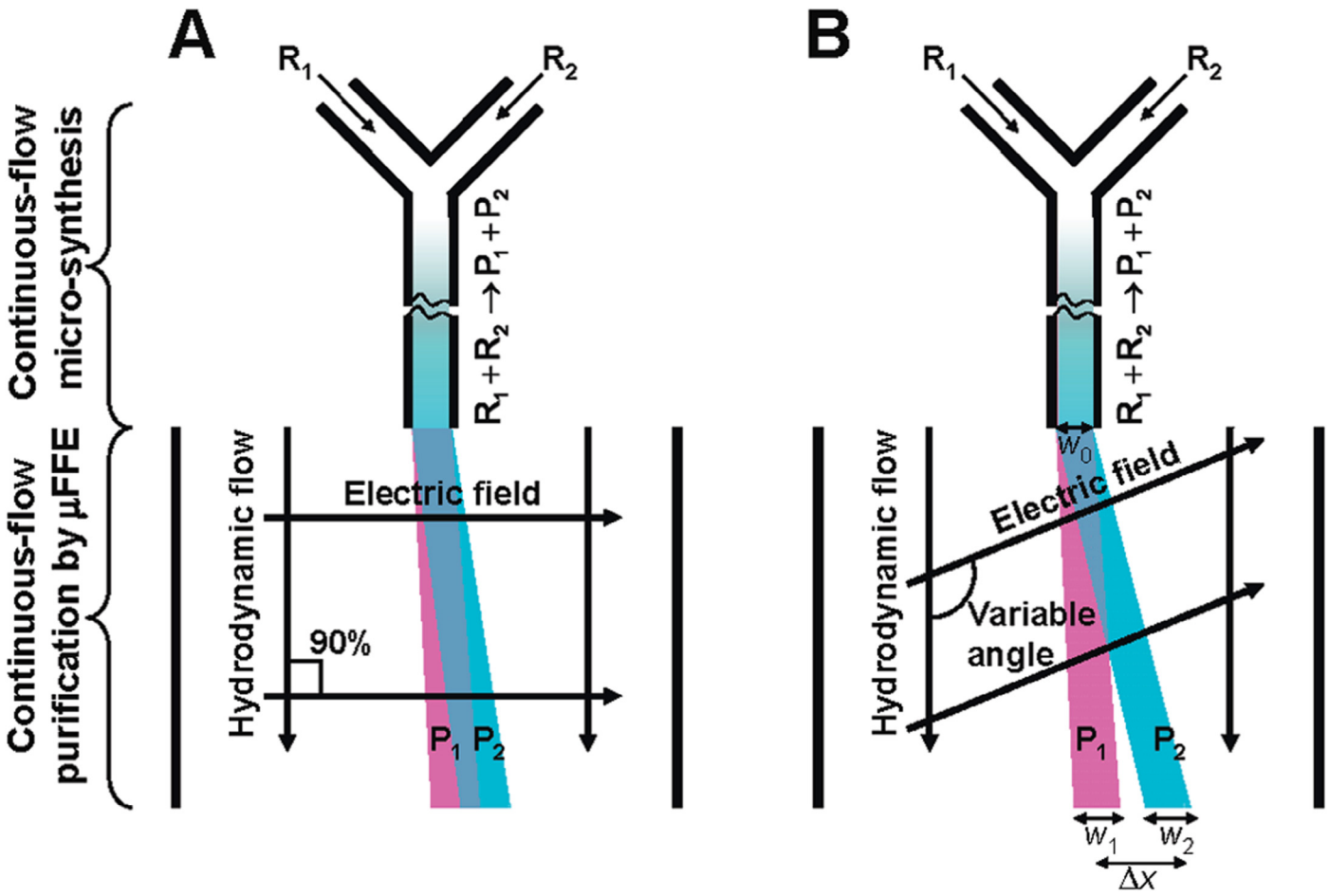


**Fig. 10.** Separation of products P1 and P2 in an integrated system for in-flow microsynthesis followed by micropurification by micro-free-flow electrophoresis (μFFE) with orthogonal (**A**) and non-orthogonal (**B**) orientations of the electric field and hydrodynamic flow. {Reprinted with permission from [73], ©2010, American Chemical Society}.

*3.3.3. Organic samples*

There has been very little work performed on the separation of hydrophobic organic molecules using FFE, or FFE separations performed in non-aqueous solvents. Organic solutes, in general, are difficult to separate by electrophoresis for obvious reasons:

- reduced solubility;
- abundance of neutral species; and,
- lack of compatible organic electrolytes for FFE.

Yang et al. experimented with diluting the electrolyte in a water/methanol mixture to increase the solubility of organic solutes. As a result, they were able to purify organic solutes that have low aqueous solubility [94]. However, water-soluble organic dyes are widely used as tracer molecules and model analytes for proof-of-concept experiments.

In general, electrophoresis is incompatible with organic solvents used in most chemical syntheses. However, modern approaches to synthesis are being developed to minimize the use of organic solvents [95]. There are even micro-reactors that avoid organic solvents and perform chemical transformations at high temperatures and pressures in aqueous solvents [96]. Ionic liquids are also being investigated as potential replacements for organic solvents [97,98]. A shift from organic liquids would facilitate the use of electrophoresis as a purification approach. Until then, there are two other purification approaches that are compatible with FFE.

Micellar electrokinetic chromatography (MEKC) in FFE format is a potential solution to separate neutral and hydrophobic molecules. MEKC is typically used in capillaries as a discontinuous method of separating neutral analytes that differ in hydrophobicity [99]. Briefly, a capillary is filled with a solution containing a charged detergent at a concentration higher than its critical micellar concentration. Analytes with different hydrophobicities can partition at different ratios between free solution and the micelles. In an electric field, charged micelles begin to migrate and to shift molecules with greater hydrophobicity across further distances, thus spatially resolving them from less hydrophobic molecules. Though the principles of MEKC could easily be adapted to an FFE design, such a design has yet to be applied in FFE.

Neutral dipole species can be separated by dielectrophoresis, which is limited to large analytes (larger than 1 nm in size). The theory of dielectrophoresis states that neutral particles can be resolved by a non-uniform electric field due to differences in dipole moments. A free-flow dielectrophoretic device was proposed [100], and recently implemented in the purification of tumor-circulating cells [101]. However, the separation of smaller neutral molecules by dielectrophoresis is challenging, as small molecules have small dipole moments and require impractically high electric field gradients.

## 4. Concluding remarks

The future of CFC streamlining lies in the success of steady-state continuous-flow purification. There are a limited number of purification platforms that could complement continuous-flow

synthesis. In particular, FFE is a method that can efficiently purify a target analyte from contaminants.

We have discussed the limitations associated with FFE, the most significant of which is its inability to maintain steady-state purification at the current stage of development for small-scale devices. Joule heating and electrolysis are mainly responsible for the reduced operating time, as well as the deteriorating separation quality of mid-scale, mFFE and μFFE.

Promising strategies were recently developed to circumvent the effects caused by Joule heating and electrolysis. With an effective, optimized combination of these strategies, it is conceivable to have indefinitely long steady-state continuous-flow purification by all scales of FFE. With further advances in separation power and the ability to purify neutral samples, FFE could become more versatile and more suitable for practical combination with continuous-flow synthesis. Where the past 150 years were devoted to industrial and continuous-flow synthesis, the near future may well be devoted to complete CFC integration.

## Acknowledgement

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