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Micro Free Flow Electrophoresis

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Abstract

Micro free-flow electrophoresis (μ FFE) is a continuous separation technique in which analytes are streamed through a perpendicularly applied electric field in a planar separation channel. Analyte streams are deflected laterally based on their electrophoretic mobilities as they flow through the separation channel. A number of μ FFE separations modes have been demonstrated, including free zone (FZ), micellar electrokinetic chromatography (MEKC), isoelectric focusing (IEF) and isotachopheresis (ITP). Approximately 60 articles have been published since the first μ FFE device was fabricated in 1994. We anticipate that recent advances in device design, detection, and fabrication, will allow μ FFE to be applied to a much wider range of applications. Applications particularly well suited for μ FFE analysis include continuous, real time monitoring and microscale purifications.

Graphical abstract

This review focuses on the fabrication methods, theory, and applications of micro free flow electrophoresis (μ FFE), a continuous separation technique particularly well suited for microscale purifications and online monitoring applications.

Introduction

Free flow electrophoresis (FFE) was introduced by Barriollier *et al.*, and later Hannig, almost 60 years ago.^{1, 2} A continuous sample stream is introduced into buffer flowing through a planar separation channel.^{3–5} An electric field is applied perpendicularly to the buffer flow, separating streams in space according to analyte size and charge (see Figure 1). Electrodes are isolated from the channel using ion-permeable membranes to prevent electrolysis bubbles from interfering with the separation. Sample introduction, separation and collection are performed continuously. Analytes are separated spatially rather than in

Conflicts of Interest

There are no conflicts of interest to declare.

time. The continuous nature of FFE makes the separation technique particularly well suited for preparative scale fractionation and purification of biomolecules.⁵ FFE has been demonstrated using a number of electrophoresis modes including zone electrophoresis (ZE), isotachopheresis (ITP), isoelectric focusing (IEF), and micellar electrokinetic chromatography (MEKC).^{4, 6, 7}

Preparative scale FFE does present a number of challenges. The relatively large dimensions of the separation channel results in significant current, which in turn leads to significant Joule heating and electrolysis, even at low potentials.^{4, 8} Laminar flow must be maintained to achieve predictable analyte streams. Careful control of separation channel depth and buffer flow is therefore necessary. The scale of FFE separations makes online detection methods challenging. Instead, offline UV absorbance analysis of collected fractions is usually performed. This can be time consuming since 90 or more fractions are commonly collected. These challenges make FFE an attractive candidate for miniaturization into a micro free flow electrophoresis (μ FFE) system. Operating on the microscale increases surface area to volume ratios which improves heat dissipation, shortens separation times, minimizes buffer and sample volumes, promotes consistent laminar flow, facilitates on device detection, and lends itself to integration with other microfluidic techniques for a total analysis system.⁹

Raymond *et al.* were the first to successfully fabricate a μ FFE device in 1994.¹⁰ The device significantly reduced Joule heating while incorporating on device, real time detection of analyte streams. The device was limited to 75 V due to the electrical breakdown potential of the silicon substrate used for fabrication.¹⁰ Since then a number of groups have continued to optimize μ FFE through improved fabrication methods and instrument designs. The introduction of novel μ FFE applications has increased as the performance and reliability of these has improved. In the current manuscript we review the state of the μ FFE field, critically assessing separation fundamentals and theory, fabrication strategies and unique applications.

Fabrication and Design

Fabrication Substrates

Raymond *et al.* fabricated their initial μ FFE device in a silicon substrate to leverage existing micromachining techniques developed in the electronics industry.¹⁰ However, silicon is not an ideal substrate for μ FFE due to its low electric breakdown potential, which in turn limits the separation potential that can be applied. Silicon substrates were therefore quickly replaced in favor of more suitable materials such as poly(dimethylsiloxane) (PDMS),^{11–14} glass,^{15–18} polystyrene,¹⁹ and various rigid polymers.^{20–22} When choosing a fabrication strategy, it is important to consider challenges that are unique to μ FFE. For example, the aspect ratio of the open separation channel is much higher than normally encountered in microfluidic devices. Eliminating electrolysis bubbles generated at electrodes also presents a significant design challenge. Figure 2 shows images of a number of μ FFE devices reported in the literature, demonstrating various design approaches to address these challenges.

Glass is a rigid material well suited for μ FFE fabrication since it can withstand high voltages and pressure, is stable over a wide range of pH, and is relatively chemically inert.²³ Fabrication in glass uses standard photolithography and wet etching techniques to pattern the electrode channel, separation channel, and electrodes in a bottom wafer.^{9, 15, 24} The featured wafer is then bonded to a second unpatterned glass wafer to enclose the channel. Thermal bonding and anodic bonding have been used to bond the two wafers together.^{15, 25, 26} While thermal bonding is much more commonly used in general microfluidics fabrication, the large open channel in μ FFE devices makes this approach challenging. Care must be taken during the annealing step to prevent sagging in the separation channel. Additionally, the low contact area between wafers can lead to significant interfacial stress between the wafers upon cooling, which can make the final devices brittle and prone to fractures. Anodic bonding addresses these issues.²⁷ In anodic bonding, amorphous silicon is deposited on the featureless wafer. The two wafers are aligned, brought into contact with electrodes, and a voltage is applied through the wafers. Current through the glass oxidizes the amorphous silicon, forming covalent bonds between the oxidized amorphous silicon and the glass.^{27, 28}

More recently, Jezierski *et al.* reported a method for fabrication of a glass μ FFE device using liquid phase photolithography between two glass wafers.²⁹ They were able to use a spacerless design to achieve channel heights of 25 μ m while eliminating bonding steps. Another interesting way to bond glass wafers is through the use of a polymer bonding layer. Ding *et al.* used a photocurable monomer Norland Optical Adhesive 81 (NOA 81) as the bonding layer between two glass wafers in a μ FFE device (see Figure 3).³⁰ The layer is dissolvable, making the glass wafers reusable and easier to maintain if the device is clogged.

Since the introduction of soft photolithography by Whitesides *et al.*, PDMS has become a standard substrate for microfluidics fabrication.^{31–34} PDMS fabrication follows a simple and inexpensive replica molding strategy which is able to quickly produce devices without the need for specialized facilities or instrumentation.³⁵ However, the inherent flexibility of PDMS is a challenge for fabrication of μ FFE devices. Using standard μ FFE designs, the large open area of a separation channel fabricated in PDMS would bow out when under pressure. Zhang and Manz addressed this issue by using vacuum to pull buffer through a PDMS μ FFE device.¹⁴ Support pillars were incorporated to prevent the separation channel from collapsing under the applied vacuum.¹⁴ While successful, precise fluidic control through an applied vacuum is significantly more challenging than using pressure driven flow. There was also some concern regarding what effect multiple paths around the pillars have on stream broadening and the homogeneity of the electric field.

More recent μ FFE fabrication strategies have aimed to combine the performance of glass devices with the simplicity and cost effectiveness of PDMS devices. Most successful strategies have utilized low-cost, hard polymer substrates. Walowski *et al.* fabricated low-cost μ FFE devices using a multistep lamination process in poly(methyl methacrylate) (PMMA).³⁶ Devices were fabricated in 1 h using transfer tape as a spacer to generate a separation channel between two PMMA wafers. Devices supported field strengths as high as 422 V cm⁻¹ without any degradation. Akagi *et al.* used a computer numerical controlled (CNC) milling machine to fabricate a PMMA device.²⁰ The two PMMA substrate wafers were sealed using masking tape. Kohler *et al.* were able to mass produce μ FFE devices using

an injection molding fabrication method.³⁷ An 80 ton clamping force injection molding machine was used to fabricate devices with a separation channel depth of 20 μm . de Jesus *et al.* used laser printer toner to make raised channels on a glass substrate.³⁸ Devices with channel depths as small as 8 μm were fabricated in 1 h. Anciaux *et al.* were able to produce a μFFE device in ABS (acrylonitrile butadiene styrene) using an off the shelf, consumer grade 3D printer (see Figure 4).²¹ They were able to produce a working μFFE device for less than \$0.25 in material cost. The two printed wafers were bonded using an acetone vapor bath and platinum wires were inserted as the electrodes. While the fabrication throughput was slower than other approaches, the ease with which design modifications could be implemented suggests that 3D printing would be an excellent strategy for fast prototype development.

Electrolysis Mediation

The relatively large cross sectional area of the separation channel gives rise to higher currents than typically observed in microfluidic systems, even at low potentials. This enhanced current generates significant electrolysis and bubble formation at the electrodes. Bubble formation is particularly troublesome since they disrupt flow paths and distort the electric field, giving rise to unstable separations. The continuous nature of the separation is one of μFFE 's strongest attributes. Being unable to apply a separation potential over extended periods of time without significant bubble formation negates this advantage. As such, significant effort has been spent developing design improvements that mediate the effect of electrolysis bubble formation on μFFE stream stability. These designs can be classified as open or closed channel devices (see Figure 5).

Early devices aimed to replicate the closed channel, ion permeable membranes commonly used in conventional FFE to isolate the electrodes from the separation channel. Microfabricated channels were integrated as a pseudo membrane between the separation channel and electrode channels.^{10, 14, 39} Unfortunately, these side channels proved largely ineffective. Long, narrow side channels are necessary to effectively isolate flow in the electrode channels from the separation channel. These channels increased electrical resistance, resulting in a significant proportion of the applied potential being realized in the membrane channels, not the separation channel. In some designs it was reported that <5% of applied potential drop occurred across the separation channel.³⁹ Subsequent closed channel designs have proven more effective. Several groups have used *in situ* polymerization to generate ion permeable membranes.^{20, 29, 30} These ion permeable membranes effectively segregate bubbles from the separation channel, but lacked chemical stability and still introduced a resistive barrier that reduces the applied potential realized in the separation channel. Köhler *et al.* took a unique approach by creating a wall between the electrode channel and separation channel with a 20 μm gap at the top of the device allowing voltage to be applied through a liquid bridge.²² While electric field strength improved, the device acted as a closed system causing electrodynamic broadening. Other closed design devices include electrostatic induction,⁴⁰ and salt bridge connections.⁴¹

Open channel μFFE devices operate without any physical barrier between the electrodes and the separation channel. While open channel devices are simpler to fabricate and do not introduce an electrically resistive barrier, they are potentially less effective at isolating

electrolysis bubbles than closed channel devices. Lu *et al.* prevented the formation of bubbles in an open channel μ FFE device by operating below the potential required for water electrolysis.⁴² This strategy limited the separation potential to <2 V, greatly increasing the time necessary to achieve reasonable separation power. Kohlheyer *et al.* introduced a competing redox pair to suppress bubble formation.⁴³ Streaming a mixture of hydroquinone and p-benzoquinone over the electrodes suppressed bubble formation at field strengths as high as 215 Vcm^{-1} . Fonslow *et al.* demonstrated that electrolysis bubbles could be effectively cleared by simply fabricating electrodes in channels that are deeper than the separation channel.⁴⁴ Lubrication theory predicts that linear velocity in a planar channel is proportional to the square of the channel depth.⁴⁵ An electrode channel that was $4\times$ deeper than the separation channel generated a 16-fold increase in linear velocity, effectively clearing electrolysis bubbles while keeping the buffer velocity in the separation channel low enough to allow the separation to occur. An electric field as high as 589 Vcm^{-1} could be applied without interference from electrolysis bubbles. It was estimated that approximately 91% of the applied potential was experienced in the separation channel. Frost *et al.* found that adding a surfactant (e.g. 300 μM Triton X-100) or organic modifier (e.g. 50:50 water-methanol) to the separation buffer improved stream stability 8-fold.⁴⁶ The buffer additives lowered the surface tension of the water, resulting in smaller electrolysis bubbles, which were more easily cleared from the μ FFE device. A separation of fluorescent dyes at an electric field strength of 75 Vcm^{-1} was stable for 2 hours. Herzog *et al.* used a nanoporous PTFE membrane as a top cover for the μ FFE chip to purge the channels of bubbles.⁴⁷ The PTFE membranes performed well, and were stable in electric fields as high as 500 Vcm^{-1} for continuous separations over 3 hours. Without the membrane however, the separation became unstable after 7 min of continuous operation.

Over the past several decades there have been tremendous advances in μ FFE fabrication and design. Fabrication is now possible in a range of chemically diverse substrates and costs have dropped dramatically. Both open and closed channel designs are available that support continuous operation over several hours at reasonable separation potentials. The choice between open and closed channel designs is largely driven by the fabrication strategies available. Open channel designs are simpler to fabricate and easier to operate. Closed channel designs are more effective at isolating electrolysis bubbles but require more complex fabrication procedures, may introduce resistive barriers that lower the effective separation potential and can introduce electrodynamic broadening.

μ FFE Separation Theory

While μ FFE appears in many ways analogous to other microfluidic separations, the relative direction of flow and field introduce a number of unique differences that influence separation optimization. To this end, μ FFE is able to draw on established FFE separation theory.^{6, 48, 49} The position where an analyte crosses the detection zone, or deflection distance (d), is determined by the analyte's overall mobility (μ_{total}), the electric field in the separation channel (E) and the time the analyte spends in the electric field (t)^{48, 50}:

$$d = \mu_{\text{total}} E t \quad (1)$$

The product (Et) is referred to as the separation power of the FFE separation. A notable difference from other electrophoretic separations is that in FFE the electric field and the time an analyte spends in the electric field are independent. The time an analyte spends in the electric field can be manipulated in FFE by changing the buffer flow rate (v), or the separation channel length (L), neither of which impact, or are impacted by, the electric field. Multiple combinations of electric field and time are possible that give the same separation power and consequently the same deflection distance. The electric field and buffer velocity can therefore be changed independently to optimize μ FFE resolution.

Factors that contribute to broadening in FFE have been discussed previously.^{6, 51} The contributing factors to overall peak variance (σ_{FFE}^2) in FFE are injection stream width (σ_{inj}^2), analyte diffusion (σ_D^2), electrodynamic broadening (σ_{ED}^2), hydrodynamic broadening (σ_{HD}^2), and electrohydrodynamic broadening (σ_{EHD}^2):⁶

$$\sigma_{FFE}^2 = \sigma_{inj}^2 + \sigma_D^2 + \sigma_{ED}^2 + \sigma_{HD}^2 + \sigma_{EHD}^2 \quad (2)$$

The variance due to injection stream width is simply determined by the width of the sample stream as it enters the FFE separation channel. The variance caused by diffusion can be quantified according to:

$$\sigma_D^2 = 2Dt \quad (3)$$

where D is the diffusion coefficient of the analyte and t is the transit time through the FFE separation channel.⁵² Electrodynamic broadening is a phenomenon observed in closed devices where the electrodes are physically segregated from the separation channel. The physical barriers prevent bulk flow generated by EOF from exiting the separation channel. Instead, EOF recirculates back through the center of the channel, introducing a crescent shaped flow profile.^{53, 54}

Hydrodynamic broadening is a consequence of the difference in the profiles of the pressure driven flow acting on the analyte longitudinally while the electric field moves the analyte laterally.⁴⁹ The pressure driven flow generates a parabolic flow profile. Analytes near the wall of the separation channel move slower and therefore spend more in the electric field than analytes transiting the center of the channel. Increased time in the electric field increases the deflection distance. A crescent shaped profile is generated where analytes travelling through the center of the channel are deflected less than analytes nearer the separation channel walls.⁵³ Hydrodynamic broadening (σ_{HD}^2) is determined by:

$$\sigma_{HD}^2 = \frac{h^2 d^2 v}{105 DL} \quad (4)$$

where h is height of the channel, d is the deflection distance, v is the buffer linear velocity, D is the diffusion constant of the analyte and L is the length of the separation channel.⁴⁹ Hydrodynamic broadening is more prevalent for analytes that are deflected most in the separation channel.

Electrohydrodynamic broadening is caused when the conductivity of the buffer solution is higher than that of the sample stream.⁶ A difference in conductivity between the sample stream and the separation buffer generates a discontinuity in the electric field, which causes analytes to either speed up or slow down as they exit the sample stream. If the conductivity of the sample stream is higher than that of the separation buffer, analyte migration velocity increases as it crosses the boundary, generating broader stream widths through de-stacking.

Fonslow and Bowser were able to demonstrate that in an open channel μ FFE device, where the sample matrix was well matched with the separation buffer, stream width was primarily determined by injection width, diffusion and hydrodynamic broadening:⁴⁹

$$\sigma_{\mu FFE}^2 = \sigma_{inj}^2 + \sigma_D^2 + \sigma_{HD}^2 \quad (5)$$

The expression could be further expanded to predict the effect of various experimental parameters on broadening:

$$\sigma_{\mu FFE}^2 = \frac{w_{inj}^2}{12} + \frac{2DL}{v} + \frac{h^2 d^2 v}{105DL} \quad (6)$$

where w_{inj} is the baseline width of the injection stream, D is the diffusion coefficient of the analyte, L is the length of the μ FFE separation channel and v is the linear velocity of the separation buffer.⁴⁹ Equation 6 is analogous to the Van Deemter equation that predicts the effect of mobile phase velocity on peak width in chromatography.⁵⁵ Fonslow and Bowser demonstrated that stream width could be minimized at a buffer linear velocity that balanced diffusion and hydrodynamic broadening (see Figure 6).⁴⁹ It should also be noted that hydrodynamic broadening scales with the square of the separation channel depth, reinforcing an important performance advantage of μ FFE over conventional scale FFE.

Combining equations for stream position (eq. 3) and width (eq. 6) allows the effect of experimental parameters on the resolution of a pair of streams to be predicted⁴⁹:

$$R_s = \frac{d_1 - d_2}{(w_1 + w_2)/2} = \frac{(\mu_{total,1} - \mu_{total,2})Et}{2\left(\sqrt{\frac{w_{inj}^2}{12} + \frac{2D_1L}{v} + \frac{h^2 d_1^2 v}{105D_1L}} + \sqrt{\frac{w_{inj}^2}{12} + \frac{2D_2L}{v} + \frac{h^2 d_2^2 v}{105D_2L}}\right)} \quad (7)$$

At lower buffer velocities, where diffusion is the dominant factor for broadening, equation 7 can be approximated by⁴⁹:

$$R_s \approx \Delta\mu E \sqrt{\frac{L}{32Dv}} \quad (8)$$

Under these conditions resolution scales linearly with the electric field and inversely with the square root of the buffer linear velocity.

At higher buffer velocities and deflection distances, where hydrodynamic broadening dominates, equation 7 can be approximated by⁴⁹:

$$R_s \approx \frac{\Delta\mu}{4h\mu} \sqrt{\frac{105L}{v}} \quad (9)$$

In this case, resolution is independent of the electric field and again inversely proportional to the square root of the buffer linear velocity. These equations suggest that in μ FFE, high combinations of electric field and buffer linear velocity are not necessary to achieve optimal resolution. Fonslow and Bowser used equation 7 to demonstrate that in μ FFE a practically achievable resolution maximum occurs at a relatively low combination of buffer linear velocity and electric field.⁴⁹ They suggested that the separation power should be maximized to spread analyte streams across the full width of the separation channel. The linear velocity of the buffer should then be adjusted to minimize stream width while also adjusting the electric field to keep the separation power, and therefore stream positions, constant. The low combination of electric field and buffer linear velocity necessary to optimize μ FFE separations is an important contrast with most microscale electrophoretic separations where high electric fields and shorter analysis times almost always improve resolution.^{56, 57} It is also of practical importance since it demonstrates that high pressure pumps and high voltage power supplies are not required for optimum μ FFE performance.

By analogy to capillary electrophoresis, early μ FFE publications included broadening terms for wall adsorption and Joule heating.^{10, 39} More recently, Geiger *et al.* have demonstrated that adsorption does not broaden stream width in μ FFE separations.⁵⁸ Analytes with no affinity for the wall stream directly through the separation channel. If an analyte adsorbs onto the surface it is fixed in position, moving neither in the pressure driven flow or electric field. When the analyte desorbs it returns to its original flow path determined by its mobility and the linear velocity of the separation buffer. Geiger *et al.* were even able to demonstrate effective μ FFE separations of proteins that strongly adsorbed onto the glass surface of the separation channel (see Figure 7).⁵⁸ It is important to note that while surface adsorption does not contribute to μ FFE broadening in the spatial dimension, it does affect the time required for an analyte to travel through the separation channel. This temporal broadening is an important consideration for monitoring applications where μ FFE is used to continuously assay a changing analyte stream over time.

Recent modeling by Okhonin *et al.* has suggested that performing FFE separations with electric fields that are not perpendicular to the separation channel (i.e. non-orthogonal) can

increase resolution at the expense of sample residence time.⁵⁹ However, fabrication challenges have prevented this approach from being confirmed experimentally at the time of this publication.

Detection

Due to size and volume constraints, conventional scale FFE detection is usually performed offline via UV analysis of collected fractions.^{6, 8} Using a similar offline analysis strategy for μ FFE is generally infeasible due to the reduced volumes and complex channels necessary to collect a large number of fractions from discrete positions across the separation channel. Conversely, fabrication of a separation channel with a smaller detection zone in an optically clear substrate facilitates direct online detection. Consequently, μ FFE devices are usually used in analytical separations while conventional scale FFE is more often used as a preparative separation.

Fluorescence

Early μ FFE utilized a point source detector with translational movement of the device to scan across the width of the separation channel.^{10, 39, 60} Unfortunately, scanning limits the sampling rate of the separation and physical translation complicates reliable alignment of the optical system. μ FFE systems quickly migrated to imaging detectors with a large field of view to continuously image the entire width of the separation channel.^{40–42, 44, 46, 61, 62} Fluorescence detection is ideal due to its high sensitivity and the wide availability of fluorescence microscopes. Excitation with a lamp is easily performed in an epifluorescence alignment. Limits of detection can be further improved using laser-induced fluorescence (LIF).^{63, 64} Integration of LIF can be as simple as focusing laser output into a line that extends across the width of the separation channel. Emission is recorded using a standard fluorescence microscope. Kochmann and Krylov have recently developed an open-source software suite in an effort to normalize image collection and analysis conditions for μ FFE separations in conjunction with a homebuilt portable fluorescence detection system.⁶⁵ The software provides detailed information on stream trajectory, width, and resolution of the separation on the whole chip.

Optical detection in μ FFE is more analogous to imaging than a typical separation detector. In chromatography or CE separations the detector only has a short period of time to measure the intensity of a peak as it exits the column. In μ FFE, continuous injection of a sample presents a static sample stream that can be imaged over longer time periods. Turgeon *et. al.* used signal averaging over relatively long exposure times to improve the detection limit of a simple μ FFE LIF detector.⁶⁴ The limit of detection improved 20 to 24-fold by collecting and averaging 500 sequential images over 2 min. The observed limit of detection for fluorescein was 14 pM.

Label-Free Detection

While extremely sensitive, the drawbacks of fluorescence detection are well known. Most analytes are not natively fluorescent and must be labeled with a fluorophore in order to be detected, which can dramatically affect the chemical properties of the sample. Expanded

strategies for label free μ FFE detection are needed. Unfortunately, few examples have been reported in the literature to date. An obvious candidate for label free detection would be UV-Vis absorbance. This is challenging in microscale devices due to the short optical path length.⁶⁶ Köler *et al.* fabricated a fused silica μ FFE device that allowed deep UV fluorescence scanning for the detection of aromatics and proteins.⁶⁰ Lysozyme and chymotrypsinogen, were separated and detected using a single point scanning method and a 266 nm Nd:YAG laser. Challenges included low sensitivity due to large autofluorescence background signal from the buffer components. Wavelength selection was limited by the availability of deep UV laser sources. Poehler *et al.* used UV laser fluorescence in conjunction with a near-infrared fluorescent sensor layer under the separation bed to perform biomolecular isoelectric point analysis in near real-time.⁶⁷ With this μ FFIEF method, the pH gradient and biomolecule separation could be simultaneously observed. The separation was validated with offline mass spectrometric analysis.

Jezierski *et al.* used cells adhered to the separation channel walls as biosensors for ATP.⁶⁸ The position of a 20 μ M stream of ATP was successfully visualized using human embryonic kidney cells doped with a calcium-sensitive fluorescent probe. The cells could be subjected to linear buffer velocities as high as 4.6 mm s⁻¹ and an electric field of 100 V cm⁻¹ before they would detach from the walls.

Becker *et al.* reported using surface enhanced Raman spectroscopy (SERS) as a label free detection mode for an isotachophoretic μ FFE separation of myoglobin.⁶⁹ However, the silver colloids used in SERS frequently blocked channel flow, and more experimentation is necessary to determine the feasibility of the technique.

Mass Spectrometry

Coupling the planar μ FFE separation channel to mass spectrometry (MS) remains a significant technical challenge. Benz *et al.* recently coupled μ FFE to nanospray MS by manipulating the buffer flow velocity from different inlets to sequentially shift analyte streams to an outlet channel (see Figure 8).⁷⁰ A sample stream of 4 fluorescent dyes were visualized via fluorescence to assist directing the dye of interest into the MS outlet connection. The authors then applied this detection scheme to analyze the products of a multicomponent [3+2]-cycloannulation reaction. Only 2.5 min was necessary to fully scan the width of the separation channel.

Applications

Design and technical improvements in μ FFE devices have made continuous, stable operation over long time periods possible. The majority of μ FFE applications reported to date have been static, proof of concept separations used to assess the progress of this design development or replicate separations similar to those employed in conventional scale FFE. μ FFE separations for a wide range of analytes have been reported including: fluorescent dyes,^{18, 27, 49} peptides,^{71, 72} amino acids,⁷³ and proteins.^{41, 74} μ FFE separation modes demonstrated to date include zone electrophoresis,^{10, 15, 21, 39, 58, 75, 76} isoelectric focusing,^{42, 77–82} isotachopheresis,^{40, 83} and field step electrophoresis.⁸⁴ While these applications demonstrate the breadth and flexibility of μ FFE separations, they generally don't perform

significantly better than existing microscale separations. For μ FFE to find widespread use, applications that take advantage of its unique properties must be developed. The continuous nature of μ FFE separations allows it to be utilized in applications not typically associated with separations. Specifically, μ FFE is particularly well suited for microscale purification and online monitoring applications.

Microscale Purification

Sample flow rates into μ FFE devices are typically 100-500 nL/min.^{73, 76, 85-88} These flow rates make purifications on a low microliter scale possible in a reasonable time period. Fractionation is as simple as directing flow streams to specific outlet channels for collection. Purifications on this volume scale are challenging with limited competing technologies available.

Chartogne *et al.* used μ FFE as an online bridging step between capillary isoelectric focusing (cIEF) and electrospray ionization mass spectrometry (ESI-MS).⁸⁹ High concentrations of carrier ampholytes in cIEF cause sensitivity issues in ESI-MS due to ion suppression of proteins.^{90, 91} Ampholytes also cause salt buildup in the ESI interface which in turn leads to electrospray instability. μ FFE was shown to effectively remove carrier ampholytes from a cIEF separation prior to ESI-MS analysis. A separation of myoglobin, carbonic anhydrase 1, and β -lactoglobulin B was demonstrated.

Jing *et al.* used μ FFE to isolate aptamers for human IgE by Systematic Evolution of Ligands by EXponential enrichment (SELEX).⁷⁶ High affinity aptamers ($K_d = 20$ nM) were isolated within 2-4 rounds of selection. The continuous nature of the μ FFE separation greatly simplified fraction collection. Significantly larger volumes could be enriched using μ FFE selections (3 μ L) than was possible in similar CE based selections (~8 nL). The increased sample volume allowed 1.8×10^{14} ssDNA ligands to be assessed in every round of selection, a 300-fold increase over CE-SELEX.

Podszun *et al.* used μ FFE to concentrate viable bacteria from mL to μ L volumes.⁸⁷ Charged bacteria samples were concentrated in a tris-borate-EDTA buffer at a constant current of 250 μ A (see Figure 9). Bacteria were continuously streamed into the μ FFE device at 300 nL/min where they were deflected toward the electrode, which was blocked by a gel barrier. Bacteria were collected at the barrier for 30 min. The polarity of the electrode was then reversed to migrate bacteria back into the separation channel for collection. Enrichment efficiencies of up to 80% were reported. Concentration factors of 13 and 25 were reported for 100 μ L and 200 μ L samples, respectively.

Kostal *et al.* used μ FFE to assess the electrophoretic properties of mitochondria isolated from rat myoblasts.⁸⁵ The distribution of mitochondria mobilities were assessed in less than 30 s. The mobility distributions correlated well with CE-LIF measurements of the same sample. The benefits of the μ FFE separation included less reagents, lower electric field, and shorter analysis time when compared to the CE method. The lower electric field of the μ FFE separation was particularly important since it was determined that extrapolation to account for particle polarization was not necessary, eliminating the need to make measurements across a range of separation potentials.

A challenge associated with quantum dot (QD) synthesis is extracting pure QDs from unreacted impurities after the reaction is completed. Purification is particularly challenging in mass production of QDs. Kim *et al.* used μ FFE as a platform for continuous separation of QDs from reaction impurities.⁹² A simple open channel device with two inlets and two outlets was fabricated in glass. The QD solution was injected in one inlet and the target solvent of ethanol flowed through the other. Voltage was applied, and the QDs were collected in the target solvent outlet, while the impurities did not migrate. A yield of 60% was reported.

Continuous Monitoring

The ability of μ FFE to separate and analyze a continuous analyte stream makes the technique ideally suited for online monitoring applications. The temporal response of the separation is only limited by the exposure time of the detection system, which can be 100 ms or faster.⁶⁴ μ FFE analyses offer the potential to combine the selectivity of a separation with a time response typically associated with an electrochemical or optical sensor.

Fonslow and Bowser used gradient μ FFE as an efficient way to optimize electrophoretic separation conditions.⁹³ μ FFE was used to continuously monitor a separation of NBD-F labeled amino acids as the separation buffer was increased from 0 to 50 mM hydroxypropyl- β -cyclodextrin (HP- β -CD) over a 5 min period (see Figure 10). μ FFE separations were recorded every 5 s, allowing the resolution to be assessed at 60 different HP- β -CD concentrations. This nearly continuous coverage allowed the optimum HP- β -CD concentration to be unambiguously identified in 5 min. A similar optimization using CE would take several hours.

Turgeon *et al.* used gradient μ FFE to measure aptamer affinities for their targets across a range of ligand concentrations.⁸⁶ A constant concentration of aptamer was mixed with an increasing concentration of ligand in an online reaction tee. μ FFE was used to continuously measure the ratio of bound to free aptamer as the ligand concentration was increased over a 5 min period. μ FFE separations were recorded every 1 s allowing the equilibrium to be assessed at 300 distinct ligand concentrations in 5 min. Aptamers for IgE and HIV-RT were assessed. The nearly continuous coverage of the binding isotherm allowed the affinity and stoichiometry of the equilibrium to be assessed with greater confidence.

Cheng and Chang reported a novel method for the generation of a pH gradient in μ FFIEF.⁹⁴ Rather than create several inlet channels for the introduction of several ampholyte solutions, a pH gradient was created with electrically switchable pH actuators and 3D integrated polyurethane based salt bridges. The pH actuator is achieved with bipolar membranes that inject excess OH^- or H^+ ions which are produced by electric field water dissociation at the membrane junction. The device was able to create pH gradients from pH 2 to pH 10 without ampholytes in proof of concept testing.

Two Dimensional Separations

Two dimensional (2D) separations offer a powerful approach for analyzing complex samples. Under ideal conditions the peak capacity of a 2D separation approaches the product of the peak capacity of each individual separation.⁹⁵ Unfortunately, the speed of hyphenated

2D separations is limited by the sampling rate of the 2nd dimension separation.⁹⁶ Peak capacity is lost if the 2nd dimension separation undersamples peaks as they elute off the first dimension column.⁹⁷ To overcome this undersampling limitation hyphenated 2D separations often couple a very slow first dimension separation with a faster than optimum second dimension separation.

μ FFE is ideally suited to act as the second dimension of a hyphenated 2D separation. Continuous μ FFE separation effectively eliminates undersampling as a limitation, allowing peak capacity to be increased significantly without increasing the first dimension separation time. Geiger *et al.* coupled nano-liquid chromatography (nLC) with μ FFE to perform 2D separations of peptides and amino acids (see Figure 11).^{73, 98} Coupling was achieved via a connection capillary introduced directly into the μ FFE separation channel to make a zero dead volume connection. Analyte peaks streamed directly into the μ FFE separation channel as they eluted from the nLC column. No complicated modulation or valving was necessary to couple the two separations. A separation of a fluorescently labeled BSA tryptic digest yielded an ideal 2D peak capacity of 1,320 in a 5 min separation window (264 peaks/min).⁷³

Johnson and Bowser coupled CE and μ FFE to perform 2D separations.⁸⁸ An ideal 2D peak capacity of 2,592 was observed in a 9 min CE $\times$ μ FFE separation of a fluorescently labeled BSA digest (7.6 min separation window, 342 peaks/min). Adding the second dimension μ FFE separation increased the peak capacity 24-fold with no increase in the first dimension CE separation time. μ FFE separations were recorded every 250–500 ms, generating 8 data points/peak as they migrated off the CE capillary, effectively eliminating under sampling as a source of band broadening. CE performed better than nLC at faster separation times allowing even faster first dimension analyses to be executed. For example, CE $\times$ μ FFE generated an ideal peak capacity of 1,885 in a 2.7 min separation of fluorescently labeled small molecule bioamines (1.8 min separation window, 1053 peaks/min).

Conclusions

Dramatic improvements have been made in μ FFE over the past several decades. A number of fabrication strategies have been developed with recent emphasis on producing lower cost μ FFE devices in hard plastic substrates. Design improvements have effectively addressed performance issues that arise from electrolysis bubble generation, allowing continuous operation over several hours. μ FFE separation theory has been developed allowing rational optimization of separation parameters. Simple detection schemes have been demonstrated but further development in this area is needed. Label-free detection strategies and MS interfaces would dramatically increase the applicability of μ FFE separations. Unique applications that take advantage of the continuous nature of μ FFE separations continue to be introduced. Applications of particular interest include microscale purifications, continuous monitoring and high peak capacity 2D separations.

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Biographies

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Michael T. Bowser is a professor of Chemistry at the University of Minnesota. He received his Ph.D. in Chemistry from the University of British Columbia and was a postdoctoral fellow at the University of Florida under Dr. Robert Kennedy. Prof. Bowser's group has made important contributions in the areas of online high-speed microdialysis-capillary electrophoresis assays, microfluidic strategies for aptamer selection and microscale free-flow electrophoresis (μ FFE). Applications of interest include adipocyte signaling in obesity, nucleic acid modulation of heart function, aptamers to facilitate cell delivery, high-speed microdialysis assays, and multidimensional separations.

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Figure 1.

FFE separation mechanism. A sample is continuously introduced into the separation channel where it is exposed to a perpendicular electric field. Analyte streams are deflected according to their mobility. Adapted with permission from Fonslow, B. R.; Bowser, M. T. *Anal Chem* 2006, 78, 8236–8244. Copyright (2006) American Chemical Society.

Figure 2.

A) A Pyrex based μ FEE device used for bacteria concentration. Adapted with permission from Ref.⁸⁷ from the Royal Society of Chemistry. B) A Borofloat wafer based μ FEE device created for bubble free electrophoresis. Adapted with permission from Ref.²⁶ from the Royal Society of Chemistry. C) A glass slide μ FEE device for the fluorescent determination of pIs in proteins. Adapted with permission from Ref.¹⁷ from the Royal Society of Chemistry. D) A Borofloat wafer based μ FEE design for high peak capacity separations of peptides. Adapted with permission from Johnson, A. C.; Bowser, M. T. *Anal. Chem.* 2017, 89, 1665–1673. Copyright (2017) American Chemical Society. E) An injection molded μ FEE device in cycloolefin polymer for low cost fast mass production of devices. Adapted with permission from Ref.³⁷ from the Royal Society of Chemistry.

Figure 3.

Schematic diagram of fabrication: (1) standard photolithography, wet etching and drilling; (2) fixing and alignment; (3) filling UV-curable monomer (NOA 81); (4) protecting the functional domain; (5) standard photolithography; (6) removing uncured monomer (NOA 81). Reproduced from Ref.³³ with permission from The Royal Society of Chemistry.

Figure 4.

Images of a (a) glass μ FFE device, (b) 3D-printed μ FFE device taken from the channel/detection side, and (c) 3D-printed μ FFE device taken from the fluidic connection side. Labels highlight the (1) buffer inlets, (2) sample inlet, (3) electrode connections, and (4) buffer outlets. Channels were filled with food dye in panel b to demonstrate that no leaking was observed after solvent vapor bonding. Reproduced with permission from Anciaux, S. K.; Geiger, M.; Bowser, M. T. *Anal. Chem.* 2016, 88, 7675–7682. Copyright (2016) American Chemical Society.

Figure 5.

(A) Side view of closed style device, where the electrodes (yellow) are segregated from the separation channel (white) by some kind of structure (purple) usually an ion-permeable membrane, insulator, or wall. (B) Side view of an open style device in which the separation channel is not segregated from the electrodes by any kind of structure.

Figure 6.

Plots of variance (σ^2) versus linear velocity. (A) and (B) are plots for rhodamine 123 and fluorescein, respectively in the presence of EOF ($\mu_{\text{eo}} = 3.80 \pm 0.05 \times 10^{-4} \text{ cm}^2/\text{Vsec}$) (C) and (D) are plots for rhodamine 123 and fluorescein, respectively with EOF suppressed by PEO ($\mu_{\text{eo}} = 8.63 \pm 2.25 \times 10^{-5} \text{ cm}^2/\text{Vsec}$). Variances are plotted for increasing linear velocities (v) with the electric field adjusted to keep the separation power constant: 0 (red), 100 (brown), 200 (yellow), 300 (green), 400 (blue), 500 (violet), 600 (black), 700 (maroon) Vsec/cm. Only the best-fit lines for the highest and lowest separation powers are plotted in (B), (C) and (D) for clarity. Reproduced with permission from Fonslow, B. R.; Bowser, M. T. *Anal Chem* 2006, 78, 8236–8244. Copyright (2006) American Chemical Society.

Figure 7.

A) CE electropherogram of Chromeo™ P503 labeled cytochrome c and myoglobin. B) nLC chromatograms measured on-column. C) Extracted chromatograms recorded in the μ FFE separation channel 12.5 mm from the sample inlet. D) Extracted μ FFE linescan recorded at 4.2 minutes. Reproduced with permission from Geiger M.; Harstad, R. K; Bowser, M. T. *Anal. Chem.* 2015, 87, 11682-11690. Copyright (2015) American Chemical Society.

Figure 8.

a) Layout of a microfluidic FFE-MS chip (I=left). b) The analysis principle. The separated analytes are directed towards the mass spectrometric outlet by alteration of the buffers' hydrodynamic flow. Arrows indicate relative flow rates and the rectangle (*) labels the area visualized by fluorescence imaging. Reproduced from Ref.⁷⁰ with permission from John Wiley and Sons.

Figure 9.

Illustration of the μ FDE device for bacteria concentration. As charged bacteria are injected, they migrate toward the electrode. The gel barrier stops the bacteria migration effectively trapping the bacteria. The polarity of the electrodes are flipped to release the bacteria, and the buffer eluent is collected at the outlet at the bottom of the device. Reproduced from Ref. ⁸⁷ with permission from the Royal Society of Chemistry.

Figure 10.

Schematic of the μ FFE pumping setup used for generating a buffer additive gradient. Pump 1 and 2 were controlled using LabView to ramp the flow rates to create the gradient while keeping the flow rate constant. Reproduced with permission from Fonslow, B. R.; Bowser, M. T. *Anal. Chem.* 2008, 80, 3182-3189. Copyright 2008 American Chemical Society.

Figure 11. 1D nLC chromatograms of A) Chromeo™ P503, B) NBD-F and C) Alexa Fluor® 488 labeled BSA tryptic peptides. 2D nLC × μFFE separations of D) Chromeo™ P503, E) NBD-F and F) Alexa Fluor® 488 labeled BSA tryptic peptides. Data shown are representative separations from n = 3 replicates. Adapted with permission from Geiger, M.; Bowser, M. T. *Anal Chem* 2016, 88, 2177-2187. Copyright 2016 American Chemical Society.