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Review

Recent developments in electrophoretic separations on microfluidic devices

Research combining the areas of separation science and microfluidics has gained popularity, driven by the increasing need to create portable, fast, and low analyte-consumption devices. Much of this research has focused on the developments in electrophoretic separations, which use the electrokinetic properties of analytes to overcome many of the problems encountered during system scale-down. In addition, new physical phenomenon can be exploited on the microscale not available in standard techniques. In this study, the innovative developments, including electrophoretic concentration, sample preparation/conditioning, and separation on-chip are reviewed, along with some introductory discussions, from January 2008 to July 2010.

Keywords:
IEF / ITP / Microfluidic / Separation

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1 Introduction

Separation science is often a necessary first step in performing an analysis. It has received significant attention as older, more established techniques such as chromatography are leveraged, or entirely new techniques are developed. Separation techniques that exploit the electrostatic and electrodynamic properties of analytes have become increasingly popular. Many of these focus on unique properties of the analytes that have not been fully examined for separations. Several of the new methods are related to electrophoresis and some of the standard bearers include isoelectric focusing (IEF) [1–3] and free-flow electrophoresis (FFE) [4, 5]. Various processes, such as FFE, are well known on the preparative-scale, whereas others, such as IEF, have been well characterized for analytical-scale separations. There have been many innovative areas of development on the smaller scale, including taking advantage of hydrodynamic counterflow [6, 7], different channel designs [8], and applying electric fields perpendicular to the flow of the sample [9–13].

Several groups continue to develop variations on standard electrophoretic separation techniques, including Astorga-Wells [14], Gebauer [15, 16], Hayes [17, 18], and Ivory [19, 20], but the current article will center on elements that are essentially new or are significant advances in strategies that uniquely exploit microfluidic formats. The advantages of microfluidic devices include lower sample consumption, portability, and shorter analysis times. These qualities are desirable for studying complex samples, especially in the current age where onsite and quick analyses are being sought. One of the advantages of microfluidic devices is the small sample size; however, this creates LOD issues, as analytes of interest may actually not be present at concentration or mass levels high enough to detect. This review will discuss the techniques that develop ways to overcome this limitation either through continuous sampling or through concentration enhancement on-chip before separation, or a combination of both.

Several topics are excluded for clarity and focus. These include chromatographic techniques, as well as channels that contain particle packing, membranes, and gels. As the review aims to describe the separation methods themselves, fabrication methods and new chip and electrode materials will not be addressed. Other areas of interest with clear connectivity to the current study will also be omitted, such as carbon nanotubes, electrophoresis in nanochannels, electrophoretic separations used for immunoassays, and dielectrophoretic separations. The topics that are addressed are categorized into three topics: (i) external field directly acting on analytes, (ii) behaviors defined by the physical

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Abbreviations: **CESE,** concentration element and solution element; **EKS,** electrokinetic supercharging; **FEKS,** floating EKS; **FFE,** free-flow electrophoresis; **GEITP,** gradient elution isotachophoresis; **GEMBE,** gradient elution moving boundary electrophoresis; **H_2Q,** hydroquinone; **LE,** leading electrolyte; **MGE,** microchip gel electrophoresis; **PET,** poly(ethyleneterephthalate); **Q,** *p*-benzoquinone; **QH,** quinhydrone; **TE,** terminating electrolyte; **TGF,** temperature gradient focusing

Colour Online: See the article online to view Figs. 1,2,3,5 and 6 in colour.

structure of the microdevice, and (iii) separations defined by local solution properties. Although the articles have been divided into categories to aid in organization, not all categories are mutually exclusive and many techniques could be placed in more than one designation. Finally, the time frame that will be discussed is between January 2008 and July 2010.

2 External field acting directly on target

In this section, processes that involve the direct interaction of the electric field on the analyte for focusing and differentiation are discussed. Some of the techniques described here include FFE and its variations, temperature gradient focusing (TGF), and methods that simultaneously separate anions and cations.

2.1 FFE

FFE is a continuous separation technique that utilizes two components: hydrodynamic flow and an applied electric field [4], where the electric field is applied perpendicularly to the flow. The sample is introduced into the flow via an inlet at one end and is separated perpendicular to the flow based on the species' electrophoretic mobilities. At the opposite end, the separated species exit through individual outlets. This technique has more recently been applied to microscale devices [21].

The Bowser group from the University of Minnesota has made several contributions to FFE since 2008 [10–12, 22]. In one study, Fonslow and Bowser used a microchip with varying depths to study the effects of a buffer concentration gradient on separations [10] and in another study, to separate mitochondria [11]. In the first article, a concentration gradient in cyclodextrin was created, and the effect on amino acid separation was examined, as well as an efficient determination of the ideal separation conditions [10]. Figure 1 shows the FFE system used by Kostal et al. In that article, mitochondria were separated using less sample and in less time than traditional FFE systems [11]. In both examples, the channel was on a microchip; however, the channel length was 5 cm and the width was 3 cm.

Kohlheyer et al. described a new method for preventing electrolysis in a microfluidic free-flow device [23]. Quinhydrone (QH), a complex of hydroquinone (H_2Q) and *p*-benzoquinone (Q), was added to the system as strategy to electrochemically quench hydrolysis. Instead of the typical generation of oxygen and hydrogen when water is oxidized and reduced at their respective electrodes, H_2Q was oxidized and Q was reduced, which prevented the formation of bubbles. Aside from the addition of QH, the chip design was also slightly modified. Rather than a single inlet channel, there were five inlet channels, two of which were used for injecting QH solution, another for sample introduction, and the other two for sample focusing as shown in Fig. 2. Fluorescein, rhodamine B, and rhodamine 6G were successfully separated; however, this technique was effective only with low-current densities, limited by the depletion of QH.

A variation of FFE was demonstrated in 2009 by the Janasek group from Germany [9]. In their apparatus, a microfluidic glass chip with nine outlet channels was designed and used for the separation of proteins. Instead of a single inlet, there were a total of 67 inlet channels used for buffer and sample introduction. The 67 channels were formed by branching from two main channels that were

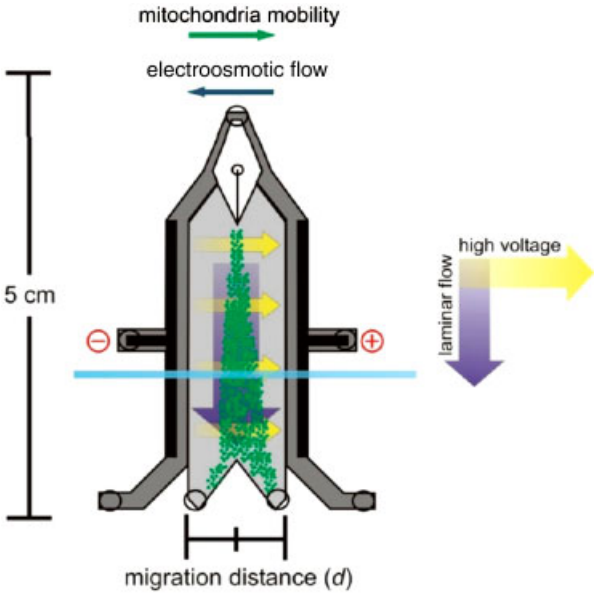

Figure 1. Schematic diagram of the flow and mobility in a micro-FFE device. The dark arrow represents laminar flow, whereas the lighter arrow represents the direction of the voltage.

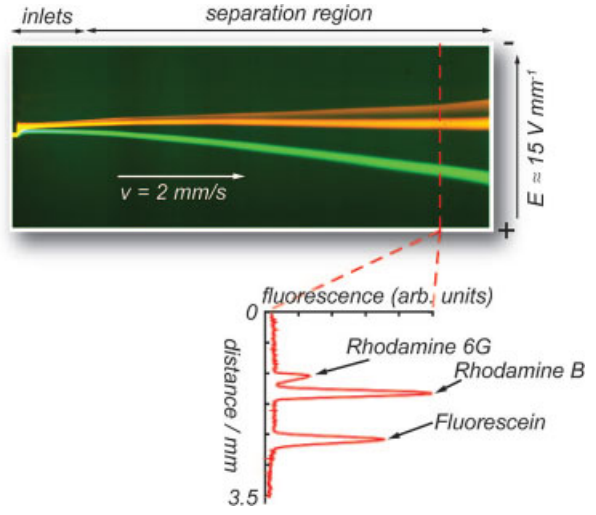

Figure 2. Photographic image of three fluorescent dyes diverging in the free-flow zone electrophoresis microdevice (top), and an intensity profile of the separated dyes (bottom).

connected to syringe pumps. The separation channel had posts incorporated to prevent channel collapse and to effectively increase the path length. The main separation channel had 222 shallow side conduits connected on each side. These areas were used to join the main channel to a large buffer reservoir on each side, where the electrodes were placed, as well as prevented bubble formation in the main separation chamber by acting like membranes. Myoglobin and trypsin inhibitor proteins were labeled with fluorescein isothiocyanate (FITC) and TGF was used to demonstrate separation. The species were focused to different outlets based on their different electrophoretic mobilities and two concentrated peaks were visible.

Zalewski et al. performed the first example of synchronized, continuous-flow zone electrophoresis on a microfluidic device [13]. This technique is also related to FFE, except that hydrodynamic flow, as well as separation, is electrokinetically driven. The borosilicate glass chip contained three inlets and three outlets connected to the separation channel. The sample was introduced into the chamber from the center inlet and was focused by buffer streams from the inlets on either side. The position of the sample stream, was adjusted by manipulating the buffer streams, and electric potential was applied perpendicularly. The combination of a varying position of sample stream and the axial electric field created a wavelike sample stream path. The path was then manipulated to separate species with different apparent electrophoretic mobilities. Theoretical and experimental data were presented and indicate that this method was successful. Rhodamine B and fluorescein were separated, as well as a three-component mixture of fluorescein, rhodamine B, and rhodamine 6G.

2.2 TGF

TGF is part of a novel group of separation techniques that differentiates and concentrates species in a channel based on their electrophoretic velocities varying with temperature [24]. In these counterflow techniques, a bulk flow opposes, or counters, the electrophoretic velocity of the species. When the bulk flow is equal to and opposite of the electrophoretic velocity, the species are retarded and focused. Species move from both directions in the channel to reach this focusing point. As the electrophoretic velocity of a species is the product of the electrophoretic mobility and the electric field, an electric field gradient must be created in the channel to allow for the movement and eventual retardation of the species. In TGF, the electric field gradient is created by employing buffers whose conductivities vary with temperature. Typically, one end of the channel is heated, whereas the other is cooled to create a temperature gradient, which results in an electric field gradient.

Studies in this area since 2008 have covered theoretical, simulated, and experimental aspects, as well as large-scale and microscale devices. The Ross group at NIST has reported on several applications of TGF. One article emphasized the effects of high ion concentration on separation and focusing [25] and another described using scanning TGF for the separation of chiral amino acids [26]. All these devices, however, utilized capillaries that were several centimeters in length. Also from NIST were applications of TGF that used the technique to prevent species from entering a channel, instead of solely for sample concentration [27, 28]. In these examples, biological samples that can damage devices by adsorption and those with high-concentration sample matrices were being used. By preventing species from entering the channel, reusable devices and less sample interference were achieved. However, these were also larger scale devices, as separations took place in capillaries that were several centimeters in length.

Although TGF commonly utilizes external physical heating or cooling of the capillary, there have also been reports of using Joule heating to induce a temperature gradient [6, 7]. In these articles, the separation channel had a change in width at one location. Tang and Yang numerically demonstrated this phenomenon in a PDMS microfluidic device [6]. When the channel narrowed, the heat density increased in the presence of applied potential, which caused an increase in the temperature in the narrow part of the channel. The group simulated this effect and indicated that concentration slowly increases at this interface (Fig. 3). Results indicated that after 190 s of applied potential, concentration increased by 350-fold (original concentration of 0.280 M). Results were compared with the study performed by Ross et al. [24] and were in good agreement.

Ge et al. also presented experimental and numerical studies of Joule heating-induced TGF in microchannels [7]. Several factors were investigated using fluorescein-Na, including channel width ratio, applied potential, and buffer concentration on TGF in PDMS/glass and PDMS/PDMS devices. It was found that increasing the applied potential, buffer concentration, and channel width ratio all lead to greater concentration enhancement. Overall, it was found that the PDMS/PDMS device required lower potential and shorter time to accomplish the same concentration enhancement as the PDMS/glass device, due to the lower thermal conductivity of the PDMS/PDMS device. In addition to the experimental data, numerical data using COMSOL Multiphysics were presented and were supported by the experimental data.

2.3 Separation of anions and cations

Reschke et al. from West Virginia University described devices that were used for the separation of ions [29, 30]. In the first article, a glass microfluidic device where flow is controlled by electrophoresis was used for anion and cation separation and detection [29]. The results showed that both theoretical and experimental ions could be ''electrophoretically extracted'' from the hydrodynamic flow stream at the intersection between two channels. A sample was pumped through the main channel and a potential was then applied

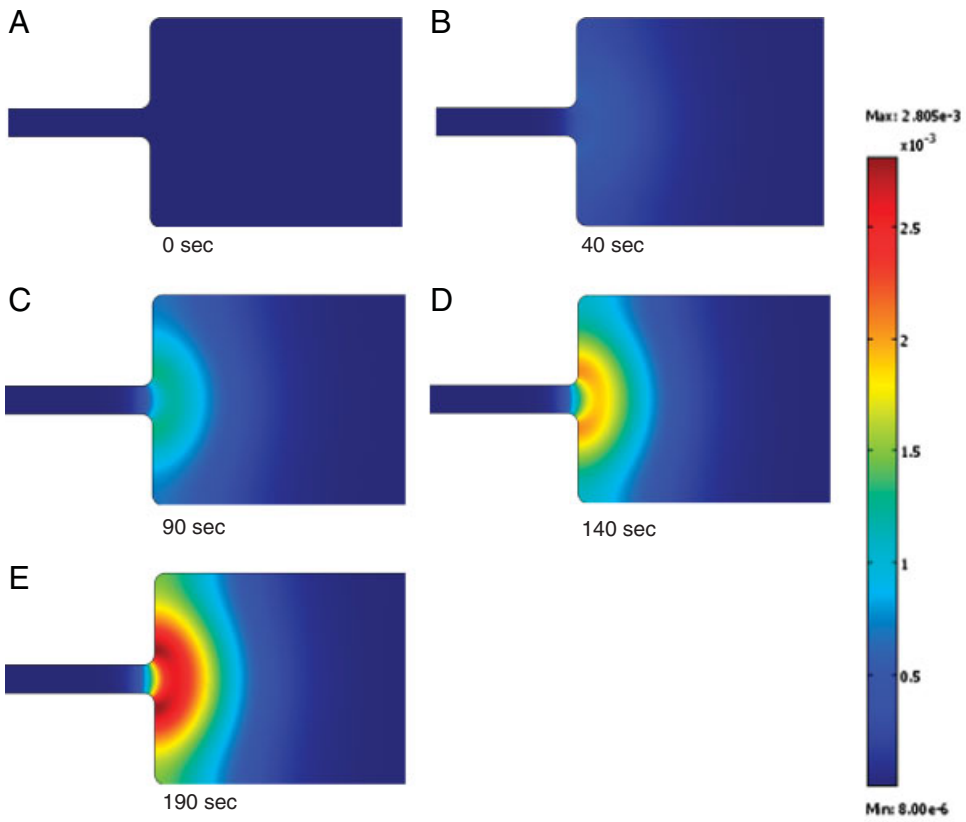

Figure 3. Simulation of sample concentrations at the junction between wide and narrow microchannels is demonstrated at various times for a TGF experiment that uses Joule heating to induce a temperature gradient.

at the intersecting channel. When the charged species came under the influence of the electric field, they were extracted from the main channel to the intersection channel based on their electrophoretic mobilities. Cations moved toward one side of the intersecting channel, whereas anions moved toward the opposite side. The behavior of fluorescein was analyzed and had nearly complete extraction.

In a subsequent article by Reschke et al. [30], the glass microfluidic device for simultaneous cation and anion detection was modified. The device retained the single, hydrodynamically pumped sample stream, but separation and detection occurred in two separate channels, one for anions and one for cations. When the sample stream passed the intersection where the injection channel meets the separation channels, the anions traveled toward the anode, whereas the cations traveled toward the cathode in different channels with separate outlets. A sample containing rhodamine 123 (cation), 6-[fluorescein-5(6)-carboxamido]hexanoic acid (anion), and fluorescein (anion) was separated in the individual channels, and the analytes were detected only in the appropriate channels, indicating that the ions were successfully ''extracted.'' Additional experiments with a positively charged peptide (TMRIA, tetramethylrhodamine-5-iodoacetamide dihydroiodide) and negatively charged proteins (bovine serum albumin (BSA), casein, and avidin) showed similar results. Separation efficiencies were all greater than 300 and at least 87% of the ions were extracted.

2.4 Other designs

In the study by Kawamata et al., a new design was investigated that employs electroosmotic pumping for particle separation and collection [31]. The method, termed pinched flow fractionation, uses a microdevice with two inlets and five outlets. With the method, flow rates can be controlled and adjusted by varying the voltages of the inlets and outlets of the channels. Using the multi-channel scheme, 0.50–3.0 μm diameter particles were separated.

Baker and Roper presented an article on the development of a glass microfluidic chip that incorporated continuous electrophoretic separation of an amino acid mixture followed by collection of the species [32]. The chip was designed with a separation channel, connected to seven small columns used for collection of the separated samples. At the end of the separation channel, there were two sheath flow channels and two shaping channels used to focus the separated species into the collection columns. It was demonstrated that the amino acids could be separated and detected after optimization of the device design with COMSOL Multiphysics.

3 Physical structure of the microdevice

Here, we will summarize the techniques that rely on the actual structure of the device for concentration enhancement

and separation. In some cases, a physical element was used to prevent samples from entering the channel, whereas in other instances, electrode placement and/or channel shape influenced the concentration enhancement.

3.1 Physical elements for trapping

As stated in Section 1, this review focuses on recently developed electrophoretic techniques on the microfluidic format that both concentrate and separate in free solution (in the absence of gels, particle packing, etc.). Recently, device designs have been developed that utilize physical features in the device to aid in concentration enhancement before separation. These chip modifications include valves, nanofissures, and ion-selective membranes.

An example of a nanofissure used for preconcentration is described by Yu et al. [33]. The poly(ethyleneterephthalate) (PET)-toner microfluidic device consisted of two mirror image V-shaped channel designs that were printed on transparency film with a laser printer (PET-toner chip). Between the mirror images was a 100-μm gap. Additional PET films were then laminated over the toner chip. The nanofissures were formed at the gap between the two mirror images. When potential was applied across the mirror image V-shaped channels, protein was concentrated in the gap. At the pH used in these experiments, the channels and nanofissures were negatively charged, causing them to be selective for cations. As the proteins were negatively charged, they were excluded from entering and were concentrated. An enhancement of 10^3- to 10^5-fold was achieved for a FITC-labeled protein in 8 min. Additional experiments using rhodamine B (positively charged) and fluorescein (negatively charged) demonstrated that there was no concentration increase of the positively charged species because it was allowed to travel through the nanofissures. Fluorescein, however, was not allowed to enter the nanofissures, and hence it was concentrated near the entrance of the structures. Further experiments with FITC-DSA and rhodamine B demonstrated that the device could be used for sample purification. The positively charged rhodamine B was allowed to pass through the nanofissures, whereas the negatively charged FITC-DSA was concentrated in front of the fissures.

Kuo et al. fabricated a PDMS chip for electrophoretic DNA separations that contained a PDMS valve to concentrate the DNA before electrophoresis [34]. The chip had a sample reservoir, buffer reservoir, buffer waste reservoir, a DNA preconcentration area (nanoscale channel), a valve, and a separation channel. The valve was connected to an area linked to a pneumatic pump. For preconcentration, DNA was first introduced into the reservoir and then potential was applied between the sample reservoir (ground) and the buffer waste (anode). During this step, the DNA migrated toward the anode, but the valve remained closed, and thus the DNA became concentrated in front of the closed valve. After the DNA was concentrated, the normally closed valve was opened via pneumatic suction and the concentrated DNA flowed to the separation channel. The valve was then closed, potential was applied between the buffer reservoir (ground) and the buffer waste (anode), and separation of DNA occurred according to size in the separation channel. Laser-induced fluorescence (LIF) detection was used, and a 3750-fold enhancement of all DNA fragments (initial concentration of 5 μg/mL) was achieved in under 2 min of preconcentration time. Separation of eleven DNA fragments took approximately 2.8 min in a 40 mm separation channel. Preconcentration times for separations ranged from 20 to 100 s. Signal enhancement was observed for all preconcentrated fragments and an inverse relationship between concentration time and separation time was observed.

Finally, a proteomic sample electrophoretic preconcentrator using PDMS and a surface patterned ion-selective membrane was developed by Lee et al. [35]. A thin-printed Nafion membrane was integrated between a PDMS chip and a glass substrate to create a simple means for preconcentration. The ability of the chip to concentrate species was dependent on the voltage difference across the sample channel, with higher voltages resulting in larger preconcentrations. The chip was able to concentrate β-phycoerythrin almost 1000 times in 5 min.

3.2 Physical structure used to define local fields

Borofloat glass microfluidic chips were used to separate and trap particles of interest in two different types of channels [8]. One study used straight channels with a uniform diameter to better understand the behavior of the particles, followed by the use of elements with converging and diverging dimensions. The technique presented is referred to as flow-induced electrokinetic trapping (FIET), and particles were trapped with pressure-induced flow, electroosmotic flow (EOF), and their electrophoretic motion. In both channel designs, the cathode was at the inlet and EOF transport was toward the inlet, whereas pressure-induced flow was in the opposing direction. Experimental data were gathered with polystyrene microspheres that were similar in size but had varying ζ-potentials. All particles had a negative ζ-potential, and hence their electrophoretic migration was opposite to EOF. Particles with a given ζ-potential were trapped, whereas those with a higher ζ-potential were carried through the device by EOF. In the channels with converging and diverging elements, most trapping occurred in the diverging areas. The main advantage of this technique is that no physical barriers were needed for particle trapping.

The Henry group at Colorado State University implemented an expanded detection area, or a bubble cell, during electrophoretic separations with contact conductivity detection [36]. The bubble cell allowed for increased separation field strengths, which lead to shorter separation times. Initial experiments included testing the separation efficiency with fluorescein, followed by experiments with inorganic anions. Results indicated that separation efficiency remains statistically the same with or without the bubble cell three times the

diameter of the capillary. Bubble cell size was also investigated using sulfamate, percholorate, and iodate. Results indicated that as the size increased above four-fold, separation efficiency decreased proportionally with the bubble size. Among other experiments, three-fold bubble cells were used with dilute background electrolyte concentrations, allowing for field-amplified stacking. LODs for dithionate (9 ± 1 nM), perchlorate (22 ± 5 nM), and sulfamate (44 ± 10 nM) were lower than the nonstacked methods.

Discontinuous bipolar electrodes (BPEs) were used for both concentration and separation in a glass/PDMS microfluidic device [37]. In this technique, anions were both concentrated and separated when their electrophoretic velocities were equal to and opposite of the EOF. Once immobilized, the focused species were then moved through the channel. Fluorescence was used to determine the concentration enhancement of BODIPY disulfonate. The electric field was monitored in each experiment to ensure that it remained constant and to determine where and how much concentration takes place. Current was monitored through the bipolar electrodes, noting that when current increased, concentration enhancement began. Concentration increased approximately 70 times in 180 s. Once the anion was concentrated, it was directed within the channel by switching the electrodes where potential was applied.

A technique used for sample concentration in a straight closed-end microchannel is presented by the Li group from the University of Waterloo in Canada [38, 39]. The device was fabricated with a straight channel connected by two reservoirs and three electrodes. Two electrodes were placed at the ends of the reservoirs, whereas the remaining electrode was located at the exit of the first reservoir/entrance of the channel. EOF and fluid velocity variation at the closed end of the channel all contributed to fluid movement. Daghighi and Li presented a theoretical model and experimental data on separation and concentration in the microchannel [38]. Initially, potential was applied to the device so that species were collected and concentrated near one end of the channel. After concentration, a different potential scheme was applied, causing collected species to migrate down the channel and separate based on their electrophoretic mobilities. After theoretical studies were conducted, two types of DNA molecules were concentrated and separated. The combined processes of concentration and separation took just over 200 s, with a concentration increase of over 90 times in 115 s. Using the same channel design, Jiang et al. also described the concentration and separation of a fluorescent dye experimentally and theoretically (Fig. 4) [39]. Similar results were obtained as concentration enhancements of 90 times were achieved in 110 s.

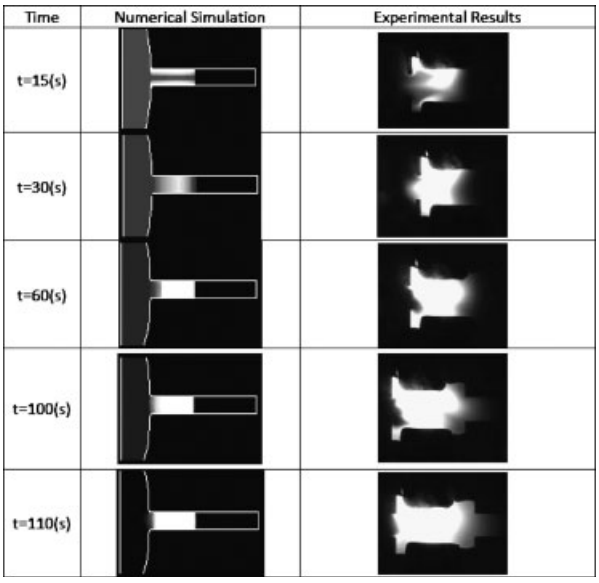

Figure 4. Comparison of the concentration of fluorescent dye molecules near an electrode in a straight microchannel between simulated (left) and experimental results (right) with increasing times of applied potential. In both cases, the fluorescence intensity increases as the time of applied potential increases.

4 Solution properties influencing local field

Designs that allow for sample separation when an electric field acts directly on the analyte of interest and methods that allow for sample concentration and separation based on some physical features of the device were described in the above sections. In this section, IEF and isotachophoresis (ITP) and its variations will be described. These two formats exploit solution properties to influence the electric fields.

4.1 IEF

IEF is an electrophoretic technique that is used to separate and concentrate molecules, most notably proteins and peptides [3]. Proteins and peptides are examples of amphoteric molecules, or those that are either positively or negatively charged depending on the pH of the solution. During operation, a pH gradient is created in the separation channel, and when potential is applied, the amphoteric molecules move under the influence of the electric field until they reach their isoelectric points (p*I*s), or the pH at which they have a net neutral charge. At this point, species are focused. This technique has been adapted to capillaries (cIEF) [40] and microdevices [1]. Capillary IEF designs from the last 2 years utilized capillaries in the tens of centimeters range [41, 42], falling outside the scope of this review.

Although there are several applications of IEF in microdevices in the last 2 years, these designs still use channels whose lengths are in the centimeter range [43–48]. Shimura et al. presented a microfluidic chip for IEF that incorporated a valve for loading each solution in individual channels, followed by selective injection, to eventually be incorporated with sample preparation before IEF separations [48]. Four tetramethylrhodamine-labeled peptide p*I* markers were used to test the chip and focusing took from 2

to 4 min, depending on the marker. A comparison between a glass and a PDMS chip design for separation of allergenic whey protein was outlined by Poitevin et al. out of Paris [47]. Two coatings, hydroxypropyl cellulose and poly (dimethylacrylamide-*co*-allyl glycidyl ether), were tested on both chips, and it was determined that glass chips coated with hydroxypropyl cellulose resulted in the best IEF separations. Ou et al. presented an article on a hybrid microfluidic device for IEF, using ultraviolet whole-channel image detection (UV-WCID) [46]. This design was intended to eliminate the step of placing in a metal optical slit when using whole-channel detection. It was determined that the device successfully separated p*I* markers and protein samples of myoglobin and hemoglobin and the fabrication process was more simple and less costly than the chips typically used for whole-channel detection. Chou and Yang performed IEF simulations using the space–time conservation element and solution element (CESE) and Courant–-Friedrichs–Lewy number insensitive CESE (CNI-CESE) for two different types of channels: one with a varying cross-width (contraction-expansion channel) and one with a constant width [43]. It was found that performing the simplified 1-D model was much faster than the previous 2-D simulations [49].

Cong et al. presented a modified IEF technique that changes the electric field strength during the separation process [44]. In the short communication, proteins from *Escherichia coli* were focused on a glass IEF microchip using a stepwise increase in electric field strength. Once proteins were separated, the electric field was decreased so that future increases in field strength could be incorporated later for more separations. This step technique resulted in better separations than standard IEF. Dauriac et al. also presented a modified IEF design [45]. The group developed a PDMS microfluidic device for separations containing PDMS micropillars. The micropillars were created as part of the original casting of PDMS and the pillar size and arrangement were studied. The separation of a mixture of seven proteins with p*I*'s ranging from 4.7 to 10.6 took less than 10 min. Although the pillars were part of the original PDMS casting, they behaved as a dilute gel; therefore the results were compared with IEF minigel electrophoresis. The minigel separations took 20 min, but resulted in less band broadening than the micropillar separations.

4.2 ITP

A common separation technique used for stacking is ITP [50]. In this technique, there are three different zones: a leading zone with higher mobility ions (LE, leading electrolyte), a sample zone, and a terminating zone of lower mobility ions (TE, terminating electrolyte). When a voltage is applied, an electric field gradient is created, and the field strength in each zone is inversely related to the ion mobility, which results in separated zones of ions of decreasing mobility. Each zone is defined by a sharp steady-state boundary, and these zones are sustained by the differing field strengths. Although ITP is a separation method, it is mostly used as a preconcentration technique for other electrophoretic methods. When ITP is used for preconcentration, it is referred to as transient ITP, or tITP. For a successful ITP enhancement, the ITP step must be completed (i.e. all of the analytes must be stacked) before the other separation technique is employed.

Nagata et al. investigated a modified form of tITP, or heterogeneous buffer combination, on a microchip [51]. In the method, the DNA sample is mixed with the TE, which contains taurine anions. As the mobility of the taurine ions is lower than that of the acetate ions in the LE, tITP occurs. In addition, hydroxyethylcellulose (HEC) is utilized in the LE buffer. Hydroxyethylcellulose is commonly used as a sieving matrix, but for this technique it is used to limit the diffusion of the sample plug. The separation length was 10 mm, which is three times shorter than the average microchip separation length and DNA ladders, where 10–100 bp were separated. The 10-bp ladders were separated within 60 s, whereas the 100-bp ladders were separated within 50 s and resolution was comparable to the chips with longer channels.

In the study by Goet et al., they developed a microfluidic contractor based on ITP [52]. It operates similarly to micromixers in that it brings samples into contact in order to assist chemical reactions, receptor–ligand interactions, or similar processes. However, micromixers typically use complex channel designs. This novel method utilizes two connected cross-style designs, and several types of experiments were performed. After characterizing the ITP zone transportation in a simple cross-channel chip, ITP zone synchronization was demonstrated using the more complex chip. Using the same sample at different injection sites, it was shown that the zones were able to merge using the method. Next, the group employed two different samples (bromophenol blue and fluorescein-Na). It was found that even if the fluorescein-Na enters the main chamber first, the bromophenol blue can overtake it (Fig. 5). Finally, to demonstrate the utility of such a device, the dyes were replaced with two complementary DNA oligonucleotide strands. To image the hybridization, one strand was tagged with a fluorophore, whereas the other was tagged with its corresponding quencher. Again, the zones appeared to overlap, and in this case, interact, resulting in the hybridization of DNA. Through these series of unique experiments, the group has demonstrated a simple means to bring samples into contact using an ITP microcontractor.

The group of Hirokawa from Japan contributed several new electrophoretic microchip articles, all investigating a method termed electrokinetic supercharging (EKS) [53, 54]. EKS is a preconcentration method that combines electrokinetic injection with tITP. In a 2008 contribution, Hirokawa et al. investigated a novel injection approach

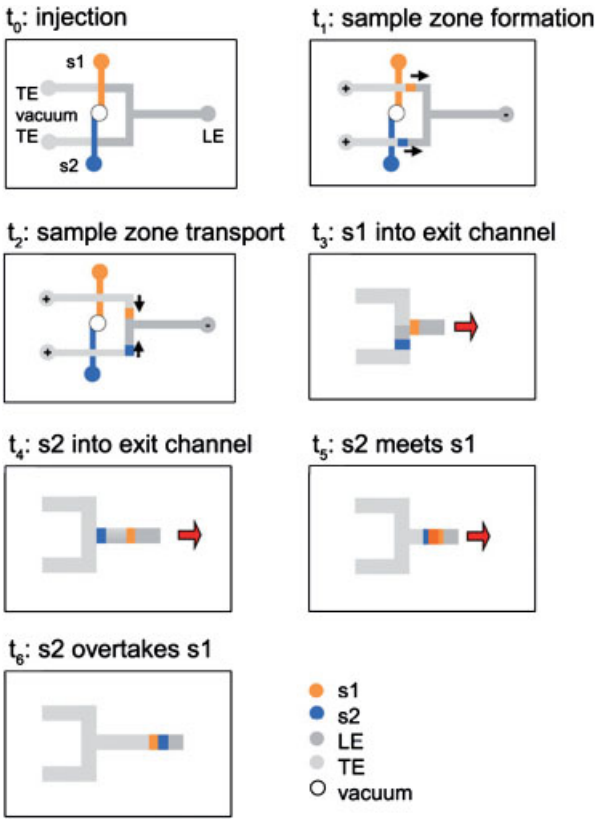

Figure 5. Example of two different samples (fluorescein-Na and bromophenol blue) contacting in a microchannel. The two samples enter the chamber from separate sample ports, and then come together in one separation channel.

using floating electrodes, termed floating EKS (FEKS) [53]. Standard Shimadzu electrophoresis microchips were employed; however, rather than pinched injection, two ports were utilized to facilitate a rapid switch from ITP to microchip gel electrophoresis (MGE). Models of the system were developed and examined using a mixture of 50-bp step ladder DNA. It was found that this strategy improved LODs ten-fold as compared with conventional pinched injection. Furthermore, resolution was improved over EKS-MGE from 0.77 to 1.62 for 50- to 100-bp DNA fragments and 0.89–1.32 for 200- to 250-bp DNA fragments.

The FEKS technique was further studied by Xu et al. [54]. In this contribution, the microchip for FEKS was modified so that the EKS concentration occurred in a curved channel with five U-shaped turns. This curved design allowed for longer ITP prior to MGE. Some modeling was performed, followed by experiments using DNA fragments. Overall, by extending the ITP steps by incorporating a curved channel, LOD was improved to 9.7, 5.0, and 5.5 ng/mL for 100, 300, and 500-bp DNA fragments, respectively. These LODs are a significant improvement over pinched injection EKS and cross-chip EKS.

Various aspects of microchip ITP are under investigation by the Treves Brown research group [55–58]. A novel means of sample injection is introduced for microchip ITP devices [55]. Although many sample injections are traditionally cross- or double-T configurations, their work presents a modified four-channel injection. The injection scheme has a wide bore sample loop and narrower side arm channels for separation and injection. The device enables variable volumes to be delivered, including smaller volumes (for highly concentrated samples) or larger volumes (for dilute samples). Another ITP microchip modification introduced by the Treves Brown group includes a low-cost, robust polystyrene chip that includes both integrated drive and detection electrodes [57]. The microchip design contains polystyrene as well as 40% carbon fiber-loaded polystyrene electrodes. These electrodes are utilized to drive the separations and for conductivity detection.

Other ITP microchip research by the group includes detecting magnesium as well as chlorate, chloride, and perchlorate anions in inorganic explosive residues [56, 57]. For the magnesium studies, various complexing agents were employed in the LE, which impacted the mobilities of the cations. It was determined that malonic acid was most effective as a complexing agent in microchip ITP for magnesium [56]. In order to analyze chloride, chlorate, and perchlorate, various electrolytes were investigated. These ions are difficult to analyze with ITP because of their very high electrophoretic mobilities (often making them suitable LEs). In order to overcome these challenges, a nitrate-based LE was employed with indium (III) and α-cyclodextrin as complexing agents. Inorganic explosive residues were analyzed with the method, and the results obtained were confirmed with ion chromatography [57].

The Santiago group has published numerous interesting articles, investigating electrophoretic methods on a microchip from 2008 to 2010 [59–77], including several that contribute to the theoretical basis of the technique [59, 64, 67, 69–72, 75, 76, 78] and some novel approaches for indirect detection [66, 77]. A selected group of these works were chosen for discussion here. In a 2009 contribution, his group demonstrated an ITP method capable of purifying nucleic acids from whole blood [60]. The LE and TE are chosen based on their compatibility with the contents of blood lysate. Figure 6 shows how the nucleic acids are focused while the proteins and other blood lysate contents move slower than the ITP interface. The nucleic acids were collected and interrogated with PCR to ensure that the fractions were purified DNA. The efficiency of the method is comparable to other microchip purification methods, obtaining 100% efficiency for λ-DNA, and between 30 and 70% for whole blood.

In another contribution, the group investigated simultaneous preconcentration and separation of analyte zones in ITP without the use of spacers or further separation steps [68]. Their studies show that carbonate ions formed from dissolved atmospheric CO_2 and carbamate ions formed from the CO_2 and its reactions with primary and secondary amines in the buffer create zones during ITP separation. The carbonate ions were found to interfere with the ITP, resulting in a broadening of the TE-LE interface. Although

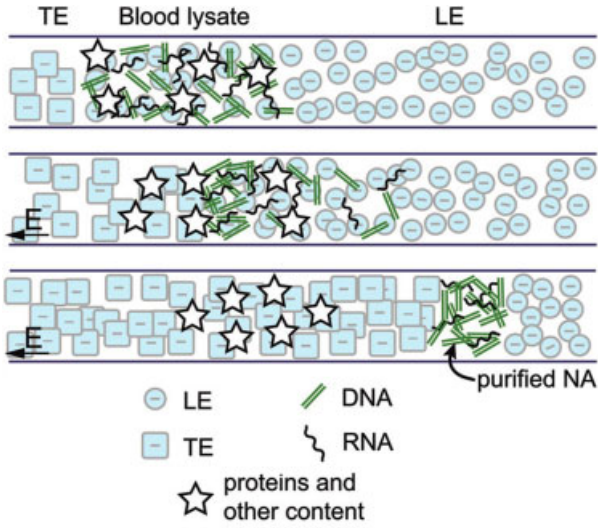

Figure 6. ITP schematic diagram showing a nucleic acid purification from blood lysate. The DNA is represented by rods, whereas the proteins and other content are signified by the stars. When an electric field is applied, the nucleic acids focus between the LE (circles) and the TE (squares), whereas the proteins move slower than the ITP interface.

such zones can have adverse effects on ITP preconcentration efficiency, the group demonstrated how these zones can improve on-chip CE. For example, by utilizing these zones, both preconcentration and separation can occur simultaneously without the need for further buffer exchange steps. The benefits of these zones were demonstrated on 25-bp DNA ladders and DNA ladders with green fluorescent protein and allophycocyanin.

Masar et al. presented a commercial PMMA column-coupling device design that couples ITP for sample concentration and CZE with contact conductivity detection [79]. The chip contained two separation channels and was used for the separation of cations commonly found in drinking water: ammonium, calcium, magnesium, potassium, and sodium. It was determined that the chip was able to sensitively and reproducibly separate the cations.

Wang et al. combined ITP with microcapillary electrophoresis (MCE) for the concentration and separation of BSA and its immunocomplex with mAb [80]. A PMMA microchip with a single cross-design was utilized in the study. Six different LEs and six different TEs were studied for their ability to enhance the ITP stacking. By employing tris-H_3PO_4 as a LE and *tris*-γ-aminobutyric acid, a 2000-fold enhancement of the BSA and mAB was obtained.

A microchip ITP method capable of analyzing highly saline PCR samples was investigated in a contribution by Wang et al. [81]. The method utilized the chloride ions in the PCR buffer to act as the LE and HEPES for the TE in a cross-style microchannel. Digested DNA samples and DL-2000 DNA markers were shown to have a 20-fold concentration enhancement. Overall, the technique increased the sensitivity of the PCR samples with no loss in resolution.

Qi et al. designed a microchip that combined ITP preconcentration with gel electrophoretic separation (ITP-GE) [82]. The chip contained a negative pressure sampler composed of a three-way electromagnetic valve and a single high-voltage power supply. The ITP step occurred in free solution, and the sample stacks between the LE and the TE in less than 1 s at the interface between the gel and the solution. The samples are then separated by gel CE. The apparatus was examined using DNA fragments. This ITP preconcentration with gel electrophoretic separation method, compared with microchip GE alone, was found to enhance sensitivity by 185 times.

The theoretical basis of different electrophoretic systems is examined by Chou and Yang [83] using a space–time conservation element assimilated with an adaptive mesh redistribution scheme (AMR-CESE). By assigning initial conditions, such as parameters of analytes, applied voltage, and grid size, the end time can be ascertained, as well as the concentration, pH, profile, and conductivity distribution within the channel. Three different electrophoretic techniques were investigated: ITP, IEF in an immobilized pH gradient, and IEF of a sample within ten background ampholytes. This novel adaptive mesh redistribution scheme-CESE technique was found to resolve points of discontinuity in the concentration distribution and compared with uniform mesh methods, fewer grid points were required for a given resolution.

Danger and Ross have developed a novel isotachophoretic approach termed gradient elution ITP (GEITP) [84]. In this method, a counterflow is applied that opposes the channel entrance and is slowly varied to selectively elute the LE, analytes, and TE into the capillary. Using the GEITP method, the group performed chiral separations with fluorescently labeled amino acid mixtures. Capillary lengths of 3 cm were employed, and various parameters including electrolyte pH, pressure scan rate, and chiral selector concentration were manipulated to achieve high-resolution separations. Studies from the Shackman group at Temple University have also investigated this technique [85, 86].

A technique based on GEITP was introduced by Ross and Kralj called gradient elution moving boundary electrophoresis (GEMBE) [87]. The method utilizes a buffer reservoir with sixteen 3-mm capillaries, each with individual sample reservoirs, and conductivity detection. This combines electrophoresis with a gradient counterflow to elute species into the capillary. To demonstrate the technique, the activity of protein kinase A and the inhibition of that activity by H-89 dihydrochloride were monitored. The GEMBE technique was further studied by Ross and Romantseva both theoretically and through experiments to optimize various parameters, including channel length, electric field, and counterflow acceleration [88]. Using various organic acids, LODs of the method were found to be in the low micromolar range. Although the basic method

was 10–20 times slower than CE, fast separations (less than 1 s) can be attained, and higher field strengths could be applied with modest voltages due to the short capillary lengths.

5 Concluding remarks

In this review, we have focused on elements that are essentially new or are significant advances in strategies that uniquely exploit microfluidic formats during the time span from January 2008 to summer 2010. These articles contributed new and valuable capabilities to the field by addressing issues such as long analysis times and poor LODs. These designs kept devices simple, allow for an array of sample types, and could be incorporated with several other aspects of analysis on a chip.

In examining the literature over this relatively short period of time, there were a large number of articles published in the area of ITP. This was predominantly driven by the Santiago group and was based on its sample preconcentration properties, as well as its feasibility to be incorporated on-chip. Closely related, but with a creative twist, GEMBE and GEITP used counterflow to preconcentrate while separating species of interest, much like the ingenious TGF technique. TGF, while rather brilliant in its inception, does have the disadvantage of being tied to specific buffer systems. On the contrary, IEF a long-standing and well-established technique and can also focus while separating; however, IEF has limited applicability with protein samples, due to their low solubility at their p*I*.

Another broad category of techniques, those we classify as relying on the direct interaction of the electric field with the analyte, has been used to successfully separate diverse samples. These techniques are obviously practical, as they can be used for many different types of mixtures. One popular method in this area – based on the numbers of contributions – is on-chip FFE, given its advantage of not requiring elution after separation. Its ultimate limitation (if it could be called that) is that analytes must be spatially separated after the separative/diffusive processes, resulting in possible design and size constraints.

Many designs described here use physical elements, such as valves, nanofissures, and variable channel geometries either to trap samples or to shape electric fields. Although these devices are effective at capturing species, they must be completely reworked to accommodate small changes. However, these methods are to be applicable to a wide range of samples and represent a truly unique microfluidic approach to separations.

We have presented and summarized a very selected group of electrophoretic techniques that uniquely exploited the microfluidic format or addressed a significant obstacle of the paradigm. We believe that these methods have the potential to affect a wide variety of research fields that require complex sample analysis. Overall, this research looks to address the challenges of applying basic attributes for separation sciences to create portable, fast, and low analyte-consumption devices for better biochemical analysis.

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6 References

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