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Research Article

Microchip free-flow electrophoresis on glass substrate using laser-printing toner as structural material

In this work, a microfluidic free-flow electrophoresis device, obtained by thermal toner transferring on glass substrate, is presented. A microdevice can be manufactured in only 1 h. The layout of the microdevice was designed in order to improve the fluidic and electrical characteristics. The separation channel is 8 μm deep and presents an internal volume of 1.42 μL. The deleterious electrolysis effects were overcome by using a system that isolates the electrolysis products from the separation channel. The Joule heating dissipation in the separation channel was found to be very efficient up to a current density of 8.83 mA/mm^2 that corresponds to a power dissipation *per* unit volume of running electrolyte of 172 mW/μL. Promising results were obtained in the evaluation of the microdevices for the separation of ionic dyes. The microfluidic device can be used for a continuous sample pretreatment step for micro total analysis system.

1 Introduction

In the early 1990s, Manz *et al.* [1] coined the concept of micro-total analysis system (μTAS), comprising the microfabrication of systems containing the main steps of a chemical analysis. Since then many researchers have attempted to develop miniaturized analytical systems for sample pretreatment, separation, and detection [2, 3].

In the research of sample pretreatment systems for μTAS, the free-flow electrophoresis (FFE) has been indicated as a promising technique [4]. FFE was introduced in the late 1950s and early 1960s [5, 6] as a preparative separation technique. It consists of a continuous injection of sample in a thin electrolyte solution film that flows across two parallel plates spaced by a small distance (less than 1 mm), known as separation channel [7, 8]. An electric field is applied perpendicularly to the flow in the separation channel in order to deflect the charged compounds in different angles according to their electrophoretic mobilities. The separated compounds can be continuously col-

lected as fractions at the separation channel outlets. FFE has been applied for separations and purifications of complex mixtures [9] containing proteins [10, 11], peptides [10, 12, 13], cells [14], and small ions [15]. FFE was also used as a continuous sample preparation step [16–18] for online or offline analytical determinations.

Raymond *et al.* [19] constructed the first FFE microdevice on a silicon substrate and separated three rhodamine-B isothiocyanate-labeled amino acids. In another work [20], the same microdevice was used for the separation of high molecular mass compounds. To work with larger electric field, silicon substrate was replaced by glass [21–23] that presents a higher breakdown voltage. The miniaturization of the FFE increases the speed of separation and minimizes the Joule heating, but the hydrodynamic and electroosmotic dispersions increase with the reducing of the separation channel thickness.

In microfabricated FFE devices, the current is higher than for a conventional CE, because the cross-sectional area of the separation channel is much larger than a usual capillary. The high currents make the electrolysis very critical because it quickly changes the pH of the running electrolyte [24] and generates gaseous products (O_2 and H_2) that cause electric field perturbation [23]. Generally, in

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conventional FFE devices, the separation channel is isolated from the electrodes bed by a membrane in order to minimize the electrolysis effects. Nevertheless, in microfabricated devices, it is very difficult to implement a membrane between these compartments. Commonly, arrays of narrow channels filled with free electrolyte solution are used to connect the separation channel to the electrode compartments. This approach presents some problems such as fluidic perturbation in the separation channel and high voltage drop in the connection channels.

In this work is presented a microfabricated FFE device manufactured by an easy and fast microfabrication technique, which uses glass as substrate and laser-printing toner as structural material [25, 26]. Additionally, the deleterious electrolysis effects were eliminated with the application of the separation potential by a system which isolates the electrolysis products from the separation channel.

2 Materials and methods

2.1 Reagents, solutions, and polyacrylamide gel preparation

All reagents and solvents were of analytical grade or better and were purchased from Merck (Darmstadt, Germany). Deionized water was produced by a Nanopure UV system (Barnstead, Dubuque, IA, USA). Stock solutions (10 mM) of ionic dyes (methylene blue (MB), violet crystal (VC), brilliant blue, and bromophenol blue) were prepared by dissolving the solid reagent in deionized water. The sample solutions used in the FFE separation experiments were prepared by diluting the dye stock solutions with deionized water as required.

Cross-linked polyacrylamide gel was prepared by mixing 200 μL of a stock solution containing acrylamide (30% v/v) and bisacrylamide (2.67% v/v) with 10 μL of ammonium persulfate (initiator) and 1 μL of tetramethylethylenediamine (catalyzer). The mixture needs to react for 2 min before the application.

2.2 Microfluidic FFE device fabrication

The FFE microdevice was constructed on a glass microscope slide (26 mm × 76 mm × 1 mm) covered by a thin glass slide (18 mm × 18 × 0.15 mm), both purchased from GlassTécnica (São Paulo, SP, Brazil). Before toner deposition, the surfaces of the glass slides were chemically etched to increase the roughness, and consequently enhance the toner adherence. The substrate and the

cover glass were coated with a paste containing ammonium fluoride Matt Glass (Auge Silk & Sign, Brazil), which is commonly used to write on glass surface. After 5 min, the paste was removed with a water stream and the glass slides were dried on a hot plate (50°C).

The fabrication process, involving thermal toner transferring on glass substrate (toner-glass), was described in details elsewhere [26]. The main steps of the toner-glass microfabrication process are depicted in Fig. 1. Briefly, the layout is drawn with the software CorelDraw 7.0 (Corel, Ottawa, Canada) and laser-printed on a waxed paper that remains after labels are removed from sheets for ink jet or laser printers. A printer HP LaserJet 1300 (Hewlett-Packard, Palo Alto, CA, USA) was used, operating at 1200 dots *per* inch (dpi) in the raster mode.

The toner layer is transferred to the glass substrate with a heating press HT2020 (Ferragini, São Carlos, SP, Brazil) by heating for 2 min at approximately 100°C under pressure (0.1–0.2 MPa).
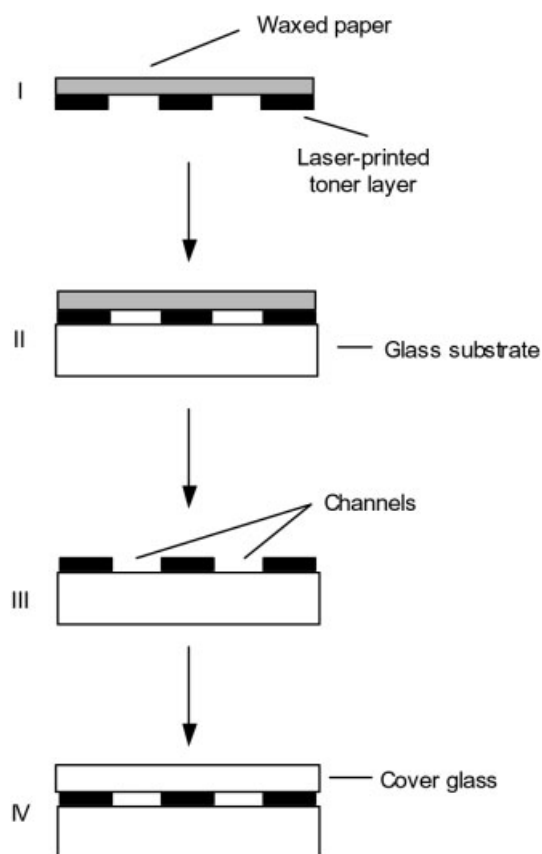

Figure 1. Diagram of the main steps involved in the toner-glass microfabrication process: (I) the layout is drawn and laser-printed on a waxed paper; (II) and (III) the toner layer is thermal transferred on the glass slide; and (IV) the cover glass is welded, leaving accessible the ends of the channels.

After depositing the structural toner layers, the cover glass is welded over them in order to seal the microdevice. This is done using the same hot press under the same conditions as the ones used for the toner transfer. The channels are accessed at the edge of the cover glass. Solution reservoirs were formed with epoxy glue or by gluing tubes made of polyethylene at the channel entrances. Epoxy glue presents a good adherence on the glass substrate.

2.3 Instrumentation setup

A simplified diagram of the instrumentation setup is shown in Fig. 2. A voltage source commonly used in gel electrophoresis was used to provide the separation voltage. This source supplies a maximum voltage and current of 500 V and 100 mA, respectively.

The pumping of the running electrolyte and the sample solutions through the separation channel was performed by connecting the outlet reservoir of the microdevice to the air entrance of a small air compressor, commonly used for oxygenation of aquarium water. In this configuration the compressor works as a vacuum pump. The flow rate of the solutions can be controlled by a valve that adjusts the pressure reducing in the outlet reservoir. This simple implementation permits a stable flow and an easy and precise flow rate control. Furthermore, it eliminates the need for a syringe or peristaltic pump. In all the experiments with the microfluidic FFE device the pumping system was adjusted to provide flow rates of 5.1 and 0.52 μL/min for the running electrolyte and sample solutions, respectively.
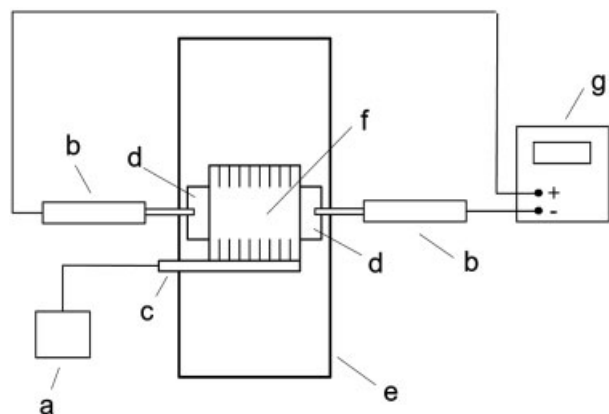
Figure 2. Diagram of the instrumentation setup used in the evaluation of the microfluidic FFE device: (a) air compressor; (b) MSE; (c) FFE microdevice outlet reservoir; (d) side reservoirs; (e) glass substrate; (f) separation channel; and (g) voltage source. The sample and running electrolyte reservoirs were omitted for sake of simplicity.

As mentioned, the electrolysis is critical in FFE and in microfluidic version the problems are enhanced, because the reservoir volumes are around a few microliters. In order to overcome the electrolysis effects, the separation potential was applied in the microdevice by a module for separated electrolysis (MSE), which separates the electrolysis products from the separation channel. The concept of the MSE was explained with details elsewhere [27], and the diagram of the module is shown in Fig. 3. A platinum wire perforates the rubber of an original syringe plunger and is bonded to the voltage source cable. The syringe barrel is filled by suction with the same electrolyte solution that flows in the separation channel. The MSE end is positioned on the side reservoirs of the microdevice. During the separation running, the electrolysis products are generated inside the MSEs and do not reach the separation channel for a short and medium term.

A CCD camera GP-KR222 (Panasonic, Japan), with a 2 × objective, was used to acquire images of the separation channel during the separation experiments. As the dyes are blue colored, the microfluidic device was illuminated with red light to increase the contrast. The red light was obtained by adapting a spectrophotometer red filter to the optic fiber end of the light source Fiber-Lite (Dolan Jenner Industries, USA). Image processing was carried out using the software Gimp 2.0.3 and Mathcad 2001 Professional (Mathsoft).
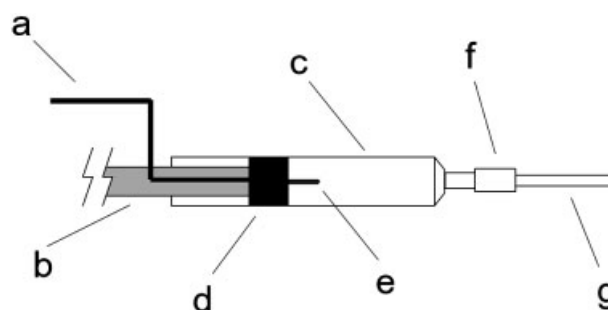
Figure 3. Diagram of the MSE used to apply the separation voltage in the microfluidic FFE device: (a) voltage source cable; (b) syringe plunger; (c) 1-mL syringe barrel; (d) syringe plunger rubber; (e) platinum wire; (f) silicone tube bushing; and (g) polyethylene tube (0.8 mm id).

2.4 Electric characterization

Electric resistances of the MSEs, separation channel, and the polyacrylamide gel film were measured by impedance analysis. The MSEs and separation channel were filled with a 30-mM sodium borate buffer solution, and an impedance analyzer HP 4194A (Hewlett-Packard) was used to obtain impedance spectra in a frequency range

from 100 to 10 kHz. The resistance of the polyacrylamide gel film was determined by subtracting the resistances of the microdevice with and without application of the gel.

To evaluate the Joule heating dissipation of the microfluidic device, a curve of current *versus* separation voltage (ohmic curve) was obtained while a 30-mM sodium borate buffer solution was pumped through the separation channel in a flow rate of 5.1 μL/min. The ohmic curve was obtained by increasing the separation voltage in 50-V steps, and the currents were measured by a digital multimeter DM 311 (Goldstar, Korea) coupled in series with the electric circuit.

3 Results and discussion

3.1 Toner-glass microfabrication

The toner-glass microfabrication technique is easy to carry out, because it involves simple handling and instrumentation. The whole microfabrication process takes around 1 h for each device, and more than one microdevice can be manufactured at the same time. Indeed, this time is much smaller than ones involving lithographic steps. The simplicity and quickness of the microfabrication technique are very important to the development and optimization of the final layout presented in this work. According to earlier studies [26], after the sealing step, a single toner layer renders 8 μm deep channels. However, it was also demonstrated that deeper channels can be obtained by deposition of multiples of toner layers, although the alignment of the layers is a critical step.

The chemical resistance of the toner-glass FFE microdevices demonstrated that they are suitable for the usual aqueous solutions used in this separation technique. On the other hand, the mechanical resistance is low and positive pressure should not be used in the separation channel, because the thin cover glass tends to bent and the microdevice sealing can be damaged. However, the resistance for positive pressure could be increased by the augmentation of the width of the cover glass.

3.2 FFE microfluidic layout

The microfluidic layout and a photo of the microdevice are shown in Fig. 4. The layout is 18 mm long and 18 mm wide, which corresponds to the same dimensions of the cover glass. The FFE microdevice has 42 inlet channels for the running electrolyte and 43 outlet channels that are 5 and 3 mm long, respectively. All channels, including the sample inlet channel, present the same width of 200 μm. Although the inlet channels are connected to a common reservoir, the layout can be modified in order to allow the different solutions to be introduced in the separation channel. The same observation can be done for the outlet channels for collection of the separated fractions. The separation channel is 10 mm × 18 mm and is filled with a matrix of 370 dots (posts) that are 100 μm wide and spaced by 500 μm. These dots play an important role, because they avoid the collapsing of the separation channel caused by a pressure reduction in the outlet reservoir. The cover glass can be easily bent because it is very thin (150 μm). Of course, a thicker cover glass could be used to overcome this drawback. However, a slim cover glass is interesting for a future implementation of an online detection system such as a contactless conductivity detection [26, 28], for example. Considering that the deepness of the separation channel is 8 μm and subtracting the area corresponding to the matrix of points (1.61%), one can calculate the internal volume of the separation channel as 1.42 μL.

The running electrolyte and the side reservoirs were confectioned with epoxy glue and can contain 20 and 300 μL, respectively. The 10-μL sample reservoir is a polyethylene
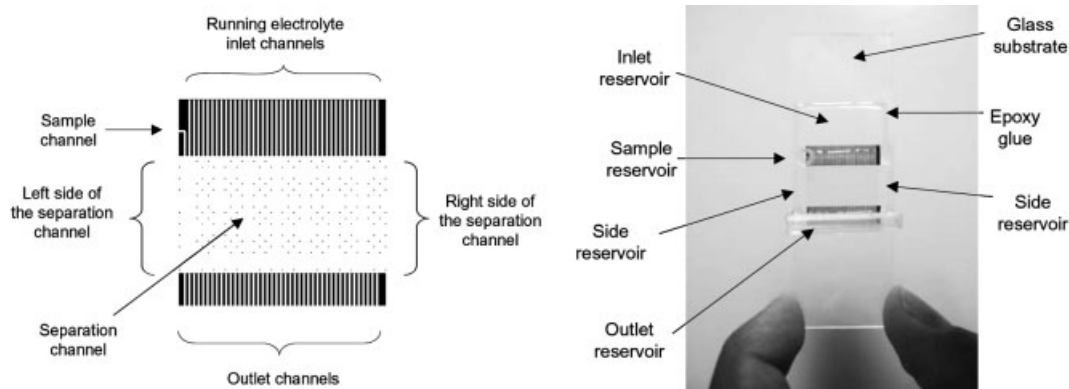

Figure 4. Layout and photo of the toner-glass microfluidic FFE device.

tube (3 mm id) glued by epoxy glue. The outlet reservoir was manufactured by cutting a rectangular (20 mm × 4 mm) aperture in a syringe needle cover made of polyethylene. This reservoir was glued so that the cut side is positioned in front of the outlet channel edge. The outlet reservoir is connected to the pumping system by a silicon bushing and a polyethylene tube.

The sides of the separation channel are connected to the side reservoirs that are filled with cross-linked polyacrylamide gel, which prevents the overflow of the running electrolyte or sample solution.

The microfluidic device can be easily filled with aqueous solution by capillarity due to the hydrophilic characteristic of the substrate. However, some air bubbles can be trapped during this procedure. To avoid air bubbles, the microdevice was first filled with ethanol, which was subsequently replaced by the aqueous solution.

3.3 Electric characterization

For a 30-mM sodium borate buffer solution as running electrolyte, the electric resistances of the MSEs, separation channel, and polyacrylamide gel film were found to be 136, 310, and 3.3 kΩ, respectively. These figures indicate that 69% of the total circuit resistance is placed in the separation channel, while 30.3% is due to the MSEs, and only 0.7% is attributed to the gel film. Even with the electric resistance of the MSEs, 69% of the potential applied by the voltage source is used to generate the electric field along the separation channel. This is the better potential for a microfluidic FFE device reported in the literature. The layout permits using crosslinked polyacrylamide gel to isolate the separation channel from the side reservoirs instead of an array of narrow microchannels. The electrical resistance of the gel film is smaller than that one for the microchannels, which decreases the voltage drop between these compartments. This low voltage drop allows one to use a low voltage power source to obtain electric fields enough for FFE separations. In this work, the maximum voltage supplied by the source was 500 V. However, this voltage can generate an electric field along the separation channel of *ca.* 192 V/cm, which is enough to separate many compounds by FFE.

The magnitude of the electric field in FFE is limited by the Joule heating caused by the high currents involved. The miniaturization of FFE decreases the cross-sectional area of the separation channel and consequently the Joule heating is reduced because the involved voltages and currents are smaller.

No positive deviation of the linearity was observed for the ohmic curve up to 706 μA, which is the current generated for the maximum voltage supplied by the source (500 V). In this case, considering the sectional area of the separation channel as 0.08 mm^2 and the internal volume as 1.42 μL, the current density and the power dissipation *per* unit volume of running electrolyte are 8.83 mA/mm^2 and 172 mW μL, respectively. According to these results, one can say that the Joule heating dissipation is efficient. It is worthwhile to note also that the electrolysis did not affect the ohmic curve, even for high currents, as it was observed for a FFE microdevice reported in a recent work [23]. The MSEs used for the application of the separation potential efficiently avoid the electric field perturbation caused by the gas bubbles generated by the electrolysis of the running electrolyte. The FFE microdevice was evaluated during 3 h under the maximum current (706 μA) and no problems with electrolysis was observed. For the FFE microdevices reported in the literature, the solution in the side reservoirs is frequently renewed by pumping to minimize the electrolysis effects. By using the MSEs, the pumping system is simplified, because it is used only to provide the running electrolyte flow in the separation channel.

3.4 FFE separations

The FFE microdevice was evaluated for the separation of ionic dyes. Although many compounds could be separated in the microdevice, dyes were chosen because their streams in the separation channel can be easily seen without a fluorescence microscope apparatus.

The flow rate of the running electrolyte and the sample solution were 5.1 and 0.52 μL/min, respectively. The sample linear speed in the separation channel was 0.54 mm/s and the residence time was 18.2 s.

Figure 5 shows images of the separation channel during the separation of the cationic dyes MB and VC. In the absence of electric field, no deflection of the sample stream, on the left side of the separation channel, is observed. Generally, in FFE microdevice with microchannels between the separation channel and the side reservoirs, the sample stream is shifted even in the absence of electric field due to the solution linkage between these compartments. After the separation voltage is applied (300 V), the sample stream bent towards the negative potential (right side of the separation channel) and quickly splits into two streams owing to the difference of electrophoretic mobility between the dyes. By comparing the color tonality, it was found that the MB stream was deflected in a larger angle which indicates its higher electrophoretic mobility. In FFE, it is well known that the
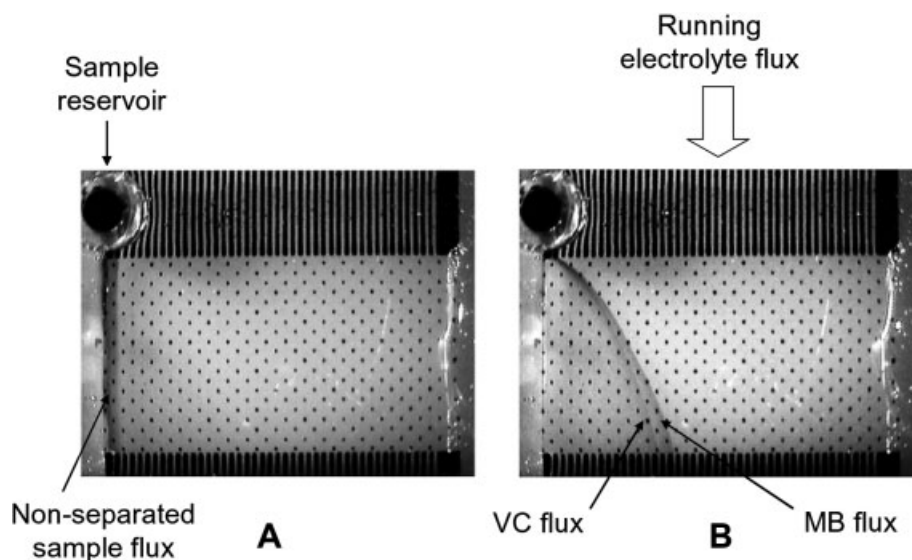
Figure 5. Images from the separation channel in the absence (A) and under a separation potential of 300 V (B). The sample reservoir was filled with a solution containing 3 mM of MB and 1.5 mM of VC. Running electrolyte was a 20-mM sodium acetate buffer solution (pH 4.7). The positive potential is on the left side of the separation channel.

laminar flow profile in the separation channel causes a band broadening that affects the separation resolution. Besides the hydrodynamic distortion, an additional flux broadening was observed for the cationic dyes, due to an adsorption of the dyes on the walls of the separation channel. This adsorption is enhanced by an electrostatic interaction between the positive charged dyes and the glass surface that is negatively charged owing to its partially dissociated silanol groups. A pH reduction of the running electrolyte (sodium acetate buffer, pH 4.7) could be used to minimize this adsorption. However, the color intensity of the dyes tends to decrease with the pH diminution, because the dyes are acid-base indicators.

Figure 6 shows images from the separation channel for the separation of the anionic dyes Bromophenol blue and brilliant blue for different separation potentials. The brilliant blue dye was found to have the highest electrophoretic mobility. It is worthwhile to remark that the brilliant blue is a three-charged anion at pH 9.3, while bromophenol blue is double-charged. The flux broadening for the separation of anionic dyes was much less than that for the cationic dyes. It can be explained by the absence of electrostatic attraction between the dyes and the walls of the separation channel.

The pixels of the separation channel images were converted to a matrix of integer numbers that are a grayscale version of the image. The rows of the matrix corresponding to the region near the outlet channels were plotted *versus* the separation channel position, providing plots similar to electropherograms. Figure 7 shows one of the plots that were used in the calculation of the separation resolution. As expected, the separation resolution

increased with the separation potential and became satisfactory for potential larger than 100 V as shown in Fig. 8.

The magnitude of the EOF in the separation channel is comparable to the silica capillary. However, no negative influences such as flux broadening or distortion were observed in these separations.

The FFE microdevice ability to separate ionic species was demonstrated. With a small modification in the microdevice layout, it is possible to collect the separated fractions or couple the microdevice to a detection system.

4 Concluding remarks

The microfabrication technique, using thermal toner transfer on glass substrate, was demonstrated to be easier and faster than those involving lithographic steps. The layout of the FFE microdevices does not use narrow microchannels to isolate the separation channel from the side reservoirs, where the potential separation is applied. A cross-linked polyacrylamide gel was used for this goal with lesser voltage drop. Thus the separation voltage is better used to generate the electric field along the separation channel. The MSEs used to apply the separation voltage work efficiently to overcome the negative effects of the electrolysis, which are critical in electrophoresis separation, especially in FFE.

The Joule heating dissipation of the device was found to be efficient, because no deviation in the ohmic curve was observed. The separation of the ionic dyes demonstrated that the microfluidic FFE devices could be applied for the
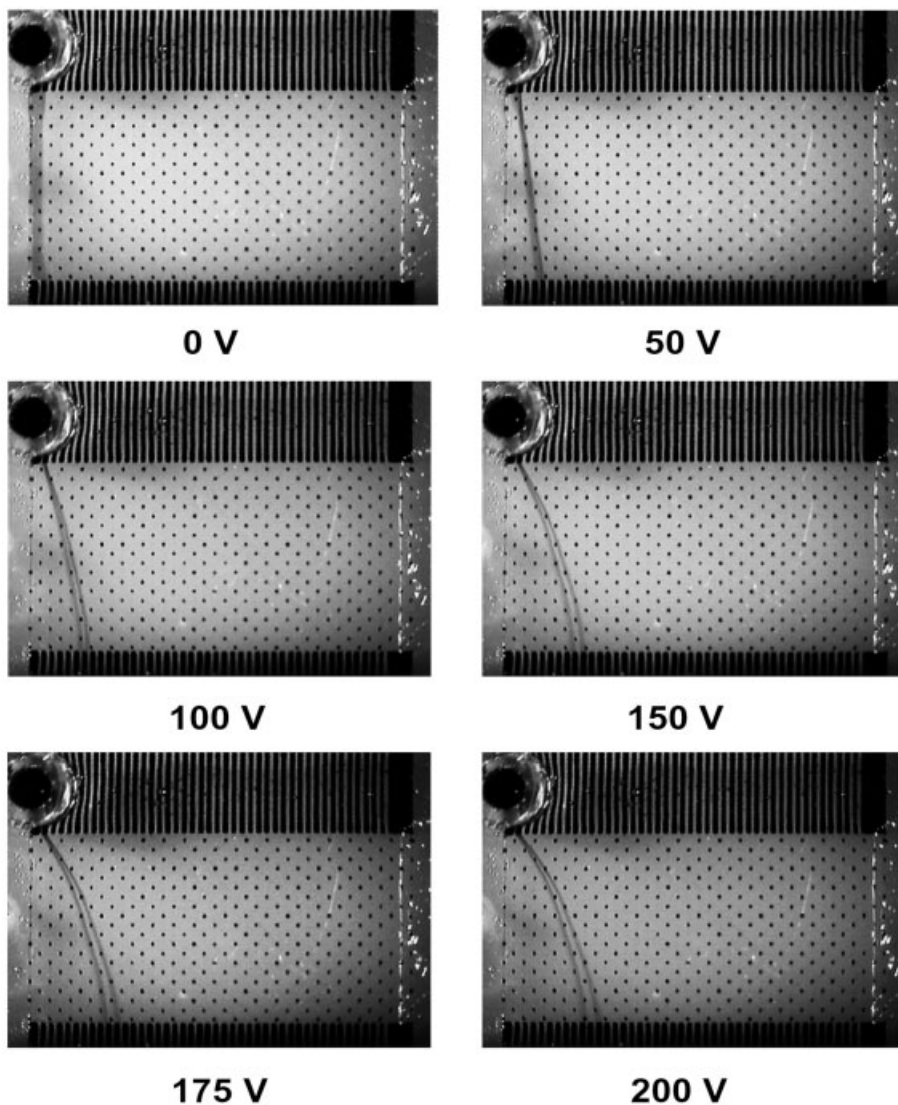
Figure 6. Images from the separation channel for the separation of bromophenol blue (2 mM) and brilliant blue (2 mM) under different separation potentials. Running electrolyte was a 30-mM sodium borate buffer solution (pH 9.3). The positive potential is on the right side of the separation channel.
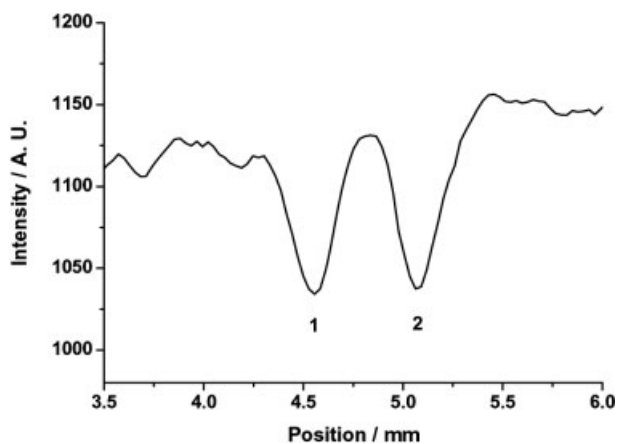

Figure 7. Pixel intensity *versus* separation channel position near the outlet channels for the separation of Bromophenol blue (1) and brilliant blue (2). Separation potential was 200 V.
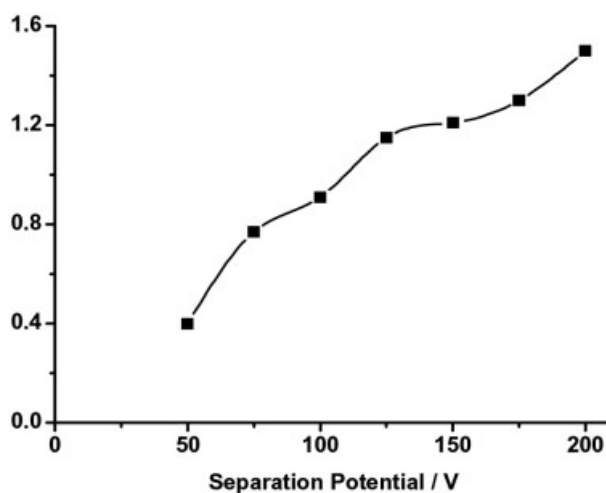

Figure 8. Resolution *versus* separation potential for the separation shown in Fig. 6.

fractionation of real samples. A layout adaptation for the fraction collection allows the microdevice to be used in a μTAS system as a continuous sample pretreatment step.

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