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Research Article

Effects of separation length and voltage on isoelectric focusing in a plastic microfluidic device

This paper describes the investigation on the effects of separation length and voltage on IEF in a plastic microfluidic device. A LIF, whole-channel imaging detection (WCID) system was developed to monitor proteins while they were moving under an electric field. IEF was carried out in a separation medium consisting of carrier ampholytes and a mixture of linear polymers (hydroxyethylcellulose and hydroxypropylcellulose). We found that the IEF separation resolution is essentially independent of separation length when the same voltage is applied, which agrees with the theory. This result supports the notion that IEF in a microfabricated device leads to more rapid analysis without sacrificing the resolving power. A higher separation voltage also brought about more rapid analysis and superior separation resolution. IEF of two proteins (green fluorescence protein and R-phycoerythrin) was achieved in 1.5 min when 500 V was applied across a 1.9-cm channel. We found that a linear relationship exists between the focusing time and the inverse of the electrical field strength. In addition, we confirmed the phenomenon in which the pH gradient was compressed to the middle of a channel, and we found that the relative amount of the gradient compression decreased with the channel length.

Keywords: IEF / Microfluidics / pH gradient compression / Whole-channel imaging
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1 Introduction

IEF is an electrophoresis technique to separate amphoteric molecules (*e.g.*, proteins and peptides) that can be either positively or negatively charged [1]. IEF is carried out in a pH gradient, which can be created by carrier ampholytes or an IPG. Under an electric field, a protein migrates along the pH gradient and is eventually focused at a location where the pH value is equal to its p*I*, which is the pH at which the net charge of the protein is zero [1, 2]. It should be noted that a dynamic equilibrium exists between IEF focusing and diffusion, and it keeps the proteins remaining at their p*I* locations [3]. As many proteins have different p*I* values, they can be separated along a pH gradient.

IEF has been adapted to the microfluidic format due to potential advantages of miniaturization [4–15]. Possible advantages include less sample amount, faster analysis, higher separation efficiency, potentially lower cost, and the ability to integrate with other components including a detector. Hofmann *et al.* [4] evaluated three mobilization methods in conjunction with IEF in a glass device, and Yager's research group electrochemically generated pH gradients for IEF in a microchannel [5]. We reported the investigation of rapid IEF in a polymethylmethacrylate (PMMA) microfluidic device and applied it for studying protein–protein interactions [6], while Li *et al.* [7] described UV-initiated grafting of polyacrylamide coating in a device made from a cyclic olefin copolymer (COC). Cui *et al.* [8] discussed their use of methylcellulose to reduce electroosmosis and peak drift for IEF in a polydimethylsiloxane (PDMS) device, and Manz's research group demonstrated sub-second separation of

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Abbreviations: COC, cyclic olefin copolymer; **GFP**, green fluorescent protein; **HEC**, 2-hydroxyethylcellulose; **HPC**, hydroxypropylcellulose; **RPE**, R-phycoerythrin; **WCID**, whole-channel imaging detection

two proteins in a free-flow IEF bed [9]. In addition, coupling IEF with a different separation mechanism (*e.g.*, free-solution electrophoresis) in an orthogonal channel has been carried out for 2-D separations [10–12]. Efforts have also been reported that integrate IEF with multiple 2-D channels; these devices have a potential to perform better separation than conventional 2-D slab gel electrophoresis [13–15].

Except for those 2-D separations, detection of the focused proteins is required immediately after IEF. Recently, we developed an LIF whole-channel imaging detection (WCID) system, in which a laser beam was expanded into a laser line, and then simultaneously illuminated an entire IEF channel in a microfabricated device, followed by imaging by a scientific-grade CCD camera [16, 17]. Similar UV detection has been reported for capillary IEF by Wu and Pawliszyn [18].

In this paper, we describe the use of the WCID system for studying IEF in a microfabricated plastic device. The question we want to address is whether IEF resolution will be reduced by miniaturization. The theoretical prediction is discussed, followed by the experimental results of the effects of separation length on the IEF resolution. We also present the investigation on the effects of the separation voltage on IEF separation and the effects of the electric field strength on the IEF focusing time, as well as the confirmation of the phenomenon in which the pH gradient was compressed to the middle of a channel.

2 Materials and methods

2.1 Reagents and materials

Carrier ampholytes (pH 3–10 and 4–6) were purchased from BioRad Laboratories (Hercules, CA) while ethanolamine, 2-hydroxyethylcellulose (HEC, *Mr* 90 000, 150 cps at 5% w/w in water), and hydroxypropylcellulose (HPC, *Mr* 80 000, 150–700 cps at 10% w/w in water) were from Sigma-Aldrich (St. Louis, MO). Green fluorescent protein (GFP, 1 mg/mL stock) was obtained from BD Biosciences Clontech (Palo Alto, CA) and R-phycoerythrin (RPE, 10 mg/mL stock) was obtained from Cyanotech (Kailua-kona, HI). TEMED, glycerol, acetic acid, and microscope slides were purchased from Fisher Scientific (Atlanta, GA). All solutions were prepared using water purified from Barnstead Nanopure Water System (Model: D11911, Dubuque, Iowa). Solutions of 10 mM acetic acid and 10 mM ethanolamine served as the anolytes and the catholytes, respectively.

2.2 Device fabrication

Plastic microfluidic devices were fabricated using modified procedures described previously [6, 19, 20]. The patterns were designed using a computer-aided design (CAD) program (AutoCAD, San Rafael, CA). Each pattern was designed to fit on a standard $3'' \times 1'$ microscope slide as shown in Fig. 1a. The CAD file was sent to the vendor (Photo Sciences, Torrance, CA) and a photomask was created. Conventional photolithography and chemical etching were used to create channels in a glass microscope slide. A layer of metal is then electroplated onto the surface of the glass plate, creating a metal mold that has inverse topology relative to the original microstructures. The metal mold is then mounted on a hydraulic press to produce plastic parts that bear the same features as the original. Hundreds of parts can be made from the metal mold. A device is completed by sealing the plastic substrate with a cover film using thermal lamination.

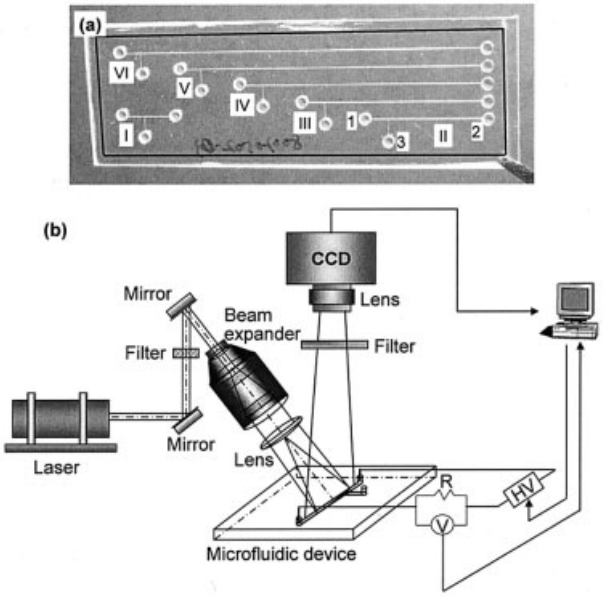

Figure 1. (a) Picture of a microfluidic device consisting of six IEF channels with different separation lengths. The device was made from COC. The size of the device is $1' \times 3''$ and all channels are 74 μm wide and 27 μm deep. (b) Schematic of the experimental setup. A laser was transformed into a line beam using a beam expander and cylindrical lens as discussed in the text. The entire IEF channel in the microfabricated device was illuminated by the line beam during IEF of proteins. The fluorescence emission from proteins was collected by a CCD camera. The electric current in the IEF channel was monitored by a voltage drop (V) through a resistor (R) while a high voltage (HV) is applied to the system. The adjustment of HV, current measurement, and the collection of emission signals were carried out by a computer *via* software written in Labview.

In Fig. 1a, there are six IEF channels labeled I–VI. Three wells in channel II are numbered for referencing wells. The lengths of the section between wells 1 and 2 for IEF channels I–VI are 0.8, 1.9, 3.0, 4.1, 5.2, and 6.3 cm, respectively. We primarily used channels II–V for most of the experiments due to their ease in fitting within the optical field of view. The junction connected to well 3 in each channel is 0.3 cm away from well 1. Well 3 was designed for possible introduction of sample or other reagents, but it was not used in this work. All channels are 74 μm wide and 27 μm deep.

2.3 Instrument setup

An LIF WCID system has been described elsewhere [16, 17]. The system and the measurement of electric currents are illustrated in Fig. 1b. An Ar-ion laser (488 nm, 30 mW, JD Uniphase) is directed by mirrors, passes through a beam expander (HB-20X from Newport), then converts into a line beam by a cylindrical lens. The line beam simultaneously illuminates an entire channel in a microfluidic device, which is mounted on an XYZ translation stage for accurate control of beam positioning. The fluorescence emission is collected by a cooled, scientific-grade CCD camera (Apogee) after passing through band pass filters (HQ585/40, Chroma Technology, Rockingham, VT). The CCD array has 2184×1472 pixels (14.9 mm × 10.0 mm); the pixel size is 6.8 μm × 6.8 μm. The field of view is controlled by a camera lens on the CCD. The exposure time of the CCD camera was 4 s, with a time interval (typically 30 s) between sequential images. A high-voltage power supply (Glassman High Voltage, High Bridge, NJ) was used for IEF and controlled by a computer using software written in Labview (National Instrument, Austin, TX). Continuous monitoring of current was achieved by measuring the voltage drop across a 10-kΩ resistor connected in series with the ground electrode.

2.4 Experimental procedures

All experiments were performed in plastic microfluidic devices. The channels in the device were first cleaned with 1% (0.18 M) potassium hydroxide (KOH). After 5 min inside the channel KOH was flushed out with water. The channel was then filled with IEF separation medium consisting of 5% carrier ampholyte (pH 3–10) stock solution (at 40% supplied by the vendor), 10% glycerol stock solution (80% v/v in water), and 85% HPC/HEC stock solution (1.83% HPC and 0.73% HEC in water). A mock IEF was performed with all the constituents (including proteins) in the presence of an electric field, followed by flushing out with water. This step helped to block all the active sites in the surface of channel, thereby preventing protein adsorptions in future experiments, and helped conditioning the wall surfaces under an electric field. It has been reported that most plastics, including COC, possess much smaller EOF, typically a factor of five or so smaller than silica/glass [20]. EOF was further suppressed by dynamic coating of HPC/HEC contained in the IEF separation medium, as reported in the literature [21–23]. Nevertheless, we still observed a minute amount of EOF, especially when the electric field strength was high.

3 Theoretical consideration

Separation efficiency and resolution are two terms that have been extensively used for characterizing separation in HPLC [24, 25] and electrophoresis [26]. However, Giddings and Dahlgren [27] pointed out that separation efficiency, expressed in theoretical plate number, should not be used for IEF because a steady state is reached at the end of the focusing process and the peak variance is not proportional to the position in the IEF. Therefore, we will primarily rely on separation resolution to study the effects of experimental parameters on the separation.

Separation resolution (R) can be calculated by dividing the separation distance of two adjacent peaks by their average width, that is

$$R = \frac{(x_2 - x_1)}{\frac{1}{2}(w_1 + w_2)} \tag{1}$$

where x_1 and x_2 are the location of two peaks and w_1 and w_2 are the width of the peak at the base, measured between the tangent lines of the peak sides [25, 26]. In order to maintain the separation resolution when the separation distance shortens (*i.e.*, $x_2 - x_1$ becomes smaller), peaks must be sharper (*i.e.*, w_1 and w_2 also become smaller). This is theoretically possible because a higher electric field results from a shorter channel when the separation voltage remains the same, and a higher electric field likely leads to sharper peaks.

Another way to indicate the IEF separation resolution is the minimum difference in p*I* required for two proteins to be separated, $\Delta(\mathrm{p}I)_{\min}$

$$\Delta(\mathrm{p}I)_{\min} = 3\sqrt{\frac{D}{E}\frac{\mathrm{d(pH)}/\mathrm{d}x}{(-\mathrm{d}\mu/\mathrm{d(pH)})}} \tag{2}$$

where D is the diffusion coefficient of a protein, E is the electrical field strength, d(pH)/dx is the pH gradient, and dμ/d(pH) is the mobility difference at the p*I* value. Equation (2) was first reported by Vesterberg and Svensson in

1966 [28] and has been redeveloped by Righetti and Drysdale [2] as well as others [29]. Several assumptions were made during derivation, including negligible EOF, negligible Joule heating, constant pH gradient, constant mobility difference at p*I* value, Gaussian distribution of peaks, and that the minimum peak separation distance to distinguish two peaks must be at least three times of the SD of the peaks [29, 30].

When a constant voltage, *V*, is applied and a uniform pH gradient is used, then $E = V/L$ and $\frac{d(pH)}{dx} = \frac{\Delta pH}{L}$, where L is the separation length. Equation (2) is simplified to Eq. (3)

$$\Delta(pI)_{min} = 3\sqrt{\frac{D}{V}\frac{\Delta pH}{(-d\mu/d(pH))}} \quad (3)$$

in which there is no term related to the distance. Equation (3) clearly indicates that the IEF resolution is independent of the separation length. As a result, a short focusing length is advantageous, especially for a microfabricated device, as it should provide more rapid analysis without sacrificing the resolving power. It should be noted, however, that a few assumptions are made during derivations, and they (*e.g.*, negligible EOF) may not be true in some experimental conditions. In addition, the separation voltage must remain the same while reducing the separation length. As a result, the electric field strength and current will increase, possibly producing Joule heating that becomes non-negligible.

4 Results and discussion

4.1 Effects of separation length

According to Eq. (3), the IEF resolution should be independent of the separation length if the voltage is maintained and all assumptions are met. We previously reported the experimental results confirming this theoretical prediction by using protein standards separated in five capillaries with a diameter of 800 μm [6]. Two proteins (hemoglobin and myoglobin) with a p*I* difference of 0.1 pH unit were separated in all distances ranging from 2 to 7 cm when a voltage of 500 V was used. An IEF gel was used in each capillary, and detection was obtained by staining the gels after they were retrieved.

We designed the device shown in Fig. 1a and used it to study the effects of the separation length in a microfabricated device. As it is impossible to stain the IEF gel in a device, we employed fluorescent proteins for this study. GFP is a widely used, naturally fluorescent protein produced by a jellyfish *Aequorea* [31]. RPE and B-phycoerythrin are two billiproteins; they are photosynthetic antenna pigments found in algae [32]. We also tried myoglobin and BSA, both of which were labeled with Alexa488 using the Protein Labeling Kit from Molecular Probes (Eugene, OR) [17]; and they gave broad peaks due to heterogeneity in labeling [33]. We then choose to concentrate on two proteins, GFP and RPE, to study the effects.

The experiments were started by uniformly filling a channel with a mixture containing ampholytes, linear polymers, and proteins. The ampholytes with a pH gradient of 3–10 were used. As reported previously [17], the protein peaks were observed almost immediately after an electric field was applied. These peaks traveled from the anode side toward the middle of the channel. The intensity of the protein peaks was initially very low, and then increased as they traveled along the channel while more proteins were focused and accumulated in the peaks.

Figure 2 shows the IEF electropherograms of GFP and RPE in channels II–V with different separation lengths. The separation voltage was 300 V. As reported in the literature [33], there are three peaks of GFP. The p*I* of the predominant peak (GFP-2) is 5.0, and the p*I*s of two minor peaks are 5.2 (GFP-1) and 4.9 (GFP-3). We observed all three peaks of GFP in all separation lengths; thus we can claim that all channels have the capability to separate proteins with p*I* difference of 0.1 pH units. These results suggest that the minimum p*I* difference required to separate two proteins (0.1 pH unit in this case) is independent of the focusing distance. We analyzed these results further by calculating their separation resolution as follows.

As discussed above, separation resolution (R) can be calculated by using Eq. (1). The R values between peaks 1 and 2 of GFP as a function of the separation length at different separation voltages are tabulated in Table 1. Statistical analysis using ANOVA [34] indicates that the resolution values at 300 and 500 V in Table 1 are the same at the 95% confidence level for different separation lengths. The resolution values at 200 and 400 V are statistically different based on ANOVA, but the trend of the change is ambiguous, especially for those at 200 V. Relatively large SD in these values is primarily due to the fact that each peak corresponds to 15–25 pixels of CCD. One pixel variation will result in 4–6% deviation. These results indicate that the separation resolution is independent, within the experimental errors, of the separation length, which is in agreement with the theory predicated. It should be pointed out that the conclusion is true for most of the experiments when the separation voltage changes from 200 to 500 V, which corresponds to the electric field strength of 38–263 V/cm.

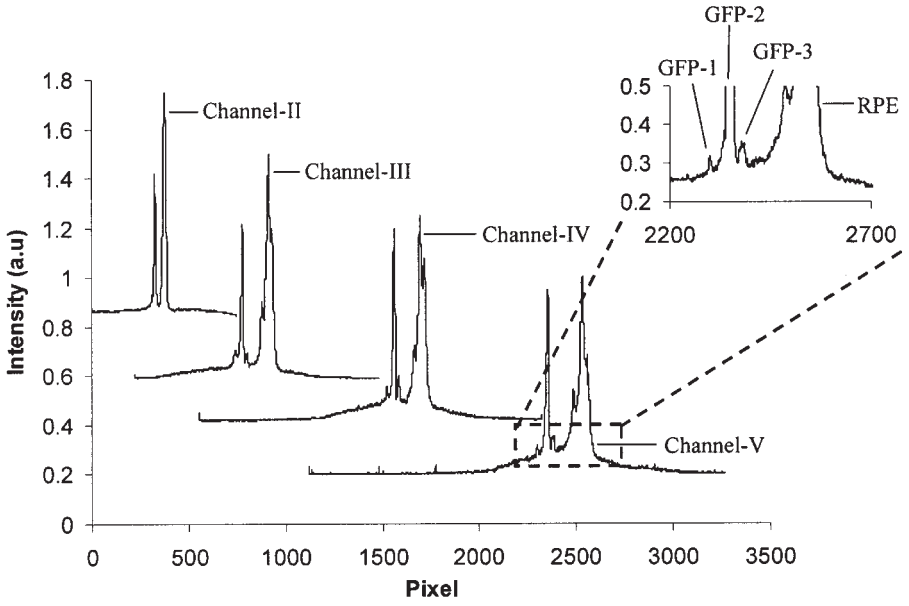

Figure 2. Effects of the separation length on IEF separation. GFP (5 ng/μL) and RPE (3.85 ng/μL) were separated in IEF channels II–V with an IEF channel length of 1.9, 3.0, 4.1, and 5.2 cm. Minor peaks of GFP are shown in the expanded views of a part of electropherograms for channel V. A voltage of 300 V was applied to all channels; the cathode was on the left and the anode was on the right. The pH gradient was 3–10, and the separation medium was prepared from 1.83% HPC/0.93% HEC stock solution. Each pixel in *x*-axis approximately corresponds to 20 μm. The IEF electropherograms were shifted in both axes for clarity, the degree of up-shift is indicated by the background intensity and that of right-shift by the starting point.

Table 1. Effects of IEF distance on separation resolution

	Channel II	Channel III	Channel IV	Channel V
Separation length (cm)	1.9	3.0	4.1	5.2
Resolution (200 V)	0.95 ± 0.07	1.1 ± 0.2	0.7 ± 0.1	1.5 ± 0.4
Resolution (300 V)	1.1 ± 0.4	1.2 ± 0.1	1.4 ± 0.2	1.6 ± 0.3
Resolution (400 V)	1.0 ± 0.1	1.5 ± 0.3	1.7 ± 0.2	2.1 ± 0.2
Resolution (500 V)	1.2 ± 0.3	1.4 ± 0.3	1.6 ± 0.9	1.7 ± 0.4

Separation resolution was calculated as discussed in the text. The SD was obtained from three repeating experiments. The separation voltage for each row is listed in the first column while other experimental conditions were the same as in Fig. 2.

4.2 Effects of the separation voltage

We investigated the effects of the separation voltage on IEF by applying different separation voltages across channel V (with a length of 5.2 cm). Figure 3a shows some representative IEF electropherograms of GFP and RPE when different separation voltages were used. According to the theory, the separation resolution is a function of the square root of the electric field strength, which is evident from the plot in Fig. 3b. The result suggests that the peak sharpened with the increase of the separation voltage, accordingly the field strength. This is the theoretical basis of the prediction that the separation resolution is independent of the separation distance according to the discussion after Eq. (1). Another key advantage of using a higher separation voltage is a shorter analysis time, as discussed in Section 4.3.

4.3 Focusing time

One of the major motivations to choose miniaturization is to achieve a shorter analysis time. Obviously, the IEF focusing time will be affected by both the separation length and the voltage. When the separation resolution is maintained, a shorter separation length and higher separation voltage, thus a higher electrical field strength, will be preferred as they will lead to a shorter analysis.

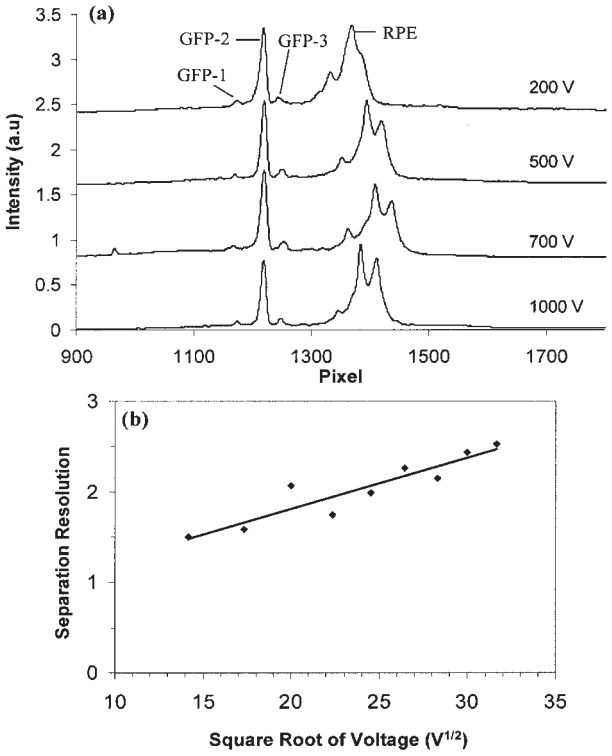

Figure 3. Effects of separation voltage on IEF. (a) IEF electropherograms were obtained at the separation voltages indicated. (b) Separation resolution between peaks of GFP-1 and GFP-2 is linearly proportional to the square root of the separation voltage (accordingly the electric field strength). Channel V (5.2 cm) was used for all experiments. Other conditions were the same as in Fig. 2.

The IEF analysis time is determined by temporal profiles of protein separations provided by the WCID system described in Section 2.4. When the peaks in the electropherograms do not move any further, IEF is considered to be completed. In addition, the drop of the IEF current gives an indication of the focusing of CA and proteins [35]. We also confirmed the completion of IEF by the expected p*I* location, assuming the pH gradient is uniform and linear in the channel.

Figure 4 shows the relationship between the focusing time and the electrical field strength when a variety of separation voltages were used in different separation lengths. The SDs represented by the error bars were obtained from three sets of repetitive experiments. A voltage range of 200–1000 V was used for channel V as in Fig. 3, whereas a smaller range of 200–500 V was used for channels II and IV. The focusing time ranged from 1.5 to 46 min depending on the separation length and electrical field used. The data suggest that the focusing time decreases with the electrical field strength, and there is a linear relationship between the time and the inverse of the

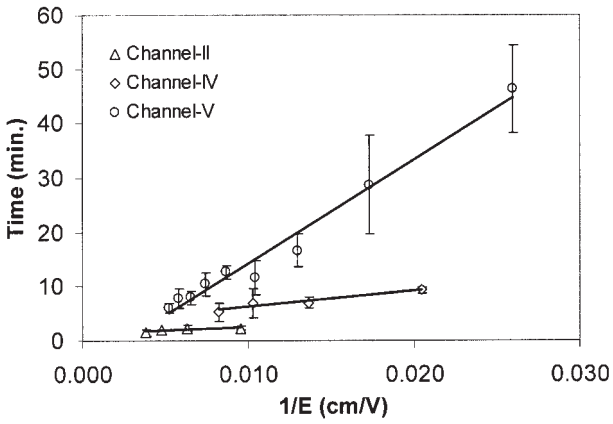

Figure 4. Relationship between the focusing time and the inverse of the electric field strength. The pH gradient used was 3–10. The separation length is represented by the channel number while the separation voltages were indicated by the electrical field strength. Other conditions were the same as in Fig. 2.

electric field strength. This relationship can be proved by IEF theory in the literature [36]. In addition, the focusing time decreased with the separation length as expected.

4.4 pH gradient compression

Recently, Cui *et al.* [8] reported that the pH gradient in a microfluidic device is compressed to the middle of a channel rather than uniformly distributed in the whole channel, starting from the anode and ending at the cathode. We examined our experimental results and found that the compression phenomenon existed. For the electropherograms in Fig. 2, the compression ratio is 83, 84, 88, and 93% for channels II–V, respectively. The compression ratio is the actual distance (in pixel) between two major peaks of GFP and RPE at the end of IEF analysis divided by the calculated distance from their p*I* difference and the pH gradient. The results suggest that catholytes and anolytes entered into IEF channel for a short distance (7–17% of the channel) to facilitate the formation of pH gradient. The degree of compression decreased with the length of separation channel.

We also investigated the effects of different pH gradients, pH 4–6 and pH 3–10. As expected, the peaks were better resolved in the pH 4–6 gradient, having larger peak separation, than in the pH 3–10 gradient, as shown in Fig. 5. Both experiments were run in channel V with the same separation length. The separation voltage was 1000 V. Their compression ratios were calculated 56 and 61% for the pH 3–10 gradient and the pH 4–6 gradient, respectively.

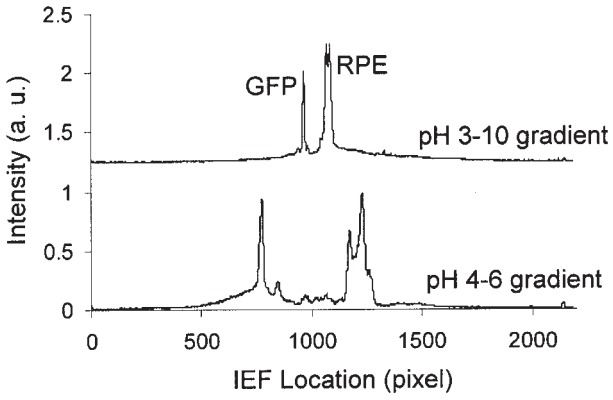

Figure 5. Comparison of IEF electropherograms of GFP and RPE between pH 3–10 and pH 4–6 gradients. Channel V was used with a separation voltage of 1000 V. Other conditions were the same as in Fig. 2.

Cui *et al.* [8] suggested that an increase in the viscosity of catholytes and anolytes may reduce the compression of the pH gradient. We studied this by adding different concentrations of glycerol in both catholytes and anolytes (glycerol concentration of 25, 50, and 65%). We failed to observe the decrease in the compression of the pH gradient. In fact, we observed the decrease in the peak distance between GFP and RPE (137, 122, and 98 pixels for 25, 50, and 65% glycerol, respectively), indicating an increase in the compression. We also observed an increase in the focusing time at a higher viscosity, suggesting that a longer time is required to form a pH gradient.

5 Concluding remarks

We investigated the effects of separation length on IEF in a plastic microfluidic device. IEF theory predicts that the IEF resolution is independent of separation length under the conditions that meet the assumptions in the derivation. The conclusion is significant as it supports the use of IEF in a microfabricated device without sacrificing the resolving power, and at the same time the analysis time is significantly reduced due to shorter channels and higher electric field strengths. Experimentally, we achieved separation of GFP peaks with a p*I* difference of 0.1 pH units, and we confirmed that the separation resolution is independent of the separation length.

The LIF WCID system is a very useful tool for studying IEF dynamics. Simultaneous illumination of an entire channel with an expanded laser line and collection of fluorescence emission by a CCD camera enable elimination of the mobilization step that is often practiced in capillary or channel IEF. In addition, WCID provides useful information about the dynamic behavior of protein migration during the process of IEF, and the temporal information could be used for determining the focusing time.

We confirmed the existence of pH gradient compression reported recently by Cui *et al.* [8], that is, the pH gradient is compressed to the middle of a channel rather than uniformly distributed in the whole channel from the anode to cathode. This phenomenon is important because it conveys the exact distance of a pH gradient actually formed, rather than the assumed capillary or slab length. In addition, presence of EOF also affects the actual distance of a pH gradient as some separation medium might flow out of the separation channel or capillary. These factors suggest that an IPG is probably a direction for future work [37–39], especially considering the claim that a true steady state in the absence of IPGs cannot be achieved experimentally at all according to theoretical simulation [3].

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