Demonstration of isoelectric focusing on an etched quartz chip with UV absorption imaging detection†

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The feasibility of isoelectric focusing (IEF) performed on-chip was demonstrated for the first time *via* absorption imaging detection. Microchannels on a quartz chip were fabricated using photolithography and a chemical etching process. The separation channels were 40 mm long, 100 μm wide and 10 μm deep, and were coated with linear polyacrylamide to reduce electroosmotic flow. A quartz chip cartridge for IEF was assembled in which two pieces of hollow fiber were glued to the two ends of the separation channel to isolate the electrolytes from the samples. Low molecular mass p*I* markers and myoglobin were selected as model samples which were mixed with 4% carrier ampholyte solution. Samples were injected into the channel via the connection capillary by pressure. A voltage of 3 kV was applied to perform IEF. The IEF current decreased from about 13.4 to 1.3 μA. The focused zones were monitored in real time by absorption imaging detection at 280 nm. The detection limit was about 0.3 μg ml^{-1} or 24 pg for p*I* marker 6.6, and 30 μg ml^{-1} or 2.4 ng for myoglobin with an optical pathlength of 10 μm. Good reproducibility and resolution were obtained for linear polyacrylamide coated channels. The total analysis time was less than 10 min. This imaged chip IEF provides a fast separation technique with quantitative ability and the potential for increasing throughput.

Introduction

The microfabrication of analytical instrumentation has attracted great interest, offering compact, reliable, and inexpensive methods for chemical and biological analysis. Chemical separation devices appear to be particularly amenable to microfabrication. The feasibility of the integration of miniaturized separation techniques into compact devices has been demonstrated for gas chromatography (GC),[1] capillary electrophoresis (CE),[2–8] micellar electrokinetic capillary chromatography (MEKC)[9,10] and open channel electrochromatography (CEC).[11] Their separation performance has been extensively evaluated. Although much work remains to be done to prove their general suitability for practical analytical applications, they have already been shown to offer some unique features with respect to separation speed, sample injection, consumption of sample and buffer solution and the use of low voltages for highly efficient separations. However, no experimental demonstration has been presented for isoelectric focusing (IEF) performed in free solutions using microfabricated devices. Although on-chip IEF is expected to have some great advantages over the conventional capillary IEF (CIEF) technique with respect to compact size, integration and throughput, the major limitations of developing this technique, as addressed later, are associated with its unique separation mode and the detection methods available.

Isoelectric focusing is a high-resolution technique for the separation of complex protein mixtures.[12,13] It is routinely used for the characterization of biological extracts, monitoring protein purification, evaluating the stability or microheterogeneity of protein therapeutics and the determination of protein isoelectric points (p*I*). As in gel IEF, proteins are separated according to their p*I*s in a pH gradient formed by carrier ampholytes when an electric potential is applied. CIEF[14–17] combines the high resolving power of conventional gel IEF with the automation and quantification advantages of CE instrumentation. The excellent heat dissipation of capillaries allows separations to be performed in free solution, without the requirement for an anticonvective gel. Typical CIEF employs 12–60 cm long capillaries with an on-column absorbance detector. Focused sample zones are moved to the detection point by electrophoretic (salt), hydrodynamic or electroosmotic mobilization. Both one-step and two-step approaches have been developed.[14,15] However, problems associated with the mobilization process may be encountered, including long analysis time, high risk of protein precipitation and distortion of pH gradient. To overcome the disadvantages caused by mobilization, imaged-CIEF has been developed by ourselves in the past few years.[18–21] A specially designed cartridge holding a short capillary was constructed. Charge-coupled devices (CCDs) were used as the imaging sensor. With such a whole column imaging technique, the focused sample zones can be monitored in their stationary state without mobilization.

With the development of this imaged-CIEF technique, IEF performed in microchip channels becomes possible. In most CE microchip systems, single-point detection including laser-induced fluorescence and UV absorbance detection has been widely adopted.[3,22] However, in the case of IEF, if performed on a microchip, it is preferable to implement whole channel imaging detection so that the focusing process takes place, as in slab gel IEF, free from any disturbance, *e.g.*, the influence of electroosmostic flow (EOF). The mobilization step to facilitate single-point detection for a short channel is expected to be less controllable than with a long capillary. Imaging detection, in which no mobilization is required, is therefore ideal for on-chip IEF. Moreover, imaged CIEF reveals the dynamic focusing process easily and accurately because of its real-time mode, and provides valuable extra information to IEF analysis, especially in fundamental investigations.

In this paper, IEF performed on a microchip is reported and the quartz chip cartridge for IEF is described in detail. To

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demonstrate the chip IEF, low molecular mass p*I* markers and myoglobin were selected as model samples. The chip channel was coated with linear ployacrylamide and the performance of both uncoated and coated channels for IEF of the model samples was explored.

Experimental

Microchip

The microchip separation devices were fabricated by Alberta Microelectronic Corporation (AMC, Edmonton, Alberta, Canada) using photolithography, chemical etching and bonding processes. The channel structures were fabricated on a piece of quartz plate. Another quartz cover plate was then thermally bonded to seal the channels.[23] The two quartz plates were 40 × 50 × 0.5 mm each. Access to the channel terminals was provided by ultrasonically drilled 0.5 mm holes. In our experiments, only one channel was selected to perform IEF each time. To minimize the EOF, the channel walls were coated with linear polyacrylamide following Hjerten's procedure.[24]

Microchip IEF cartridge

A microchip IEF cartridge was assembled to match the imaging detection as shown in Fig. 1(A). In order not to disturb the pH gradient in the separation channel, two pieces of hollow fiber (od 200 μm, id 170 μm, length 5 mm; Spectrum Medical Industries, Los Angeles, CA, USA), one at each end of the channel, were used to isolate the electrolytes from the sample solution. This special design eases the sample injection for the short separation channel. Two connection capillaries (od 160 μm, id 100 μm; Polymicro Technologies, Tucson, AZ, USA) were inserted into and glued to the two pieces of hollow fiber dialysis tubing. The sections of hollow fiber were glued to the two holes in the ends of chip channel. A piece of chemically etched metal with a 50 μm wide slit was glued to the under surface of the substrate plate. The slit ensures that UV light only passes through the channel for absorption imaging detection. Two plastic tubes, each with a volume of about 0.15 ml, were finally glued to the end of the channels as electrolyte reservoirs.

Imaged chip IEF procedures

The instrumental set-up for imaged chip IEF and sample injection is shown in Fig. 1(B). The UV source was an 80 W Xe lamp with a 280 nm bandpass filter. The UV radiation was projected on to the chip channel by an optical fiber bundle. A linear CCD array was used as detection sensor. The microchip IEF cartridge was fixed in the optical path. Samples were injected by pressure: one end of the capillary connected with the channel was inserted into a 2 ml sample vial with a rubber septum cap. A 0.5 ml volume of air was then injected into the vial using a 3 ml syringe so that sample was forced into the separation channel. After 30–60 s, the capillary was pulled out and inserted into the two balancing vials. The two vials were filled with water to the same level in order to reduce hydrodynamic flow while performing IEF. After 1 min relaxing and stabilizing, IEF was started by applying a high voltage of 3 kV. Images before and during focusing were taken by the absorption imaging detection system. The exposure time was 20 ms for each scan, and 64 scans were averaged for each electropherogram to increase the signal-to-noise ratio.

Chemicals

All chemicals were of analytical-reagent grade and solutions were prepared using de-ionized, distilled water. Solutions of 10 mM H_3PO_4 and 20 mM NaOH were used as the anolyte and catholyte, respectively. Low molecular mass p*I* markers 8.6, 7.4, 6.6, and 5.3 were purchased from Bio-Rad (Missisauga, ON, Canada). Myoglobin was purchased from Sigma (St. Louis, MO, USA). The p*I* markers or myoglobin were/was mixed with the carrier ampholyte solution (Pharmalyte pH 3–10, Sigma) to a final concentration of 2–40 μg ml^{-1} for low molecular mass p*I* markers, 400 μg ml^{-1} for myoglobin and 4% for ampholytes.

Results and discussion

IEF performed in uncoated channel

In CIEF, coating of the capillary inner wall is employed to reduce substantially the adsorption of proteins on the capillary wall to avoid poor reproducibility and detection sensitivity, and to minimize the electroosmotic flow to produce a high resolution. In the case of IEF performed on-chip, both uncoated

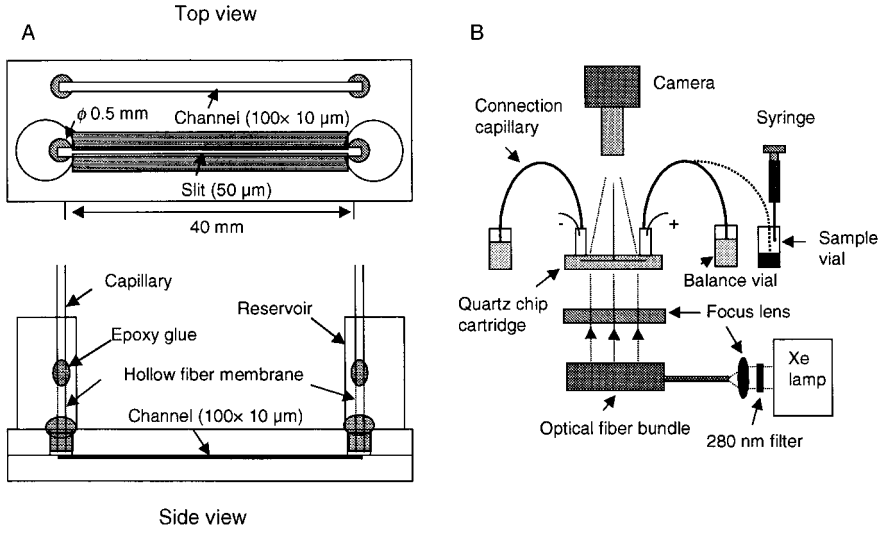

Fig. 1 (A) Schematic diagram of microchip IEF cartridge; (B) schematic diagram of the instrument set-up for chip IEF using absorption imaging detection.

and coated channels were tested. First, IEF performed on uncoated channels was investigated. Fig. 2 shows the electropherograms of p*I* markers 8.6 and 5.3 focused in the chip channel. At 2 min after focusing took place, two peaks are observed. The dynamic focusing process shows that the two peaks merged into one peak after 6 min. This suggests that the two p*I* markers were not separated. According to our previous experiments,[20] the dynamic focusing process reveals a 'double peak' focusing pattern for each component in the early focusing stage when the sample is uniformly distributed inside the separation column. This means that the component is focused towards its p*I* point from both sides. For a two-component system, four peaks are observed in the early stage of a normal focusing process. Hence the experimental results show that a large EOF drove the components out of the short channel before the focusing was complete. In this case, the focusing pattern is not reproducible, as shown by our unpresented experimental results.

IEF performed in coated channel

IEF performance was assessed in the linear polyacrylamide coated channel. Fig. 3 shows one IEF dynamic process of p*I* marker 6.6 with a concentration of 8 μg ml^{-1}. At 2 min, two focused zones of the p*I* marker appeared and focused towards the correspondent p*I* point from both sides. They joined together to form one peak after the focusing came to end. During the focusing process, the current decreased about 10-fold from 13.4 to 1.3 μA. The focusing process reached a steady state after 4 min for one p*I* marker component. The electropheorgrams show a dynamic IEF process where EOF is not obvious. It is observed that the total focusing time is <5 min. Hence the channel coating was successful, resulting in a good focusing process.

It should be noted that in our experiments the pH gradient formed inside the channel does not correspond to the exact pH range 3–10 for the chosen ampholytes. The real pH range in the channel is narrower because in the two ends of the channel, the holes to which hollow fibers were glued stored a larger amount of ampholytes than that of the channel itself. After the voltage is applied, the pH gradient is partly influenced by this volume. To minimize such an effect, this volume, which can be called the dead volume in connection, must be reduced by improving the method of gluing the hollow fiber to the ends of channel.

The reproducibility and the detection limit of chip IEF were checked. Fig. 4 shows two electropherograms of p*I* marker 6.6 with two different concentrations at 6 min. The peak position shows good reproducibility for the two runs, and a smooth baseline is observed. The noise level of absorption imaging detection in our experiments is 1×10^{-3} arbitrary units when 64 scans of CCD images are averaged and the detection limit can therefore be assumed to be 3×10^{-3} arbitrary units. When 4 μg ml^{-1} p*I* marker 6.6 was injected, a peak with a height of 0.042 arbitrary units was formed after focusing. The detection limit, therefore, corresponds to a concentration of 0.3 μg ml^{-1} of the injected p*I* marker 6.6. The channel volume is only 0.08 μl, so the amount which can be detected is only 24 pg. The quantitative performance of the IEF chip was studied on the basis of p*I* marker 6.6. In the concentration range 1–30 μg ml^{-1}, the absorption is linearly proportional to the concentration of injected p*I* marker with a correlation coefficient of 0.999.

Compared with the use of a capillary cartridge,[25] less spike noise appeared with this chip cartridge, as shown in Fig. 4. With a capillary cartridge, after applying a voltage, the capillary is subject to slight bending owing to an electrostatic force which may cause some unpredictable noise. The chip cartridge shows no such a bending owing to its solid channel structure. Moreover, the chip cartridge, in which each channel was covered by a slit, eases the requirements for optical alignment for the light passing through, and gains a high light throughput that also improves the detection limit, since the noise level in imaged chip IEF is mainly limited by the shot noise of the CCD sensor.[26] A better choice for absorption imaging detection will be a photodiode array detector (PDA), the high well capacity of which makes the shot noise insignificant in the detection signal.[26, 27]

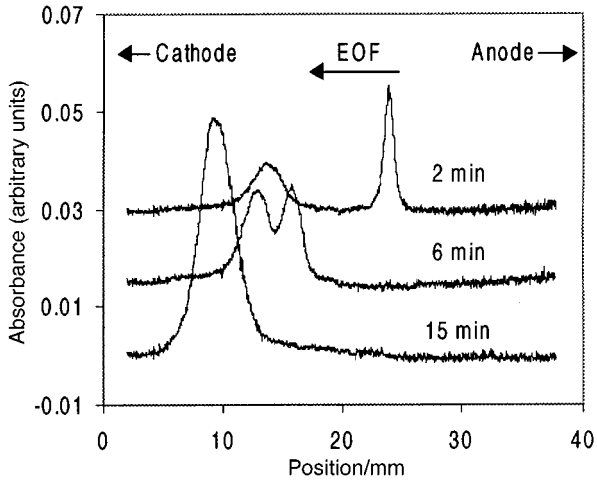

Fig. 2 Electropherograms of p*I* markers 8.6 and 5.3 at 2, 6 and 15 min. Concentration: 40 μg ml^{-1} of each p*I* marker. Applied voltage: 3 kV.

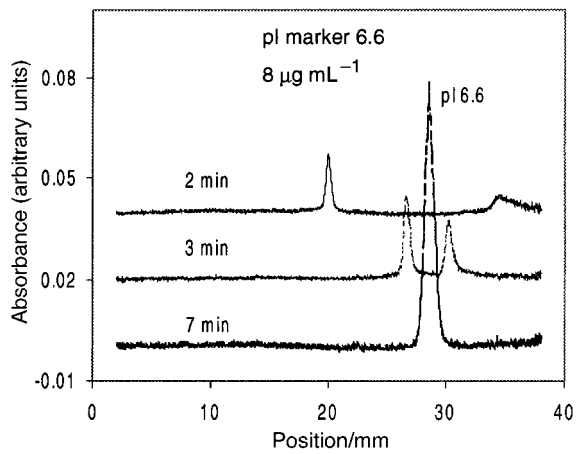

Fig. 3 The dynamic process of p*I* marker 6.6 with a concentration of 8 μg ml^{-1} recorded at 2, 3 and 7 min. Left, cathode; right, anode. Applied voltage: 3 kV.

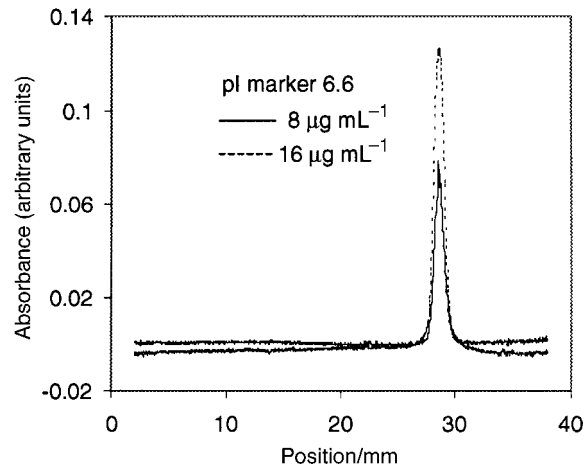

Fig. 4 Reproducibility of peak position for chip IEF of p*I* marker 6.6 at 6 min with concentrations of 8 and 16 μg ml^{-1}. Left, cathode; right, anode. Applied voltage: 3 kV.

Separation of model samples

The electropherograms of p*I* marker mixture 7.4 and 6.6 separated on a coated channel are shown in Fig. 5. At 7 min, four peaks are observed that finally focus into two peaks correspondent to p*I* marker 7.4 and 6.6 (as shown at 11 min). The molar absorptivity of p*I* marker 6.6 is higher than that of p*I* marker 7.4, and therefore the peak height is correspondingly higher. This demonstrates the successful separation of p*I* markers by chip IEF. As another example, myoglobin was separated in coated channels as shown in Fig. 6. Myoglobin has two variants: one is p*I* 7.2 and the other is p*I* 6.8. The p*I* 7.2 variant is present at higher levels than the p*I* 6.8 variant. Correspondingly, the p*I* 7.2 variant has a higher peak. For myoglobin, the detection limit is 30 μg ml^{-1} or 2.4 ng based on the p*I* 7.2 variant. The total separation time for the two examples is < 10 min.

Resolving power for chip IEF

It is feared that the short channel may cause a decrease in resolution for IEF. For this reason, theoretical considerations of the feasibility of a short channel are discussed here. For a sample zone focused in a capillary by the IEF process, concentration has a Gaussian distribution with a variance σ:[12,13]

$$C = C_0 \exp(-pEx^2)/2D \quad (1)$$

where $C = C_0$ is the maximum concentration, p the mobility slope ($-\mathrm{d}u/\mathrm{d}x$), E the field strength, D the diffusion coefficient and x the position along the capillary.

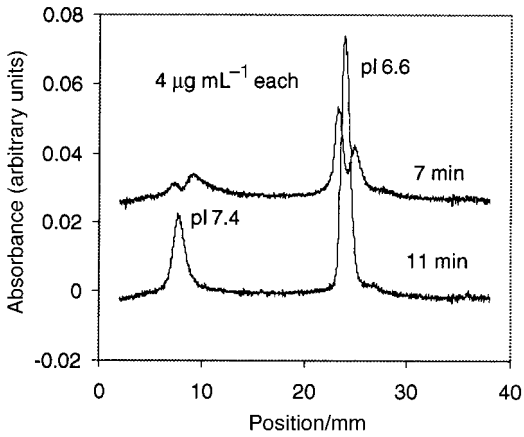

Fig. 5 Separation of low molecular mass p*I* markers 7.4 and 6.6 on a coated channel. Concentration of each component: 4 μg ml^{-1}.

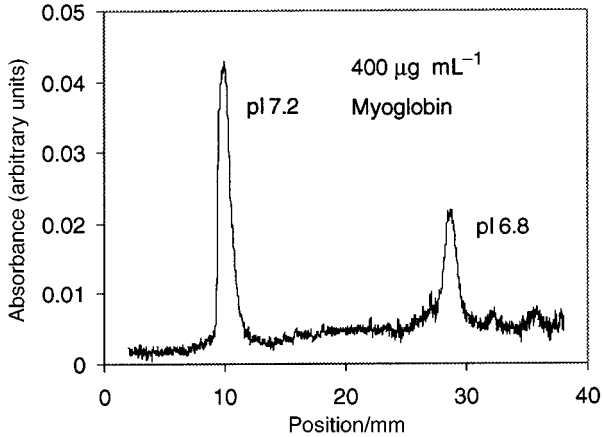

Fig. 6 Electropherogram of myoglobin with a concentration of 400 μg ml^{-1} separated by chip IEF at 11 min. Two variants are p*I* 7.2 and 6.8.

$$\sigma = \pm\sqrt{\frac{D}{E}\frac{\mathrm{d}x}{(-\mathrm{d}u)}} \quad (2)$$

Using the criterion of three times the variance σ for resolved adjacent proteins, the resolving power, Δ p*I*, of IEF in terms of σ can be expressed as[12]

$$\Delta \mathrm{p}I = 3\sqrt{\frac{D}{E}\frac{\mathrm{d(pH)}}{-\mathrm{d}u}\frac{\mathrm{d(pH)}}{\mathrm{d}x}} \quad (3)$$

Equation (3) shows that good resolution is favored by high field strength, low diffusion coefficient, high mobility slope du/d(pH) and a narrow pH gradient. Of the variables, the diffusion coefficient and the mobility slope are intrinsic properties of the analytes, so only pH gradient and the field strength can be varied experimentally.

The resolving power in imaged-CIEF employing a short capillary format can be adopted to the chip channel format. In conventional CIEF, where long capillaries (12–60 cm) are used, pH gradients are more shallow than those seen in imaged CIEF with short capillaries. Satisfactory resolution, however, still can be obtained by adopting a higher field strength. Narrow fused-silica capillaries have excellent heat dissipation and so allow high field strengths (500–800 V cm^{-1}) to be applied. When a narrow pH gradient of 6–8 and a high voltage of 3 kV are used, a resolution of about 0.03 pH unit can be achieved with imaged-CIEF performed in a 5 cm long capillary.[28] This resolution is slightly lower than that in optimized conventional single-point detection CIEF with a resolution of 0.01–0.02 pH unit,[29] but, it is good enough for clinical analysis. Owing to the short capillary, imaged-CIEF attains equilibrium faster, *e.g.*, within a few minutes (2–3 min), resulting in a faster analysis speed. For similar reasons, imaged chip IEF provides a fast separation technique with a good resolution.

Conclusions

Isoelectric focusing was successfully performed on a quartz microchip for the first time, where low molecular mass p*I* markers and myoglobin were used as model samples. Absorption imaging detection proved to be an ideal way to record the chip IEF process in real time. Coating of the inner wall of channels played an important role in the chip IEF performance. Good resolution, sensitivity and reproducibility are obtained for IEF performed on coated channels. Imaged chip IEF allows fast separations with a potential for increased throughput.

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