Talanta 116 (2013) 259–265

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

A stable and high-resolution isoelectric focusing capillary array device for micropreparative separation of proteins

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ARTICLE INFO

Article history:
Received 10 March 2013
Received in revised form
16 May 2013
Accepted 19 May 2013
Available online 24 May 2013

Keywords:
Capillary Array
Isoelectric focusing
Coating
Repeatability
Resolution

ABSTRACT

A simple capillary array IEF device was developed for high resolution and micropreparative separation of trace amounts of proteins. Based on quasi-chip-scale manufacturing, the specific capillaries (600 μm i.d., 1200 μm o.d. and 20 mm length) were integrated with the miniaturized polymethyl-methacrylate electrode trays. Electroosmotic flow was suppressed effectively by modified cross-linked polyacrylamide coating, and instability of IEF was addressed using the designed concentration of electrolytes via moving reaction boundary theory. As a prototyping, the resolution, reproducibility, throughput, speed and linearity of pH gradient were systemically evaluated with model proteins. The results revealed the following advantages: (i) the reproducibility of array was assessed as RSD values of 0.95% (intra-day) and 2.88% (inter-day); (ii) IEF could be completed in 20 min with up to 400 V/cm electric field; (iii) high resolution separation of model proteins achieved in 20 mm length column; (iv) multi-units with 48 micro-columns can be easily integrated to obtain high throughput; and (v) good linearity of pH gradient (R=0.9989). More importantly, utility of the device was tested by using hemoglobins sample from human red blood cell. HbA_0 and HbA_{1c} with only ΔpI 0.03 have been successfully separated by the developed method.

1. Introduction

The idea of isoelectric spectra, viz., the prototype of isoelectric focusing (IEF), was unveiled by Kolin [1], and yet the separation of protein and virus in line with their p*I*s could only last for about 10 min due to the lack of carrier ampholyte (CA). Svensson [2] advanced the theory of classic IEF and pointed out the crucial role of CA in IEF. Accordingly, Vesterberg [3,4] successfully synthesized CA in accordance with the classic theory, leading to the real application of gel-based IEF in biological research. As the first dimension separation in 2DE, the classic gel IEF has been used extensively in the analyses of complex protein samples [5,6]. Nevertheless, there were still a few of issues that weaken the separation performance of the IEF, such as instability of pH gradient, unsatisfactory separation of proteins with minor p*I* difference (e.g., hemoglobin A and hemoglobin A_{1c}) and time-consuming (e.g., up to 10-h run of gel IEF) [7,8].

The technique of capillary IEF (cIEF), initially reported by Hjertén et al. [9] with a salt-induced mobilization step, made significant advances in improving the resolution via high electric field thanks to the high specific surface area. The complex peptide mixture such as the lysate from yeast cytosol could be separated by cIEF in 10 min [10]. To address the problem of throughput, a series of array cIEF systems that coupled with liquid chromatography (LC) were developed for the analyses of multiple protein samples [11,12]. Remarkably, the array cIEF device with up to 60 coated capillaries was reported by Mao et al. [13]. A reagent-release capillary array device was developed by Kataoka et al., by which the mixture of standard hemoglobin AFSC have been separated [14]. The array prototype of capillary shows a promising manner for research of complex proteins by integrating high resolution and throughput. Nevertheless, the pretreatment process of traditional capillary was time-consuming. Besides, the repeated rinse between inter-assays will surely weaken the reproducibility of proteins separation.

Numerous chip-based IEF approaches that required extremely small sample volume have been proposed for the rapid separation

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http://dx.doi.org/10.1016/j.talanta.2013.05.041

of proteins [15–20]. Dauriac et al. [16] described a $5 \times 3.9\ cm^2$ array micropillar device and displayed the rapid IEF of seven visible proteins. Herr et al. [17] developed an acrylic microfluidic chip device, in which the separation time of three fluorescent proteins was less than 1 h. Zilberstein et al. [18] reported the parallel IEF microdevice using a dielectric membrane with conducting channels for protein separation in several minutes. The mobilization step in cIEF that resulted in band distortion and resolution damage could be removed by whole-column imaging strategy in chip-based IEF. For instance, Pawliszyn's group [19,20] reported the special work that was aimed at the miniaturized UV detection device and hybrid materials construction. However, the resolving power achieved by chip-based IEF was barely satisfactory. For example, the complete separation between normal hemoglobin and the glycated species was not conducted in chip-based IEF yet [21].

The array cIEF and chip-based IEF respectively have its own focus on protein research. In this work, we endeavored to combine the advantages of the two strategies for the high resolution and throughput separation of trace amount of proteins such as glycated hemoglobins. The prototyping and manufacturing of the miniaturized device was described. Briefly, the specific capillary (600 μm i.d., 1200 μm o.d. and 20 mm length) with no outer polymer coating was employed as the disposable separation channel. The designed electrolytes pair by the moving reaction boundary theory (MRB) [7], and the modified cross-linked polyacrylamide (CPA) coating were applied to generate stable pH gradient. The separation performance of the device such as throughput (up to 48 parallel runs), stability and resolution were systematically evaluated. Notably, HbA_0 and HbA_{1c} with only 0.03 p*I* difference from human blood erythrocytes were separated in 20 min with high resolution, which has not been reported in chip-based IEF research. In addition, a very rare of the phenomenon that the standard cytochrome (Cyt) C consistently presented two focused bands in IEF was shown, and a possible mechanism was discussed. The developed device was further applied for the micropreparative separation and identification of Hb species from diabetics in our accompany work [22]. Additionally, the comparison of resolution between developed device and traditional IEF method was discussed, including large-scale column and immobilized pH gradient strip (IPG) IEF.

2. Experimental section

2.1. Chemicals

Unless stated otherwise, all chemicals were of analytical grade and purchased from Sinopharm Chemical Reagent (Shanghai, China). Acrylamide (ultra-pure grade, $>99.9\%$) and methylcellulose (MC) were purchased from Aladdin Reagent (Shanghai, China). N, N'-methylen-bis-acrylamid (Bis), N, N, N′, N′-tetramethyl- ethylenediamine (TEMED) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Bio-Lyte pH 3–10 and 6–8 carrier ampholytes were purchased from Bio-Rad (Hercules, CA, USA). Fused silica capillary 600 μm i.d. without polymer coating was purchased from Ruipu Co. (Hebei, China). Ultra-pure water was prepared with a commercial ultrapure water system (SG, Wasseraufbereitung und Regenerierstation Gmbh, Germany).

2.2. Fabrication of array IEF device

The design of the IEF platform was outlined in Fig. 1A. The basic unit of polymethyl methacrylate (PMMA) device, briefly, had a pair of symmetrical electrode trays. The base plate of the tray was etched with twelve 1.2-mm wide channels for the assembling of the capillary array, which spaced 4 mm apart from each other. Corresponding to the channels, one of the side plates was manufactured as a comb-like structure, consisting of thirteen watertight shutters with 4 mm in width and 5 mm in height. Due to the surface tension of liquid and sufficiently small dimension, the gaps between the watertight shutters would not cause any leakage of the electrolytes. Fig. 1B shows the basic unit of the device. Capillaries of 20–30 mm long and 600 μm i.d. were used as disposable separation channel. The size of single-unit device was $45 \times 70\ mm^2$ (if 20 mm long capillary was used) or $55 \times 70\ mm^2$ (if 30 mm long). Capillary array could be assembled on a platform that contained multi-units of the PMMA device.

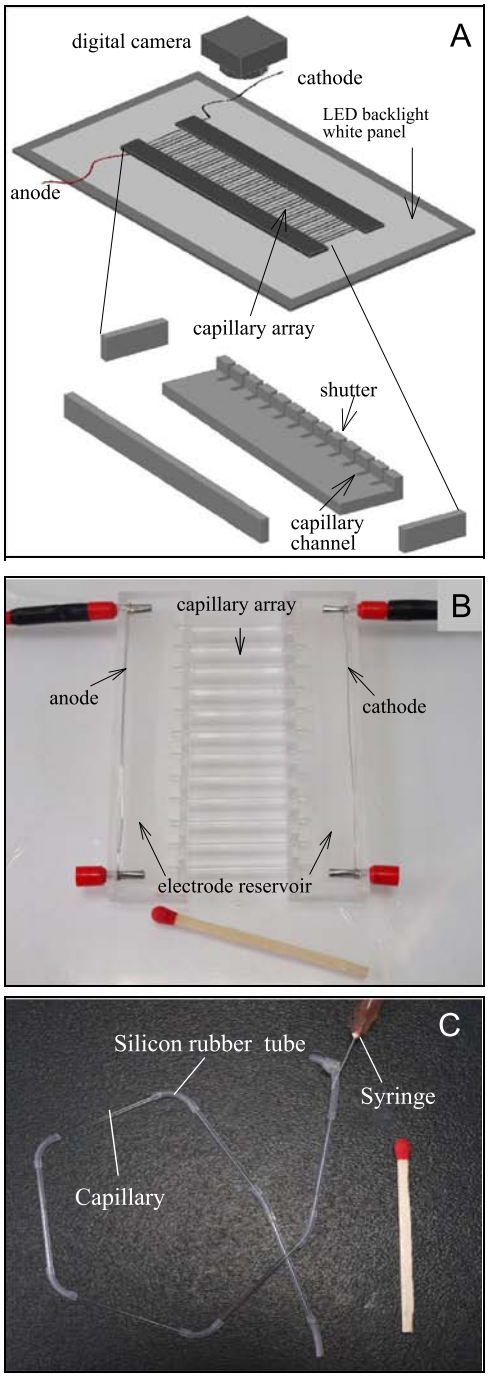

Fig. 1. (A) Schematic of the multi-unit platform for the capillary array IEF. (B) Picture of a basic-unit device with twelve capillaries. (C) The batch coating method for multiple samples.

Note herein, 75–300 μm i.d. capillary without external polymer coating was very fragile and could not be easily performed for IEF separation. However, 400–600 μm i.d. capillary column, especially a short (20–30 mm length) microcolumn, was tough enough and hardly be snapped.

2.3. Solutions and samples

The CPA stock solution containing acrylamide (10%, w/v) and Bis (5.0%, w/v) was stored at 4 °C in the dark. A mixture of 40% (v/v) 3-(trimethoxysilyl)- propylmethacrylate and 60% (v/v) acetone were prepared as the silane coupling solution. The solution of 50% methanol: water saturated with chloroform was made for the modification of tryptophan (Trp) residues in the standard protein of Cyt C. 20 mM NaOH (pH 12.2) and 20 mM sulfuric acid (pH 1.61) solutions were prepared as the catholyte and anolyte of IEF, respectively.

In order to display the separation performance of developed array IEF visually, a mixture solution of 5 μg/μL naturally-colored proteins was prepared for the IEF experiment, including Phy, Mb and Cyt C. The solution of 5 μg/μL Cyt C was prepared for the Trp residue modification. The Trp residue modification procedure of protein samples was performed according to the previous method [23].

Adult blood samples were taken from Ruijin Hospital, Shanghai Jiao Tong University School of Medicine (Shanghai). One milliliter of each samples were washed three times with isotonic saline and then centrifuged (ca. 9,000 g) for 10 min. The washed erythrocytes were lysed in a milliliter of ultrapure water. Hence hemolysates had a concentration equal to blood samples under normal physiological conditions. Ultimately, the prepared hemoglobin samples gassed with carbon monoxide for 1 min, so as to prevent auto-oxidation. The loading amount of Hb samples was 0.1 μL in each capillary.

2.4. Modified CPA coating and sample loadings

The short capillary column requires proper coating to suppress electroosmotic flow (EOF) and to prevent protein adsorption. Two sorts of commonly used coating methods, CPA coating and MC coating [24], were evaluated in our work. The corresponding effect of EOF-suppressing was further quantitatively compared. Because the disposable capillary array could be easily prepared, we used higher concentration of CPA coating solution than that of the well-established method [25]. To attach the silane coupling solution to the capillary inner wall, the solution was flushed into the capillaries with a syringe pump for 2 h in the dark, then rinsed with acetonitrile and dried. The ultimate concentration of CPA coating solution was diluted to 5%T, and the cross-link degree of gel was 5%C. The 50 μL CPA stock solution was added with 1 μL of 5% (w/v) ammonium persulfate and 0.5 μL TEMED. Then, 5 μL standard protein samples and 2% (v/v) CA were mixed into the solution, and quantitated to 200 μL with ultrapure water. The coating solution was left in the capillary and polymerized to CPA gel to achieve better controlling of EOF. If extra-pure grade of acrylamide was used, the polymerization time was less than 10 min at 40 °C or 2 h at room temperature. Capillaries were pretreated with 1 M NaOH for 30 min and ultrapure water and acetonitrile for 15 min each, according to the previous work [26].

A simple 'batch processing method' was used for capillary pretreatment and multiple samples loading. Capillary array was connected in line by short silicone rubber tubes (Fig. 1C), the CPA gel solution and protein samples could be injected by a syringe. This method guaranteed the homogeneous polymerization of CPA solution and equal amount of samples within every capillary. Dozens of capillary columns with single or multiple protein samples could be conveniently prepared at once.

2.5. Determination of EOF in capillary

Due to the special dimension of 600 μm i.d. capillary, we measured the EOF with the approach of current-monitoring [27]. The electrolyte consisting of 20 mM sodium phosphate buffer with a pH of about 7.0 was used herein. In short, the capillary and cathodic tray were filled with electrolyte at a high concentration (e.g., 15 mM), and the anodic tray was filled with the same electrolyte but at a low concentration (e.g., 10 mM). The EOF could drive the 10 mM electrolyte into capillary and gradually displace the 15 mM electrolyte during a 'blank' run of zone electrophoresis. As a result of the change of electrolyte concentration, the current simultaneously decreased in the whole system.

2.6. Array IEF experiments

Standard protein and hemoglobin samples were respectively introduced into capillary with coating solution by the 'batch processing method' mentioned above. The coated capillary array was assembled into the PMMA device. The cathode and the anode solutions were 20 mM NaOH (pH 12.2) and 20 mM sulfuric acid (pH 1.61), respectively. After the assembly of the array, the trays were covered with two plates to avoid the volatilization of electrolyte and the potential reaction between NaOH and carbon dioxide in air. Electric field was produced by a power supply (DYY-4C, Beijing Liu-Yi Instrumental Factory, Beijing, China). The power supply was set at 20 V/cm for 2 min, 40 V/cm for 2 min, and 60 V/cm for 2 min, and then set at constant voltage of 400 V/cm until the complete of run. A digital camera (Olympus E520, Olympus Co., Japan) was used to monitor the dynamic focusing in real-time.

3. Results and discussion

3.1. Stability and high throughput analysis

To achieve stable pH gradient, much attention should be paid to both the 'outer' and the 'inner' IEF instabilities. On one hand, it was demonstrated in our previous work [7] that the "inner" pH gradient instability involved with the drifting and plateau of pH gradient as well as salt-induced Hjerten's mobilization. In this case, NaOH (20 mM) and sulfuric acid (20 mM) were used as the catholyte and anolyte, respectively. Based on the certified MRB equation, the flux of hydroxyl ion in the catholyte was almost equal to that of hydrogen ion in such condition. This part of study was fully demonstrated by the experiments on long-term stable IEF run in our previous work [8].

On the other hand, previously reported work [24,25] showed the evidence that the EOF was the dominating "outer" factor, resulting in the poor reproducibility of IEF. Fig. 2 showed the comparison between uncoated capillary but with CPA gel-filled and CPA coated capillary via the current plots as a function of running time. If the whole IEF system (including the capillary column and electrode trays) contained the 10 mM phosphate buffer, the current was about 127 μA (the pentagram mark). The current reached 200 μA when 15 mM phosphate buffer was used (the round mark). Note herein, the current was determined by the concentration of solution buffer, but not associated with the factor that if the capillary was coated.

While the uncoated capillary and cathodic tray were filled with 15 mM phosphate buffer, and the anodic tray was filled with 10 mM phosphate buffer, the current was gradually decreased to 136 μA in 20 min slowly reduced to 131 μA in 23 min, and

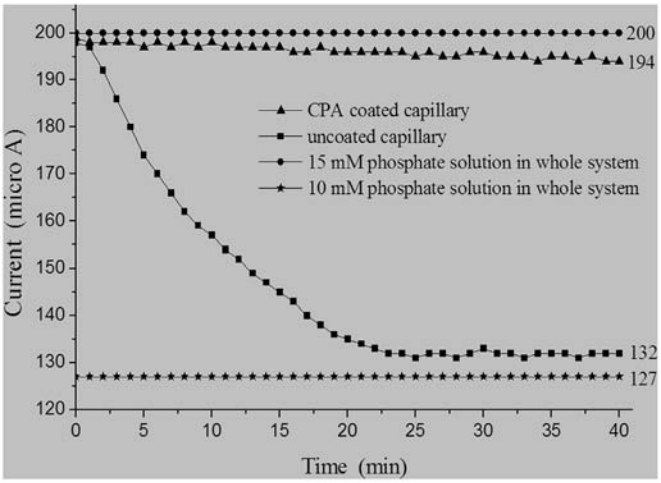

Fig. 2. Comparison of EOF in the uncoated (filled with CPA gel) and CPA coated capillary uner the experimental conditions: (-●-) uncoated capillary and electrode trays contained 15 mM phosphate buffer; (-▲-) CPA coated capillary and cathode tray contain 15 mM and anode tray contained 10 mM phosphate buffer; (-■-) uncoated capillary and cathode tray contain 15 mM and anode tray contained 10 mM phosphate buffer; and (-★-) uncoated capillary and electrode trays contained 10 mM phosphate buffer. Other conditions: 600 μm i.d. 30 mm length capillary, 5%T and 5% C CPA gel.

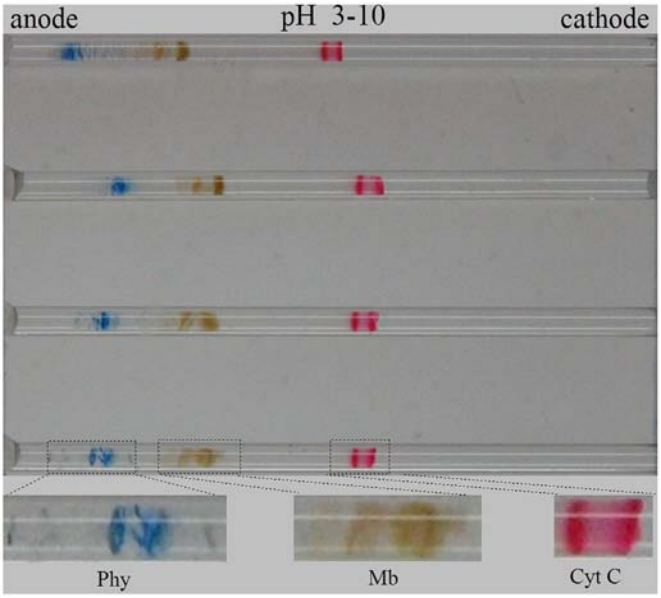

Fig. 3. IEF separation of standard proteins in 1% MC dynamic coating capillaries. Experimental conditions: 600 μm i.d. 20 mm length capillary, 1% MC coating, 2% pH 3–10 CAs, 20 mM NaOH used as the catholyte, 20 mM sulfuric acid used as the anolyte, focusing at 20 V/cm for 2 min, 40 V/cm for 2 min, and 60 V/cm for 2 min, then 300 V/cm for 30 min.

maintained between 131 μA and133 μA during 23–40 min (the square mark). The result revealed that the uncoated capillary just filled with CPA gel still had strong EOF. In contrast, the initial current of the coated capillary was 198 μA, and then gently reduced to 194 μA in 40 min (the triangle mark). It was computed that the mobility of EOF was less than 2.0×10^{-7} cm^2 V^{-1} s^{-1}, at least reducing 5 fold in comparison to the routine linear polyacrylamide coating [26,28] in cIEF.

The concentration of CPA solution also had considerable impact on EOF, especially on focusing time of proteins. It was observed that (1) less than 4%T, the concentration of CPA gel was too low to effectively control the EOF; (2) higher than 6%T CPA (e.g., 7%T and 8%T) could well suppress the EOF, but the focusing time of proteins became much longer (over 40 min if 8%T CPA gel). To prevent the EOF as much as possible and achieve an acceptable focusing time, ultimately, 5%T and 5%C CPA gel was chosen in our work.

The MC coating was proved to be an effective dynamic coating method [24] for suppression of analytes adsorption and EOF in cIEF. We also assessed 1% (w/v) MC solution to coat the capillary array. As evidently shown in Fig. 3, there were serious anode drift of pH gradient. The Cyt C finally focused in the middle area of the pH gradient, which should be focused in the alkaline area. Furthermore, the focused protein markers were divided into small irregular sub-bands, implying that the MC coating was not suitable for the treatment of the 600 μm i.d. capillary. The reasons, however, were not clear yet.

The developed array IEF achieved rather good stability and high throughput for the analyses of multiple samples. As shown in Fig. 4A, up to 48 capillaries array could be easily performed in the multi-unit IEF device. It could be easily calculated that the capacity of sample loading of the developed device was improved 65 times in comparison to that of traditional capillary array [13]. Panel B further revealed that the standard proteins were focused into three main zones in the first basic IEF unit, viz., the blue Phy zone, the brown Mb zone and the red Cyt C zone. As clearly shown in Fig. 4C, the relative positions of three standard proteins were constant in each channel. To accurately evaluate the stability performance of capillary array, the protein zones in Fig. 4B were transferred as the electrophoregram in Fig. 4D by the MetaMorph software (Molecular Devices, Inc., California, USA). The peak height was not representative to the amount of protein marker but was proportional to the color intensity of band. The reproducibility was presented via the CVs of focused position of standard proteins in Fig. 4D. By assessing single-unit array (12 capillaries), the CV of intra-day was 0.95% with five consecutive runs. Seven runs were performed, giving 2.88% CV value for the inter-day IEF run.

3.2. Resolution

The developed array IEF, using the model protein samples, showed a series of high resolution separation. Generally, a long separation channel has high resolution and a short one is of poor resolution. Herein, a shorter capillary (20 mm length) was used as the separation channel. However, quite high resolution of standard proteins IEF was obtained via the developed method. Fig. 5A shows the dynamic focusing process of the standard proteins IEF within 20 min. Fig. 5B further unveils the high resolution of standard proteins. It should be mentioned that a total of three brown Mb sub-bands could be distinctly observed. Two notable brown ones were the known p*I* 6.8 and 7.0. Furthermore, the last one was focused between the microbands of p*I* 6.8 and 7.0. Due to Mb consists of a single polypeptide chain of about 153 amino acid residues and an iron-porphyrin complex [29,30], the unknown microband could not be the subunit dissociated from the protein quaternary structure. Hence, it might be Mb variant.

In comparison to the traditional IEF (online supporting materials), we performed the separation of standard proteins in 70 mm (Fig. S1A) and 110 mm pH 3–10 IPG gel strip (Fig. S1B) as well as 170 mm pH 3–10 large-scale column (2 mm i.d.) IEF (Figure S2). The comparison of focused protein zones between Figs. 3 and 4 and S1 and S2 clearly indicated the improvement of resolution in the developed array IEF. We further calculated the resolution (R_s) of IEF with the equation $R_s=[2\,(T_2-T_1)]/(W_1+W_2)$ (where, T_1 and T_2 are the center positions of zone 1 and 2, respectively, W_1 and W_2 are the widths of zone 1 and 2 at baseline, respectively). The calculation results showed that the R_s value in the developed IEF was about 2.5, approximately five times as high as that in the 170 mm traditional IEF (Fig. S2), but about half of that in the IPG strip IEF (Fig. S1). Considering the lengths of IPG strips (7 cm in

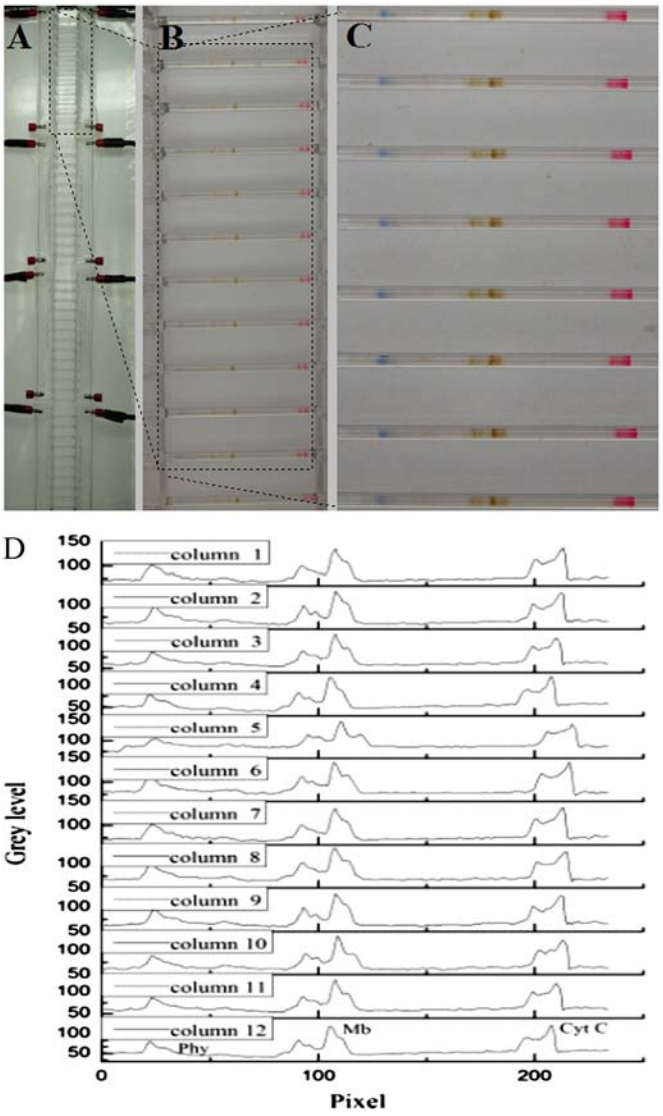

Fig. 4. Forty-eight capillaries array IEF of standard proteins (Phy p*I* 4.75 and 4.85; Mb p*I* 6.8 and 7.0; Cyt C p*I* 9.6). (A) Picture of the array IEF with 48 capillaries in four assembled units, (B) Zoomed imaging of the array IEF in the first basic unit, Zoomed insertion (C) and electropherograms (D) of the first basic unit. The capillaries were treated by the CPA coating. The other experimental conditions are the same as those in Fig. 3.

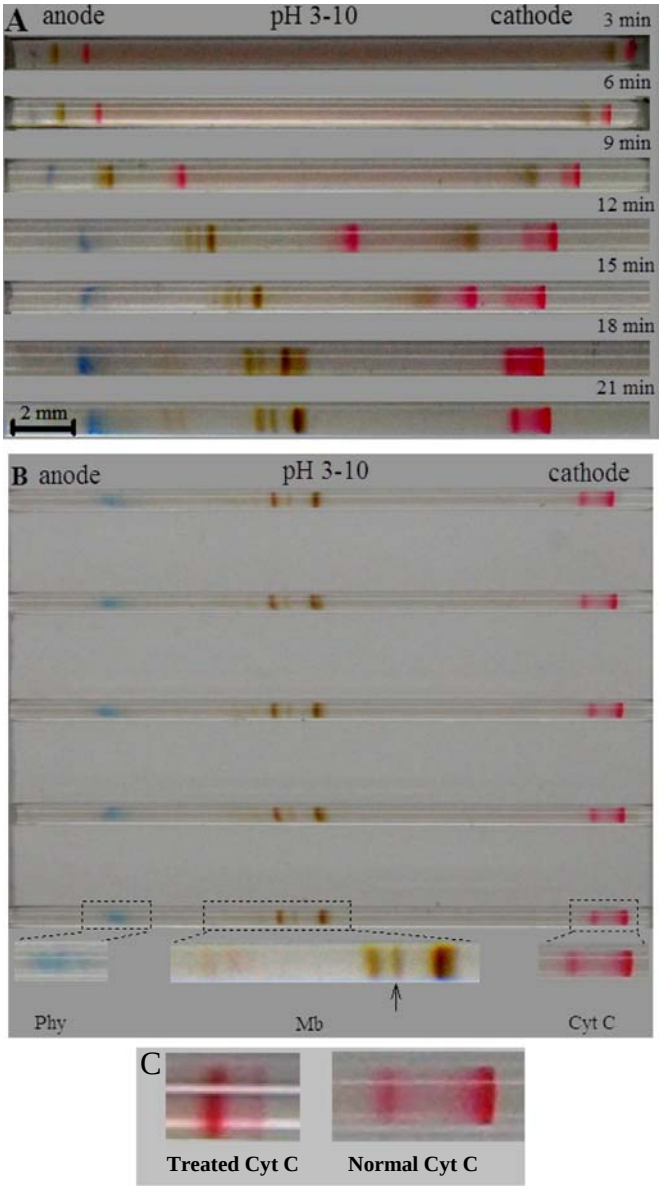

Fig. 5. Standard proteins of IEF. (A) Dynamic IEF process of the standard proteins (Phy, Mb and Cyt C). The time given at the right side indicated the running time. (B) Zoomed image of the focused bands of the standard proteins, the unknown Mb band was marked by the arrow. (C) Comparison of the focused bands of treated Cyt C and normal Cyt C. The experimental conditions are the same as those in Fig. 4.

Fig. S1A and 11 cm in Fig. S1B) are 3.5 and 5.5 times as long as that of capillary used herein, the developed IEF has still achieved rather good resolution.

An interesting phenomenon was observed that the focused Cyt C showed two peculiar bands. It is shown in Figs. 4 and 5B that the zoomed insertion of Cyt C has two distinct parts. In addition, there was some 'dispersed Cyt C' between the two ones. Such phenomenon has not been observed in the previous work [8] as well as Fig. S1 and S2 in the online supporting materials. It was well known that Cyt C is an indispensable protein on the electron transport chain, the amino acid sequence of which was comparatively conserved [31,32]. It has 104 amino acid residues without quaternary structure. Besides, the earlier work reported by Mines et al. [33] indicated that oxidized and reduced forms of Cyt C could transform into each other in the process of electron transport. Therefore, a possible explanation on the phenomenon might be as follows. Cyt C could carry and release the mobilized electron during IEF, the corresponding combination and dissociation states of Cyt C might result in tiny p*I* difference.

In order to rule out the possibility of variant, the Cyt C protein sample was analyzed by the commercial 2D LC-linear ion trap quadrupole (LTQ)-mass spectrometry (MS) (Michrom LC & LTQ XL, Michrom BioResources Inc., USA). The sequences of three detected core peptides of Cyt C were specifically matched the standard Cyt C from horse heart of *Equus caballus* (MS data and conditions please see the online supporting materials).

3.3. Speed of separation

Theoretically, the focusing time (t) was simply defined as the time of protein electromigration from the position of sample loading to its p*I* point (L). Assuming the average mobility of protein during its focusing ($\overline{\mu}$) and the uniform electric field ($\overline{E}$), one could approximately evaluate the time via the following

equation:

$$t = \frac{L}{\bar{\mu}\bar{E}} \quad (1)$$

Eq. (1) means that (1) high electric field can reduce the focusing time, and (2) a short separation channel can also decrease the time. In a chip-based IEF, up to 350 V/cm electric field can be used and the separation channel length is always less than 100 mm. Hence the focusing time is short (1.0–10 min), especially the electrophoresis performed in dynamic coating microchannel (no molecular sieve effect). However, in a traditional gel-based IEF, the focusing time is long (generally 3–6 h), because (1) the length of IPG gel is about 17–24 cm, (2) the electric field is about 150–220 V/cm, and (3) the molecular sieve effect of CPA gel.

Fig. 5A reveals the dynamic focusing of three standard proteins in a 20 mm long capillary. The real time focusing was recorded in every 3 min. It is unveiled that the mixed proteins are gradually focused into three sites in pH 3–10 gradient within about 20 min. The focusing time was less than one-tenth of that needed in the classic one. Despite the separation speed being slower than that of chip-based IEF, the developed method has the potential that if smaller i.d. capillary and cooling technique were employed, the electric field can be greatly increased, thus the separation speed of the array IEF may be further improved.

3.4. Linear relationship

Linear pH gradient was an ideal approach conveniently used for the detection of protein p*I* via IEF technique. We also investigated the linearity of pH gradient in the array IEF. Fig. 6 shows the focusing bands of three standard proteins of Phy, Mb and Cyt C distributed among the 20 mm length capillary. It was apparently manifested that (1) two known bands of Mb of p*I* 6.8 and p*I* 7.0 were focused 1.2 mm apart; (2) the center distance of two Phy bands of p*I* 4.75 and p*I* 4.85 were respectively at the distances of 6.4 mm and 6.1 mm from p*I* 6.8 band of Mb; and (3) the main band of Cyt C of p*I* 9.6 was focused at the distance of 9.4 mm from the Mb band of p*I* 7.0.The linearity of pH gradient was assessed by calculated the distance between the lowest p*I* (viz., pI 4.75 of Phy) and the highest p*I* (viz., p*I* 9.6 of Cyt C). It was shown that the linear equation was: $pI = 0.2852D_x + 4.8178$ (where, D was the distance between the unknown protein of p*I* focused at position D and the known zero position of p*I* 4.75 Phy), and the correlation coefficient was 0.9989, indicating quite good linear pH gradient. The high accuracy could be attributed to the introduction of the CPA coating on the inner wall of capillary, which might greatly decrease the EOF

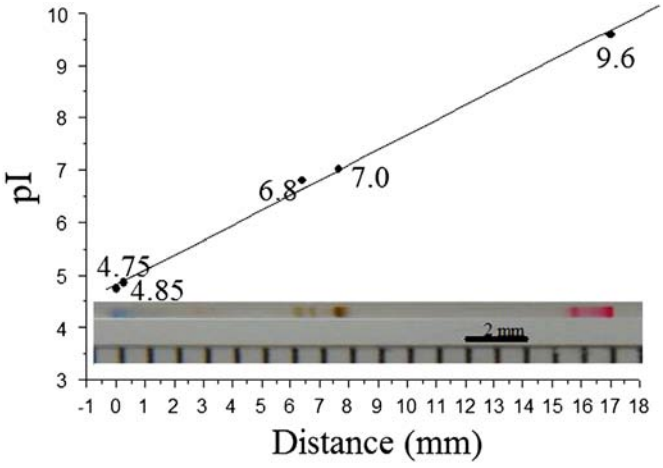

Fig. 6. Linear relationship of p*I* versus distance of standard proteins. The experimental conditions are the same as in Figs. 4 and 5.

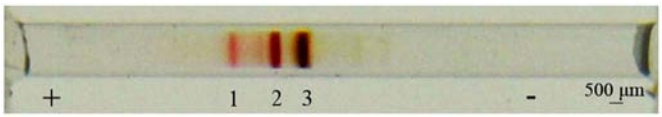

Fig. 7. Hemoglobin IEF from human adult blood cell. The numbers designate the following fractions: (1) HbA_3; (2) HbA_{1c} and (3) HbA_0, according to the previous work [32]. The final applied voltage was set at 400 V/cm. The capillary of 30 mm length was used. The other experimental conditions are the same as those in Fig. 4.

and simultaneously provide the necessary support and sieving matrix for the stabilization of the focused bands.

3.5. Separation of Hb samples

In order to simply demonstrate the use of the developed IEF device on the protein mixture, the separation of hemoglobin sample from human erythrocytes was conducted in the pH 6–8 range. A capillary assay of 30 mm length was used. The three main Hb species, HbA_3, HbA_{1c} and HbA_0, were separated in ~25 min. It should be noting that the p*I* difference between HbA_0 and its glycated form, viz., HbA_{1c}, was less than 0.03 pH units. Fig. 7 shows the images of Hb samples with 5.4% HbA_{1c} value in the developed array IEF. From the anode side to cathode side, three main Hb species was represented as HbA_3, HbA_{1c} and HbA_0 according to the previous work [21,34]. HbA_0 and HbA_{1c}, the two species with ΔpI 0.03 difference, were evidently detached within the 500 μm space. It was calculated that the resolution values of the developed and the traditional IEF methods [34] for the separation of Hb samples were about 2.0 and 0.3, respectively, obtaining a 7 fold performance improvement. To the best of our knowledge, the complete separation of the Hb fractions in such quasi-chip-scale IEF system has not been reported yet.

4. Conclusion

From the results discussed above, we could draw the following conclusions: (i) for analytical application separation of proteins, the most important performance characteristics of resolution, reproducibility and throughput were systematically displayed on the developed array IEF. Firstly, rather stable separation of proteins was successfully obtained due to the modified CPA coating and electrolyte design ensured the stabilization of pH gradient. Secondly, the resolving power of the developed method was fully unveiled by the high resolution separation of hemoglobin species from blood sample, in which the proteins with only ΔpI 0.03 were separated in such a miniaturized system; (ii) the utilization of the developed method for micropreparative separations of proteins was also exposed. It was apparent that the improved sample loading capacity was achieved by the use of 0.6 mm i.d. column. More importantly, the purification and extraction of trace amounts of interested proteins such as hemoglobin variants could be fulfilled by the integration of multi-unit array. Coupling to the further analysis techniques such as mass spectrometry or liquid chromatography, the developed device holds great potential for the micropreparative separation and in-depth analysis of proteins.

Acknowledgments

The authors are grateful for the funding provided by the National Natural Science Foundation of China (No. 21035004), the National Key Program of Scientific Instrument Development (No. 2011YQ030139) and Shanghai Jiao Tong University (No. YG2010ZD209). The LTQ MS

test was accomplished by Dr. Jing-Li Hou and Lei Feng in Instrumental Analysis Center of SJTU.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.05.041.

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