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Analysis of single erythrocytes by injection-based capillary isoelectric focusing with laser-induced native fluorescence detection

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Abstract

A modified version of capillary isoelectric focusing (cIEF) was developed to separate hemoglobin variants contained within single human erythrocytes. Laser-induced native fluorescence with 275 nm excitation was used to detect the separated hemoglobins. In this method, baseline fluctuations were minimized and detection sensitivity was improved by using dilute solutions of anolyte, catholyte, and carrier ampholytes (with methylcellulose). Since electroosmotic flow was used for mobilization of the focused bands, separation and detection were integrated into a single step. The capillary was first filled with only ampholyte solution, and the cell (or standard) was injected as in capillary zone electrophoresis. The ~90 fl injection volume for individual cells is 7×10^4 times lower than those previously reported. Adult (normal and elevated A_1), sickle (heterozygous), and fetal erythrocytes were analyzed, with the amounts of hemoglobins A_0, A_{1c}, S and F determined. The pH gradient for cIEF was linear (r^2=0.9984), which allowed tentative identification of Hb F_{ac}. Variants differing by as little as 0.025 p*I* units were resolved.

Keywords: Erythrocytes; Isoelectric focusing; Hemoglobin

1. Introduction

Capillary isoelectric focusing (cIEF) is a high-resolution mode of capillary electrophoresis (CE), in which amphoteric species (e.g., proteins) are separated according to their isoelectric points (p*I*). Several modes of cIEF now exist, however, the basic mechanism remains the same. The pH gradient is obtained by filling the capillary with carrier ampholytes – amphoteric substances which are neutral over a specified pH range. Acid is placed in the anodic (injection side) buffer vial, and base in the cathodic vial. Upon application of the electric field, a pH gradient is established along the capillary. Proteins migrate to the point at which pH equals p*I* and stop (i.e., focus). The focused bands are then detected, usually by a mobilization step in which they are swept toward the detector.

The high efficiency of this technique is well suited for the determination of hemoglobin (Hb) variants. Due to the difficulty in separating Hb by CE, most high resolution Hb separations are done by cIEF. However, there are a few reports in which Hb has been separated by capillary zone electrophoresis. The determination of globin chains using coated capillaries and denaturing conditions has been performed starting from Hb solutions [1–4] as well as from individual erythrocytes [5]. Intact Hb variants have been separated by reducing electroosmotic flow (EOF) with a coated capillary [6], as well as with

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uncoated capillaries using Tris, borate or barbital buffers [3,7].

Highly efficient separations of Hb variants by conventional cIEF have been demonstrated [1,2,8–15]. In these examples, the ampholytes are mixed with the sample, and the entire capillary is filled with protein. Typically, coated capillaries are used to eliminate EOF, so that when the proteins focus, they remain stationary. Subsequent mobilization is then necessary to sweep the zones past the detector. Cathodic [1,2,8–10], anodic [11,16], or pressure [16] mobilization steps are common when EOF is zero. In some cases, EOF is sufficient to focus and mobilize proteins in a single step. This has been demonstrated both in coated capillaries, in which EOF is reduced [9,14,17], and in bare fused-silica capillaries [18,19]. When bare capillaries are used, the addition of a dynamic polymer coating (e.g., methylcellulose) helps to reduce electroosmotic flow, as well as prevents adsorption of proteins to the capillary wall.

Detection for conventional cIEF is typically based on on-line absorbance, however, laser-induced fluorescence (LIF) was used by Shimura and Kasai [20] for the detection of labelled peptides. Foret et al. have used a combination of hydrodynamic flow and EOF to mobilize and elute Hb variants into a fraction collector, for subsequent analysis by mass spectrometry (MS) [13]. Wu and co-workers [21–24] have developed concentration gradient detectors in combination with short capillaries. They have extended this detection scheme to allow imaging of the focused bands with a CCD camera [25]. Recently, they have developed an imaging detector for cIEF based on fluorescence [26], and a similar method for the absorption imaging of Hb [15].

The cIEF conditions in the reports described above provide efficient separations of Hb variants for sample volumes >100 nl. However, such volumes preclude the analysis of single mammalian cells, which are around 1 pl in volume. Lower injection volumes are necessary, and several reports have demonstrated the use of sample volumes substantially less than the entire volume of the capillary. One mode of cIEF was developed by Thormann and co-workers [27–30], in which only a fraction of the capillary is filled with sample. This method, termed dynamic cIEF, utilizes an uncoated capillary and a polymeric additive, hence, EOF mobilization. Hemoglobins were successfully separated with this method. Smaller volumes (~40 nl) of Hb variants were injected and separated by Hempe and Craver [31]. However, the lowest injection volume reported thus far is 6.5 nl for the separation of RNase proteins by Chen and Wiktorawicz [32]. Both reports [31,32] utilized coated capillaries, relying on a combination of EOF and pressure to mobilize the proteins. Naturally, injection-based cIEF depends on the favorable mobility of the analyte, such that it reaches the focusing zone (pH=p*I*) before mobilization carries the zone beyond the detection window.

In this work, we report a separation scheme based on cIEF in which a single red blood cell (i.e., ~90 fl) is injected. To our knowledge, this represents the lowest volume for a sample in cIEF. Native LIF detection is used to determine hemoglobin variants in normal, diabetic, and sickle adult erythrocytes, as well as fetal (cord blood) erythrocytes.

2. Experimental

The experimental instrumentation used in this work has been described previously [5]. Briefly, a 21 μm I.D., 360 μm O.D. bare capillary was used (Polymicro Technologies, Phoenix, AZ, USA), with a total length of 40 cm (30 cm to detector). Electrophoresis was driven by a high-voltage power supply (Glassman High Voltage, Whitehorse Station, NJ, USA; EH Series; 0–40 kV). The applied voltage was +24 kV at the injection end, and was constant during the entire separation (i.e., focusing and mobilization). Before each run, the capillary was rinsed with 20 m*M* NaOH for 5 min, then rinsed (5 min) and filled with the ampholyte mixture. The ampholyte solution consisted of 0.5% Ampholine, pH 5.0–8.0 (Pharmacia, Uppsala, Sweden) and 0.1% methylcellulose, 25 cp (Aldrich, Milwaukee, WI, USA). A 24-bit A/D interface (ChromPerfect Direct, Justice Innovation, Palo Alto, CA, USA) was used to record electropherograms which were stored in a computer.

The 275.4 nm line of an argon-ion laser (Spectra Physics, Mountain View, CA, USA; Model 2045) was isolated with a prism and used as the excitation

source. The laser was focused with a 1-cm focal length quartz lens onto the capillary.

Fluorescence was collected with a 10× microscope objective (Edmund Scientific, Barrington, NJ, USA) and passed through two UG-1 color filters (Schott Glass Technologies, Duryea, PA, USA) onto a photomultiplier tube.

Unless otherwise noted, all chemicals were obtained from Fisher Scientific (Fair Lawn, NJ, USA). The anolyte and catholyte were 1 m*M* H_3PO_4 and 2 m*M* NaOH, respectively. Before each run they were prepared fresh from stock solutions of 10 m*M* H_3PO_4 and 1 *M* NaOH, and filtered with 0.22 μm cut-off cellulose acetate filters (Costar, Cambridge, MA, USA).

Hemoglobin A_0 standard (Sigma Chemicals, St. Louis, MO, USA) was injected hydrodynamically by raising the sample vial to a height of 11 cm relative to the detection end for 10 s (~0.14 nl injected volume). Sickle-Trol sickle cell hemoglobin controls (normal adult and sickle cell erythrocyte suspensions) were purchased from Dade International (Miami, FL, USA). Fetal (cord blood sample) and adult (elevated A_1) erythrocytes were obtained from Mercy Hospital (Des Moines, IA, USA). All cells were washed in the same way prior to injection: 10 μl of whole blood or erythrocyte suspensions were washed with phosphate buffered saline (PBS) which consisted of 135 m*M* NaCl and 20 m*M* NaH_2PO_4, pH 7.4. The cells were then centrifuged and the supernatant discarded. This procedure was repeated at least five times, with the cells finally suspended in a solution of PBS.

The procedure for injecting individual cells is the same as described previously [5]. Because high salt concentration can be detrimental to cIEF, approximately 200 μl of 8% (w/v) glucose was placed on a microscope slide. The inlet end of the capillary was inserted into this droplet, then 10 μl of cell solution (PBS) was placed on the slide near the capillary tip. A septum was placed over the outlet buffer vial to create an air-tight seal, into which a syringe needle was inserted. By applying gentle suction, one cell was injected and visually confirmed under the microscope. Following cell injection, the inlet end of the capillary was immersed in its buffer vial and electrophoresis initiated.

3. Results

3.1. Hemoglobin separations

The distinction between our cIEF protocol and others is the amount of sample injected into the capillary. Most previous reports have used absorption detection, where limitations in detectability have influenced the development of separation conditions. With less sensitive detection, a greater amount of sample must be introduced into the capillary (whether it is filled or injected). The separation mechanism depends critically on the integrity of the pH gradient and the ability of the ampholytes to buffer and focus the proteins. Therefore, it is not surprising that significantly altering the injected sample amount would require manipulation of the separation conditions.

In cIEF methods published thus far, anolyte and catholyte concentrations are at least 10 m*M* H_3PO_4 and 10 m*M* NaOH, respectively. These are typically used in conjunction with ampholyte concentrations of >1% [1,2,8–12,31], although lower concentrations [10–13,19,32] have been used. It has been reported that increased ampholyte concentrations lead to more stable pH gradients and higher resolution separations [10]. Likewise, when methylcellulose (MC) is used as an additive in either coated or uncoated capillaries, typical concentrations are greater than 0.2% [9,12,31,32], although 0.1% MC has been used [12,19]. When uncoated capillaries were used at near neutral pH, it was found that significant protein adsorption caused the pH gradient to deviate from linearity with decreasing MC concentration [12].

The above observations are probably indicative of the higher analyte concentrations used. Anolyte and catholyte concentrations must also be sufficient to allow a stable pH gradient to be formed. Furthermore, a certain level of ampholytes must be present to maintain the pH gradient, and to buffer and focus higher concentrations of proteins.

Native LIF detection of Hb from single red blood cells has been demonstrated previously for CE [5,33]. However, typical concentrations of ampholytes and MC in cIEF presented detection problems. For example, when 0.4% MC-25 and 1%

Ampholine (pH 5–8) were used with anolyte and catholyte concentrations of 10 mM H_3PO_4 and 20 mM NaOH, respectively, good separations of the variants from standard samples were seen (data not shown). However, baseline instability prevented us from achieving the level of detection sensitivity required for the analysis of single cells. When the concentration of the ampholyte mixture was decreased to 0.1% MC-25 and 0.5% Ampholine to improve detectability (with identical anolyte and catholyte), peak integrity was compromised and often even double peaks were seen for A_0. With the lower concentration of ampholytes in the capillary, hence, lower buffering capacity, 10 mM H_3PO_4 may have been sufficient to partially dissociate the Hb molecules, which were also present in low amounts.

In order to retain detection sensitivity, 0.1% MC-25 and 0.5% Ampholine were chosen as our final ampholyte mixture. However, to prevent Hb dissociation, the anolyte and catholyte concentrations were decreased to 1 mM H_3PO_4 and 2 mM NaOH, respectively. Anolyte and catholyte were prepared fresh before each run, to ensure that the pH of these solutions remained consistent despite the low buffering capacity. In Fig. 1, an electropherogram for Hb A_0 (standard) is shown in which these dilute conditions are used. The injected amount is about 290 amol, which corresponds to slightly less than the 450 amol of Hb in an individual erythrocyte [34]. Since this is about 10 300× less Hb injected than previously reported [31], it is reasonable that higher MC and ampholyte concentrations are not necessary to successfully focus such low amounts of Hb.

This dilute system was then applied to the analysis of individual erythrocytes containing different types of Hb variants. At least two single cell runs were performed for each cell type to confirm the peak patterns, however, only the data from the electropherograms displayed here were analyzed. Peak identification is based on migration times plus the known composition of the samples. For example, the next most abundant protein (carbonic anhydrase) is present only at 7 amol/cell and others are at least 1/10 below that amount [33]. Fig. 2 is the analysis of a normal adult erythrocyte. Fig. 3 is the separation of Hb A_0 and A_{1c} from a patient containing elevated Hb A_1. This sample was independently assayed by the hospital to give an A_1 value of 16.4%. We have identified peak 2 as A_{1c}, and determined it to be 12% of the total Hb (i.e., peaks 1+2). This differs from the hospital assay because Hb A_1 is comprised of several glycated forms of Hb, of which A_{1c} is the major component. Also, it is expected that the amount of a component determined in single cells will deviate from the average (bulk) value.

Other types of human red blood cells were also studied with these conditions. A single sickle cell

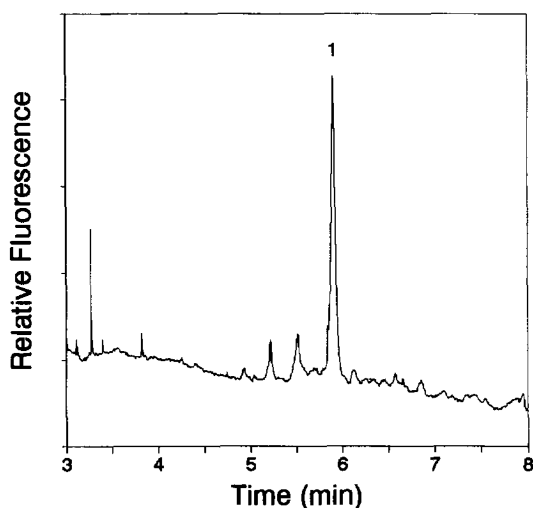

Fig. 1. Hemoglobin A_0 standard (peak 1), 2×10^{-6} M (0.14 nl injection). LOD is 3×10^{-8} M, or 4 amol (S/N=2).

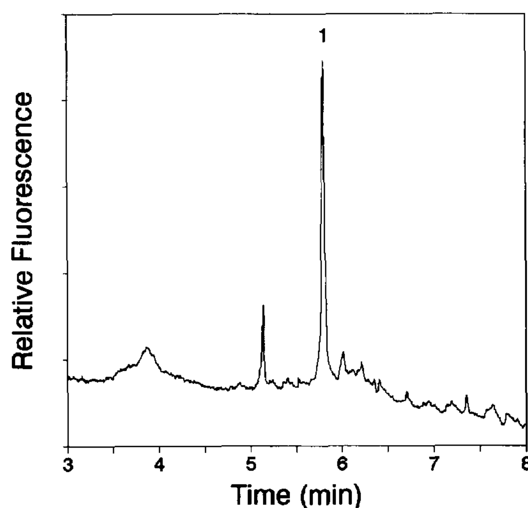

Fig. 2. Single red blood cell (normal adult). Peak 1 is Hb A_0.

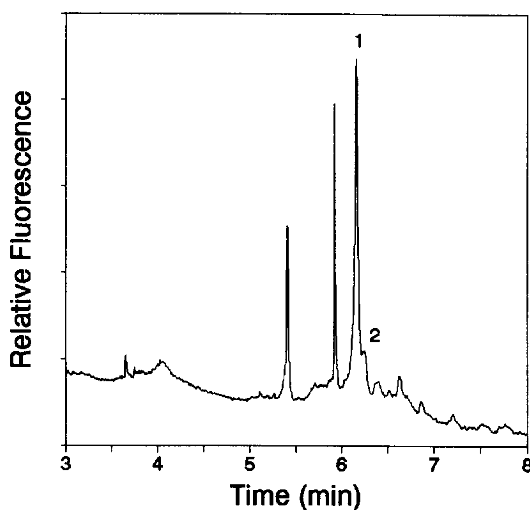

Fig. 3. Single red blood cell (elevated A_1). Hb peak identification: (1) A_0, (2) A_{1c}.

was analyzed and is shown in Fig. 4. This type of cell is heterozygous for the sickle trait, which means that the cell produces both Hb S and A_0 in approximately equal amounts. Our analysis revealed the amounts of Hb S and A_0 to be 44.7% and 55.3%, respectively. Fig. 5 shows the Hb separation from a single fetal (cord blood sample) erythrocyte in which Hb F and A_0 (peaks 1 and 2, respectively) were

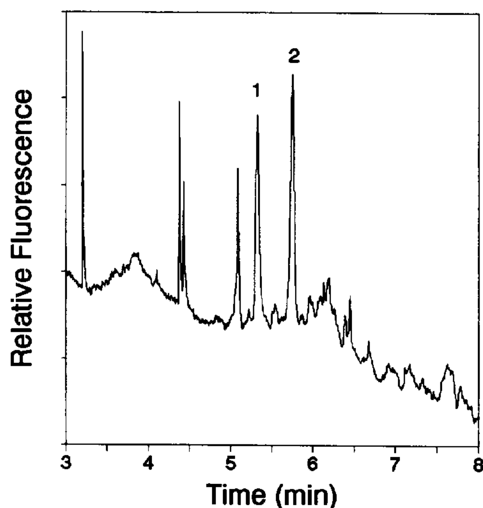

Fig. 4. Single sickle red blood cell. Hb peak identification: (1) S, (2) A_0.

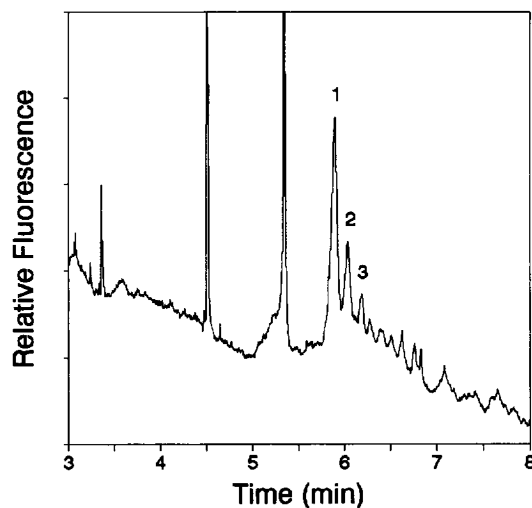

Fig. 5. Single fetal red blood cell. Hb peak identification: (1) F, (2) A_0, (3) F_{ac}.

identified. Linearity of the pH gradient is described in the following section, and is shown in Fig. 6. Based on this linear regression, peak 3 was tentative-

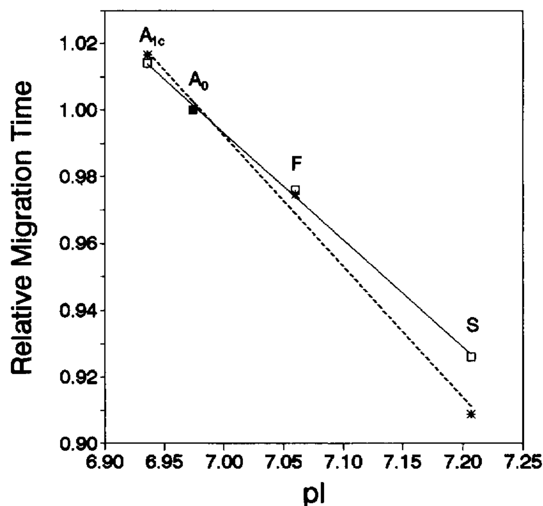

Fig. 6. Linearity of pH gradient. Relative migration times (vs. Hb A_0) are plotted as a function of literature p*I* values [31]. Dilute buffer conditions (solid line) are described in the Experimental section and gave r^2=0.9984. Concentrated (dashed line) buffer conditions gave r^2=0.9932, and are as follows: anolyte, 10 m*M* H_3PO_4; catholyte, 20 m*M* NaOH; ampholyte mixture, 0.4% methylcellulose (25 cp) with 1% Ampholine (pH 5–8); 50 μm I.D. capillary.

ly identified as acetylated fetal hemoglobin (Hb F_{ac}) with a calculated p*I* of 6.914, compared to a literature value of 6.911 [31]. In Fig. 5, total Hb F (i.e., peaks 1+3) is 73.3%, of which F_{ac} is 12.4%. Hb A_0 comprised 26.7% of the total Hb in this cell, which is in agreement with literature values.

3.2. Linearity of the pH gradient

The linearity of the pH gradient is shown in Fig. 6 (solid line). It was determined by plotting relative migration times (vs. A_0) of the Hb variants in single cells as a function of literature values of p*I* [31]. The relative migration times of S, F, A_0 and A_{1c} were 0.926, 0.976, 1.000 and 1.014, respectively. Linear regression analysis revealed respective p*I* values of 7.209 (7.207), 7.054 (7.060), 6.979 (6.974) and 6.935 (6.936); parenthetic quantities are the referenced isoelectric points [31]. A correlation coefficient (r^2) of 0.9984 was found using dilute conditions. As mentioned in the previous section, the p*I* of Hb F_{ac} was determined to be 6.914 (6.911) by this linearity plot. It is interesting to note that a peak occurs around 5.25 (±0.15) min in every electropherogram. The relative migration time of this peak is 0.884, with a calculated p*I* of 7.341. Hb A_2 has a p*I* of 7.411 [31], however it is unlikely that the peak is A_2. Its concentration is only about 3% in normal red cells, and in all cells analyzed the concentration of this component was much greater than that. Also, all other Hb variants determined with this system had a calculated p*I* within 0.006 p*I* units of the literature value. Because the difference between A_2 and our unknown peak is 0.07 p*I* units, this peak is not assigned as A_2 and remains unidentified at this time.

The linearity of a gradient with more concentrated buffer conditions and hemolysate samples (instead of single cells) is also plotted in Fig. 6 (dashed line). The correlation coefficient of this system is 0.9932. Comparing the two plots, it is apparent that more concentrated conditions lead to a steeper gradient. With such a gradient, resolution is improved because for the same Δp*I*, there is a greater difference in migration times. However, detection is compromised and these conditions do not allow Hb in individual cells to be determined.

4. Conclusions

In this work we have developed an injection-based cIEF method well suited for the analysis of very small sample volumes, such as individual cells. By diluting all buffer components, baseline stability was improved and attomole detection limits were reached, which were necessary to determine Hb in a single cell. Adult (normal and diabetic), fetal and sickle erythrocytes were analyzed. A linear pH gradient (r^2=0.9984) served to confirm the peak identities for Hb S, F, A_0 and A_{1c}. In addition, it allowed identification of Hb F_{ac} in a single fetal erythrocyte. The eluted peaks are sharp and well defined, further confirming that focusing is complete. The performance of the system is sufficient to determine analytes with a p*I* difference as low as 0.025, and may be used to identify proteins with unknown isoelectric points.

Acknowledgments

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