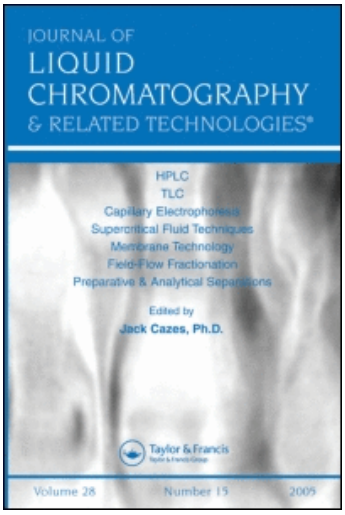

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Capillary Isoelectric Focusing with Imaging Detection

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CAPILLARY ISOELECTRIC FOCUSING WITH IMAGING DETECTION

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ABSTRACT

A simple on-line absorbance imaging detection system was constructed for detection of capillary isoelectric focusing (CIEF). Because of the use of the CIEF-imaging detection system, separation and detection of protein sample could be completed in 2 minutes. The use of the imaging detection system also allowed for studying the focusing process. When a 4 cm long, 200-μm i. d. square capillary was used as the separation column, the concentration detection limit of the detector reached 5 μg/mL. This is the first report of applying absorbance imaging detection system to capillary electrophoretic techniques. The absorbance imaging detection system was compared with the refractive index gradient imaging detection system. For the capillary with large i. d., the absorbance imaging system showed higher sensitivity than the refractive index gradient imaging detector. However, the refractive index gradient imaging system was more suitable for narrow capillaries because it applied a laser as the light source which could be easily focused into narrow capillaries. The sensitivity of the refractive index gradient imaging detector was expected to be higher for narrower capillaries than the absorbance imaging detector.

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INTRODUCTION

Capillary isoelectric focusing (CIEF)[1] is beginning to attract attention among analytical chemists and biochemists because it is an ideal tool for characterization of protein samples, such as antibodies, due to its high resolution and separation speed[2-4]. Small amounts of proteins (in nL) with isoelectric point (pI) differences as small as 0.02 can be separated and detected in 15 - 20 minutes. In conventional CIEF, all focused protein zones have to be moved inside the separation capillary, after the focusing process, through the detection point of an on-column detector which is located at one end of the capillary[1]. The mobilization is non-linear, which results in deterioration of resolution[2], long analysis times[2], and difficulty for determination of pI values for protein samples[5]. Therefore, an on-line imaging detection system will be the best detector for CIEF. The use of an on-line detector also allows for on-line optimization of experimental conditions and study of focusing mechanism in CIEF.

Several new imaging detection schemes have been developed for CIEF. An electrode array detector could be used as an on-line imaging system for CIEF for study of focusing process[6]; however, the resolution of the detector is low due to the limited number of the electrode. The focusing process of blue dye stained proteins in a capillary was monitored by photographic method under the illumination of visible light[7]. However, it is difficult to obtain quantitative information using photographic film in the method. The focused protein samples could be quantitatively determined by a whole column absorbance detection method[8]. In this method, all the focused protein zones inside the capillary were detected using a single point UV-vis absorbance detector by moving the capillary after the focusing process. This method is not a on-line detector, and can not be applied to studies of focusing process. Also, zone broadening caused by diffusion during detection is

unavoidable because the capillary is transferred to the detector after focusing[8].

A real on-line imaging detection system, which was based on the detection of the refractive index gradient generated by a concentration gradient, was developed for CIEF in our laboratory[9,10], by which separation and detection was combined into one step, and analysis of protein mixtures could be completed in 1 - 3 minutes[10]. The detection system could also be used for on-line optimization of focusing conditions and study of focusing processes[10]. This detection system can be modified to become a simple on-line absorbance imaging detector by using a visible light lamp source and a lens. In this paper, we will show preliminary results of applications of the on-line absorbance imaging detector and compare the results to those when the concentration gradient imaging detector is used. Human hemoglobin was used in the research as a model sample because of its absorption bands in the 400 - 600 nm visible light range.

MATERIALS AND METHODS

Instrumentation

A 200-μm i. d., 4-cm long square glass capillary was used as the separation column when the absorbance imaging detection was applied. A same length, 100 μm i. d. square glass capillary was used for the concentration gradient imaging detection system. The capillary inner wall was coated with non-cross-linked acrylamide to eliminate electroosmosis by the reported method[3]. The capillary cartridge used was the same as that used in our previous research[9,10]. The sample focusing was driven by high-voltage dc power supply (RE3002B, Northeast Scientific, Cambridge, Mass.). The current passing through the capillary during the focusing process was monitored at the cathodic end of the capillary.

In the absorbance imaging detector, the light beam source was a halogen lamp. The light from the lamp was collected by a paraboloidal reflector which reflected light onto a mirror. The light beam was filtered by color filter which was transparent in 400 nm - 600 nm wavelength range. The light beam was then focused into a 200 μm width slit and was focused again into the separation capillary. The image of the capillary illuminated by the light beam was projected by a 10 cm focal length lens onto a 1024 pixel charge-coupled device (CCD) sensor (S3903-1024Q, Hamamatsu, Hamamatsu City, Japan) which had a 25-mm X 0.5-mm sensing area. The changes in the light beam intensity profile due to the refractive gradient inside the capillary was eliminated by focusing the image of the capillary onto the detector plane. The data was collected by an IBM DACA board, in a PC-AT personal computer. A averaging method was applied to reduce the random noise, in which for each measurement the CCD scanned for ten times in 1 s and these scans were averaged. The background image which was recorded before the focusing voltage was turned on, was first subtracted from sample images, and the sample images were then normalized by the background image to eliminate fluctuations created by the source beam intensity distribution. All other instruments were the same as those used in previous experiments[9,10].

Reagents

Human hemoglobin sample was purchased from Sigma Chemical Co., which contained 75% of methemoglobin, balanced primarily with oxyhemoglobin. All chemicals were reagent grade, and solutions were prepared using deionized water. 10 mM H_3PO_4 and 20 mM NaOH were used as anolyte and catholyte, respectively. Protein samples were mixed with the carrier ampholytes (Pharmalyte pH3-10, Sigma) solution to a final concentration of 2% ampholytes[2]. Solutions were filtered using

0.2-μm pore size cellulose acetate filters (Sartorius, Gottingen, Germany) prior to use.

Isoelectric focusing process

The samples were loaded into the capillary by pressure. A plug of an 1% agarose gel (prepared in the anolyte) was introduced into the reservoir at anodic end of the capillary to avoid hydrodynamic flow during introduction of the catholyte and focusing process. A 2.5 kV dc voltage was applied in focusing process for the 200 μm i. d. capillary, and for the 100 μm i. d. capillary, the focusing voltage was 4 kV. The current which passed through the capillary dropped from 50 μA to 3 μA in 2 minutes before stabilizing. During the focusing process the capillary was continuously monitored by the imaging detection systems, and the detection completed in 2 - 2.5 minutes when the focusing process finished.

RESULTS AND DISCUSSION

Unlike other capillary electrophoretic techniques, in CIEF, all components in a protein sample are focused inside the capillary during the focusing process. The best way to detect those focused zones without mobilization is using an imaging detection system. Compared to conventional CIEF on-column absorbance detectors, the absorbance imaging detector with the large CCD sensor facilitates high analysis speed, good sensitivity and high resolution. The detection system combines separation and detection steps into one step. Combined with a short capillary, fast separation and detection of protein samples can be achieved in short times. Figure 1 shows focusing process of the human hemoglobin samples at total concentration of 60 μg/mL monitored by the

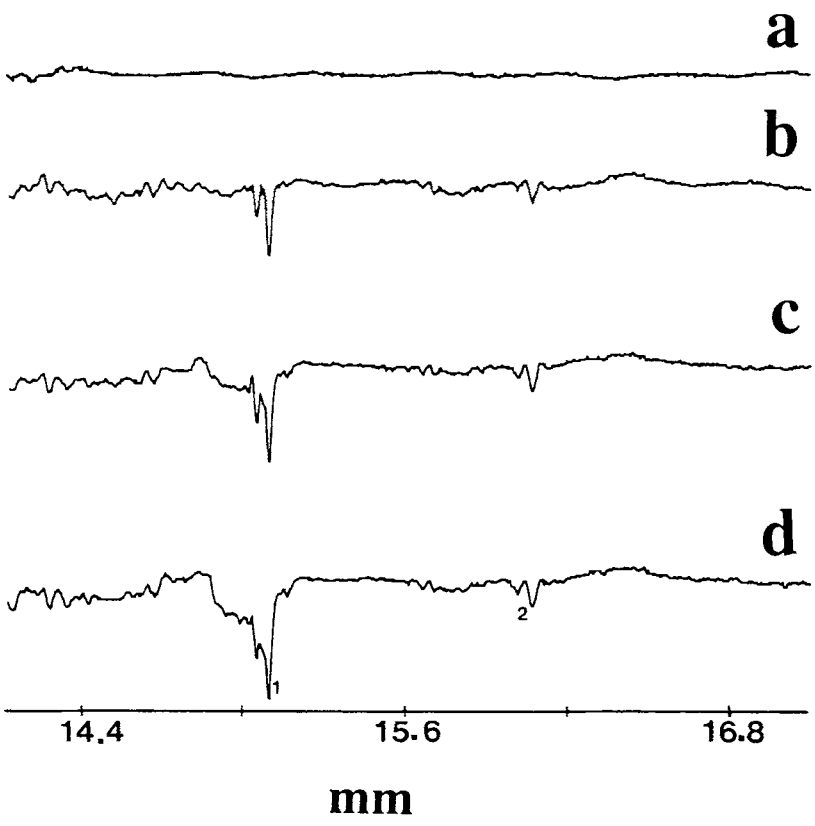

FIGURE 1. Absorbance images of focused human hemoglobin sample in the capillary. Total concentration of the sample; 60 μg/mL. (a) 3 s after focusing; (b) 0.5 minutes; (c) 1 minute; and (d) 2 minutes.

absorbance imaging system. Peaks 1 and 2 correspond to two variants of hemoglobin; methemoglobin (pI 7.2, comprises 75% of the sample) and oxyhemoglobin (pI 7.0, comprises less than 25% of the sample), respectively. Other peaks may correspond to other variants of hemoglobin[11]. The results show that separation and detection can be completed in 2 minutes, which is much faster than the speed of conventional CIEF with mobilization process, 15 - 20 minutes[1].

Although a short capillary is used in the separation, the resolution of the detection is still high due to the use of the high resolution CCD sensor. The resolution of the detector is estimated to be less than 0.02 pH units from the peak width of peak 2 and distance between peaks 1 and 2 in Fig. 1. The resolution is the same order of magnitude as that of conventional on-column single point absorbance detectors[1], and better than the whole column scanning absorbance detector[8]. In the whole column absorbance detection, the capillary needs to be removed from the focusing voltage and placed in the scanning stage for detection. In the detection, diffusion of the focused zone deteriorates the resolution. However, the on-line absorbance imaging system can detect sample zones inside the capillary while the focusing voltage is applied, which eliminates the zone broadening caused by diffusion.

The use of the imaging system also allows for study of focusing process and on-line optimization of separation conditions[10]. An interesting phenomenon is observed by the imaging system. The focusing of the hemoglobin sample is very fast, only about 0.5 minutes as shown in Figure 1b. The electropherogram at 0.5 minutes shows the best resolution, although all zones become stable after 1 minute. This observation can not be obtained with a conventional on-column detector or capillary scanning detector because they are not on-line detectors.

The detection limit of the detection system is about 5 μg/mL from the signal-to-noise ratio of peak 2 in Fig. 1, which corresponds to 7.8 X 10^{-8} M from the molecular weight of hemoglobin, 64,500. A advantage of using the fast CCD sensor is the possibility of signal-to-noise ratio enhancement by averaging method. The detection limit is expected to be lower if more images are scanned and averaged by the detection system[10]. The high speed CCD sensor can scan the capillary many times in a short time. The scanning speed of the present CCD reaches 0.3 ms/scan. The averaging method has proved to be a effective way to increase signal-to-noise ratio of the imaging detector[10]. The only

limitation for fast scanning of the CCD under the present experimental conditions is the speed of the A/D board. Although averaging method can be used in the whole column absorbance detection method[8], accumulation of many scans is impossible due to the low speed of capillary scanning by mechanical stage (more than one 1 minute per scan). Also, the scanning of the capillary adds extra noise to the detection system.

The absorbance imaging detector is compared with the concentration gradient imaging detector[10]. Because of the use of a incoherent light source in the absorbance imaging detector, it can not be applied to CIEF with narrow capillaries. Under the present experimental conditions, it is difficult to detect capillaries with an i. d. less than 200 μm. Use of narrow capillaries in the absorbance imaging detector requires a wavelength adjustable laser working in UV range since most proteins only have absorbance bands in UV range. However, the concentration gradient imaging detector can be used for narrow capillaries. Because it is based on the detection of refractive index gradient, a inexpensive, low power He-Ne laser or even a diode laser can be used as its light source[10, 12]. Figure 2 shows the electropherogram of the same hemoglobin sample as that in Fig. 1, which is focused in a 100 μm i. d. capillary and detected by the concentration gradient imaging detection system. The resolution of the concentration gradient imaging detector shown in Fig. 2 is better than that of the absorbance imaging detector due to the second derivative nature of the concentration imaging detector which eliminates broad bands and fluctuations.

The sensitivities of both of the imaging detectors are compared. The relationship between the maximum concentration in a focused zone, C_{max}, and the concentration of introduced sample, C_0, can be written as[13]:

$$C_{max}=C_0 L/\sqrt{2\pi}\sigma_x \qquad (1)$$

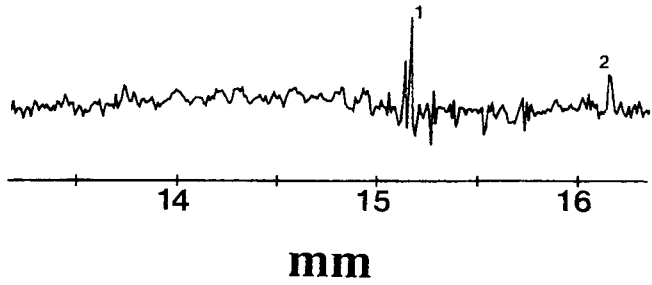

FIGURE 2. Concentration gradient image of focused human hemoglobin sample in the capillary after 2 minutes focusing. Total concentration of the sample; 100 μg/mL.

where, L is the overall length of the capillary, and σ_x is the standard deviation of the concentration distribution of the zone. The absorbance coefficient of hemoglobin[11] in 400 nm - 600 nm is less than 10^5 $cm^{-1}M^{-1}$. The absorbance detection limit of the absorbance detector can be assumed to be 1 X 10^{-3}. The detection limit for C_{max} is 5 X 10^{-7} M for the 0.02 cm i. d. capillary. By Equation 1, this value is calculated to corresponds to 2.1 X 10^{-9} M of the introduced concentration for the hemoglobin zone shown in Fig. 1d, which has a zone width of about 250 μm. The experimental detection limit, 7.8 X 10^{-8} M, is about 40 times higher than the theoretical value. This can be explained by the following two reasons. In the imaging detector, the noise level of the detected image depends mainly on the noise level of the light beam intensity profile which should be higher than the noise level of the whole beam intensity. Also there is absorbance background due to the carrier ampholytes[11]. As mentioned above, the noise in beam intensity profile can be reduced by the averaging method. As for the concentration gradient imaging detector, the theoretical detection limit under the optimum conditions is about 10^{-8} M[13]. The experimental detection limit

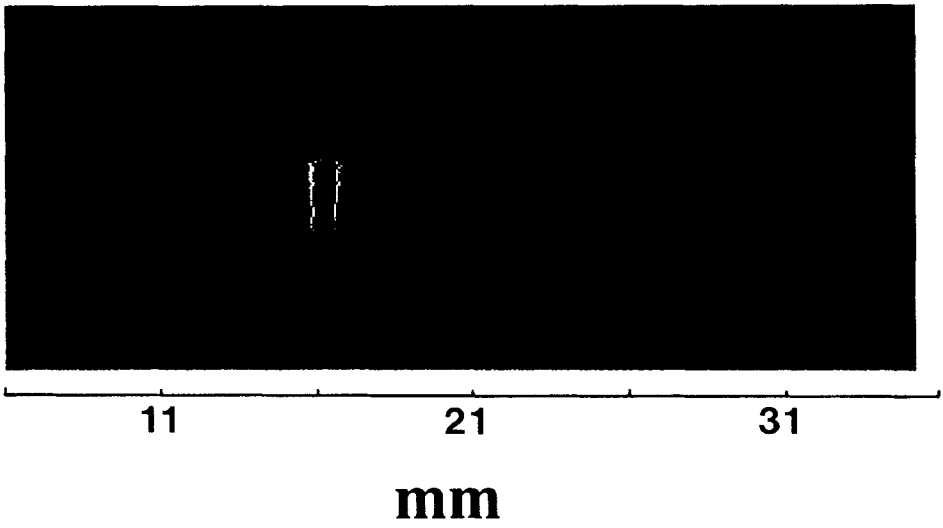

FIGURE 3. Two-dimensional image of focused human hemoglobin sample in a 100 μm i. d. capillary after 2 minutes focusing detected by the 1200 X 256 pixel two-dimensional CCD sensor. Total concentration of the sample; 300 μg/mL.

obtained from Fig. 2 is about 1.5 X 10^{-7} M, which is higher than the theoretical value. This is due to the existence of a high concentration of the carrier ampholytes stacking along the capillary[13]. This experimental detection limit is two times higher than that of the absorbance imaging detector under the conditions. These results show that the absorbance imaging detector has higher sensitivity for CIEF when capillaries with large i. d. are used. For the narrower capillaries, higher focusing voltage can be applied, which generates narrower sample zones. For the absorbance detector, its sensitivity is proportional to $1/\sigma_x$, and for the sensitivity of the concentration gradient detector[13], it is proportional to $1/\sigma_x^2$. Therefore, the detection limit of the concentration gradient imaging detector is expected to be lower than that of the absorbance imaging detector.

It should be noted that these two imaging detectors used in the research are inexpensive, each of which costs about $3,000, respectively, and they only use the commonly available components. They are also applicable to isoelectric focusing performed in capillary array[14]. In the future, the imaging detector is expected to replace the single point on-column detectors for the detection of CIEF.

When a large two-dimensional CCD is used in the imaging detectors, they can not only be applied to CIEF performed in capillary array, but also can be used to study the focusing mechanism inside the capillary. For example, when a 1200 X 256 pixel two dimensional CCD sensor is used to observe the focused hemoglobin zones, as shown in Fig. 3, the sample zone shapes inside a 100 μm i. d. capillary can be observed. Figure 3 shows that the hemoglobin zone positions along the capillary axis are not the same at the places near capillary wall and at the center of the capillary. This observation can not be made without the use of the two dimensional CCD.

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