The Analyst COMMUNICATION

Axially illuminated fluorescence imaging detection for capillary isoelectric focusing on Teflon capillary

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Received 26th April 2000, Accepted 25th May 2000
Published on the Web 14th June 2000

An axially illuminated laser induced fluorescence (LIF) whole column imaging detection (WCID) method was developed for capillary isoelectric focusing (CIEF). In this method, an argon ion laser was used as the excitation source and the isoelectric focusing process was monitored dynamically by a thermoelectrically (TE) cooled (charge coupled device) CCD camera. Low refractive index PTFE capillary was used in the experiment, and electroosmotic flow (EOF) and protein adsorption were minimized by dynamic conditioning of the capillary with methyl cellulose (MC). With the addition of a small amount of high refractive index organic solvent such as glycerol in the sample mixture, the laser beam was axially propagated with total internal reflection. Stray light and scattering light originating from the wall of the capillary were minimized. The instrumentation is simple and the method is sensitive. Two naturally fluorescent proteins (*R*-phycoerythrin and green fluorescent protein) were separated, and a concentration detection limit (LOD) of 10^{-13} M or mass LOD of 10^{-19} mol for *R*-phycoerythrin was obtained.

Introduction

Capillary isoelectric focusing (CIEF) is a high resolution capillary electrophoresis (CE) technique for the separation of proteins and other zwitterionic biomolecules.[1,2] It has most often been used to separate closely related proteins with subtle differences in their structures. In conventional single point on-column detection CIEF, the focused zone within the capillary must be moved, chemically or electroosmotically, past the point to be detected. The mobilization step in CIEF requires extra time and distorts the focused zone, thus making it difficult to obtain reproducible qualitative and quantitative results. To eliminate the mobilization step in CIEF, imaging detection has been explored in the past decade. Wu and Pawliszyn demonstrated a concentration gradient imaging system in 1992.[3] A photodiode detector driven by a micro-syringe pump and a photodiode array were used to measure the light intensity, this enabled the focusing process in 3 cm of a 5 cm separation capillary to be monitored. This on-line detector greatly reduced the analysis time of CIEF from around 20 min to less than 5 min. Whole column imaging detection (WCID) initiated by Wu and Pawliszyn has been applied to UV,[4,5] concentration gradient,[6] and LIF[7] using a (charge coupled device) CCD camera, and UV WCID has been successfully commercialised.[8,9] As the detection limit of UV WCID is normally relatively high (usually 10 μg mL^{-1} for protein), a more sensitive WCID method is needed for broader application of WCID CIEF. At present, LIF provides the lowest detection limit in CE. As low as a single molecule can be detected.[10] In LIF, the careful alignment of the excitation light with the small id capillary and the collection of emission fluorescence are very important. The optical alignment is crucial in imaging detection, because it is much more difficult to obtain an homogenous background signal when a longer capillary is used. The short excitation path of the excitation light and the ease of interference of the fluorescence signal by the scattering light coming from the wall of the capillary limits the improvement in sensitivity of the LIF method. To minimize the interfering light and to increase the path length of the incident light, axially illuminated LIF for CE has been explored. Taylor and Yeung reported an axial-beam laser excited fluorescence in CE.[11] Total internal reflection of incident light inside the capillary was realized by using a high refractive index solvent dimethyl sulfoxide (DMSO) in a fused silica capillary. An LOD of 6×10^{-12} M of rhodamine 6G was estimated. Johansson and Nilsson demonstrated fluorescence imaging of light absorption for axial-beam geometry in CE.[12] The probing UV light was introduced axially to one end of the capillary. Based on light absorption along a separation capillary, the loss of fluorescence intensity of sample peaks can be detected by a CCD camera.

In this manuscript, we report on an axially illuminated LIF WCID for CIEF. In our experiment, glycerol (20%) was added to the sample mixture, and the incident laser beam entering the capillary axially was propagated with total internal reflection. The light loss and scattering light originating from the capillary wall was sharply reduced. The developed method greatly improves the sensitivity of WCID for CIEF.

Experimental

Apparatus

A schematic of the axially illuminated LIF WCID is shown in Fig. 1. In the system, a small UV lens (A4869, Hamamatsu Inc, Hamamatsu, Japan) was coupled to an UV-sensitive CCD camera, which was controlled by a ST-130 controller (TEA/CCD-1752/PF/UV, Princeton Instrument, Trenton, NJ, USA). An edge filter (LL-550-s-577 D, Corion, Franklin, MA, USA) was placed in front of the CCD camera. The CCD camera (f = 50 mm) was thermoelectrically (TE) cooled down to −40°C and the focal length was adjusted to about 22 cm to get a clear image of the separation capillary. The electropherogram was obtained by binning perpendicular to the capillary axis to get a

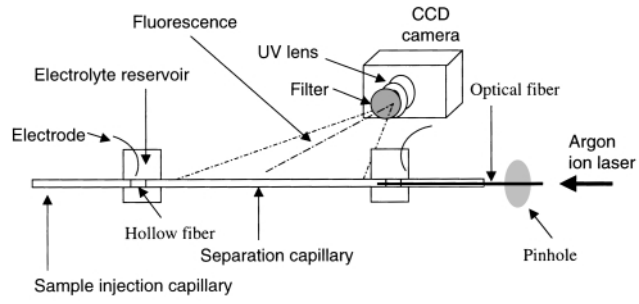

Fig. 1 Instrument set-up for the axially illuminated LIF WCID for CIEF.

better signal to noise ratio. The power supply was a RE-3002B (Regulated High-voltage supply, Mass, Northeast Scientific Corporation, Cambridge, MA, USA). An air-cooled argon ion laser (Cyonics, San Jose, CA, USA) was used as the excitation source. An *x-y* stage was used to facilitate adjustment of the capillary cartridge to let the laser light pass axially through the capillary. The cartridge of the Teflon capillary is also shown in Fig. 1. A glass plate was used as a support for the cartridge. The separation capillary was a 6 cm long polytetrafluoroethylene (PTFE) or fluorinated ethylene propylene (FEP) capillary. One end was connected with another piece of the same capillary and the other end was connected to a piece of 100 μm id UV-transparent capillary, by small pieces of hollow fiber. A piece of optical fiber was inserted into the end of the Teflon capillary to facilitate introduction of the laser beam. The sample was injected from the fused silica capillary end. Interference produced by stray light and scattering light was eliminated by use of a pinhole for holding the laser beam introduction optical fiber.

Materials and chemicals

Optical fibers with 100 μm core and 61 μm core (FVP100110125, FHP061067075A) were purchased from Polymicro Technologies Inc (Phoenix, AZ, USA). Microporous hollow fiber of 0.03 μm pore size and 383.3 μm id was obtained from Hoechst Celanese (Frankfurt, Germany). Polytetrafluoroethylene (PTFE) capillaries of 100 μm id, 229 μm od, and fluorinated ethylene propylene (FEP) capillary of 305 μm id, 620 μm od were obtained from Zeus (Raritan, NJ, USA). PTFE capillaries of 102 μm id, 406 μm od, 203 μm id, and 406 μm od were obtained from Cole-Parmer Instrument Co. (Vernon Hills, IL, USA).

R-phycoerythrin was purchased from Calbiochem-Novabiochem Corporation (La Jolla, CA, USA). The green fluorescent protein (GFP) sample solution was received as a gift from Convergent Bioscience Ltd, and the concentration was 0.5 mg mL^{-1}. Methyl cellulose, glycerol, and Pharmalyte of pH 3–10 were obtained from Sigma (St Louis, MO, USA) and were of analytical grade. Water was purified using an ultra-pure water system (Barnstead/Thermolyne, Dubuque, IA, USA), and was used for all solutions.

Sample was prepared by mixing spiked protein, carrier ampholytes (CAs) and a desired solvent.

Results and discussion

WCID is usually conducted in electroosmotic flow (EOF) controlled fused silica capillary cartridges, in which the focusing process can be monitoring in a real time mode by a CCD camera. However, total internal reflection is difficult to implement in fused silica capillaries, because of the high refractive index of fused silica (approximately 1.467). The CIEF process is a unique CE separation mode in that a high percentage of organic solvent may cause protein precipitation, or make isoelectric focusing impossible. Teflon and plastic capillaries have been investigated as separation capillaries for CE for their flexibility, durability, stability in basic buffer, and most importantly the low refractive index. EOF should be eliminated or minimized in CIEF, and surface modification and sample additive modification are commonly used for this purpose. Ren and Lee reported a method to control EOF in plastic capillary by surface modification of the capillary with cellulose.[13] Wu also recommends conditioning fluorocarbon coated capillaries with methyl cellulose (MC) solution.[8] During our experiments, if the Teflon separation capillary was not conditioned with MC solution, the EOF was so strong that focusing would not complete. It was found that EOF was virtually eliminated in the Teflon capillary after conditioning by 0.35% MC for half an hour. EOF was not observed even in the absence of MC additive in the sample mixture. The dynamic focusing of *R*-phycoerythrin (3.3×10^{-10} M) is shown in Fig. 2. To prevent sample photodegradation, the laser was turned on only during imaging. From Fig. 2, it can be seen that the fluorescence intensity was nearly homogenous across the entire capillary before applying a high voltage. Once the laser was turned on, photo bleaching was more serious closer to the inlet of the laser beam during focusing.

Two naturally fluorescent proteins, *R*-phycoerythrin and green fluorescent protein (GFP) were separated by the established system. The maximum emission wavelengths for *R*-phycoerythrin and GFP are 560 and 515 nm respectively. When no filter was used, the interference light was too strong to observe the fluorescence signal, while only GFP could be detected when using the 515 nm interference filter. Both *R*-phycoerythrin and GFP could be detected when the edge filter was used, with the sacrifice of the sensitivity of GFP. Fig. 3 shows the dynamic focusing of the two naturally fluorescent proteins (*R*-phycoerythrin and GFP were 1.7×10^{-10} M and 1.8×10^{-8} M, respectively). It can be seen that *R*-phycoerythrin was very well separated from the three peaks of GFP.

Direct illumination of the separation capillary by the laser beam was also investigated. The fluorescence intensity didn't change significantly, although the optical fiber illumination produced a better signal to noise ratio (S/N), and less interference. The laser beam was introduced by a piece of optical fiber mounted axially into the separation capillary, so that most of the scattering light was eliminated in axially illuminated LIF. Since both 488 and 514.5 nm were used to excite GFP and *R*-phycoerythrin respectively, no filter was used to select a narrow band excitation light, thus stronger incident light was obtained. Because the edge filter could not discriminate the unwanted luminescence coming from the wall of the Teflon capillary, and the scattering light by the submicron particles in the sample mixture, the background interference

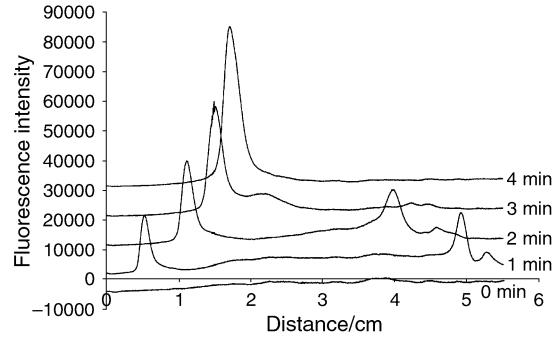

Fig. 2 Dynamic focusing of the naturally fluorescent protein *R*-phycoerythrin. The separation capillary was 200 μm id PTFE. The protein sample concentration was 3.3×10^{-10} M, containing 2% carrier ampholytes, and 20% glycerol. 20 mM of phosphoric acid and 40 mM of sodium hydroxide were used as anolyte and catholyte respectively. The exposure time of the CCD camera was 10 ms. 3 KV focusing voltage was applied.

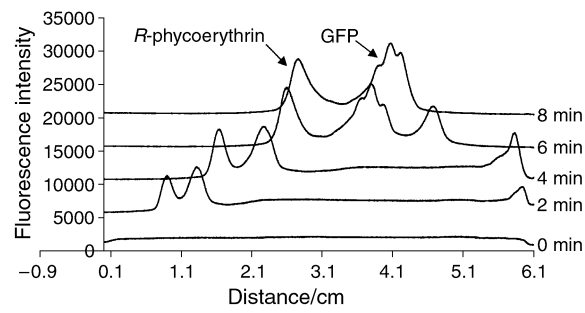

Fig. 3 Dynamic focusing of two naturally fluorescent proteins *R*-phycoerythrin (1.7×10^{-10} M) and green fluorescent protein (1.8×10^{-8} M). The separation conditions are the same as in Fig. 2, except the exposure time of the CCD camera was 250 ms.

was significant during longer exposure time. Under these conditions, an LOD (S/N = 3) of 10^{-13} M for *R*-phycoerythrin was still obtained.

CCD is an ideal imaging detector for LIF. The dark current noise (N_d) and the read noise (N_r) are very low in a TE cooled CCD camera, thus photo shot noise (N_s) is dominant. The total noise (N_T) in a CCD detector is defined as[14]

$$N_T = \sqrt{N_s^2 + N_r^2 + N_d^2} \quad (1)$$

N_s refers to photons arriving randomly at the detector, and is equal to the square root of the number of photogenerated charges; N_d refers to charge accumulating in the detector when not being exposed to radiation (a few electrons per pixel per second); N_r refers to the random movement of charge in the device and associated readout electronics (also a few electrons per pixel per second).

Because N_r ($N_r = \sqrt{kTC}$, where k is Boltzmann's constant, T is temperature, and C is capacitance) is independent of the magnitude of the signal, the S/N improves in direct proportion to the signal level. The signal can be increased by longer integration times or by an increase in the incident light intensity, which is easily accomplished in LIF CCD detection.

From the electropherogram, it was also noticed that the peak was much broader than those obtained from UV WCID. This may partly be due to the fact that the collected signal in LIF is emission (all direction), while in UV it is absorption (one direction); or due to protein adsorption as protein/capillary surface interaction is more serious in plastic capillaries than in modified fused silica capillaries. Suitable resolution may be achieved by applying a high electric field, selecting narrow pH gradient carrier ampholytes and use of a longer separation capillary, as well as by minimizing the protein/capillary surface interaction.

Conclusion

An axially illuminated WCID LIF for CIEF was developed. An LOD at the 10^{-19} mol level for a naturally fluorescent protein *R*-phycoerythrin was obtained. The developed method will facilitate the CIEF trace analysis of protein, and the study of protein interactions. By using a highly sensitive fluorescence probe and coupling with a sample preparation technique such as on-fibre derivatization solid phase microextraction (SPME), ultra high sensitivity can be expected, which may be applied in micro-analyses such as single cell investigations.

Acknowledgements

The authors gratefully acknowledge the financial support from the Natural Sciences and Engineering Research Council of Canada and editorial assistance by Heather Lord.

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