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High-sensitivity laser-induced fluorescence detection of native proteins in capillary electrophoresis

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ABSTRACT

A highly sensitive laser-induced (LIF) detection scheme for native, tryptophan- or tyrosine-containing proteins in capillary electrophoresis (CE) has been demonstrated. The 275.4 nm line from an argon-ion laser is used to excite native protein fluorescence. A limit of detection (LOD) ($S/N = 2$) of $1 \cdot 10^{-10}$ M for conalbumin represents a 140-fold improvement over earlier reports. With stacking at injection, the LOD is $3 \cdot 10^{-12}$ M. Linear dynamic ranges of at least 5 and 4 orders of magnitude for, respectively, tryptophan and bovine serum albumin are found. The practical performance and blueprint of an easily constructed, rugged, compact and user-friendly LIF detector for CE are shown.

INTRODUCTION

The high efficiency and speed of capillary electrophoresis (CE) in the resolution of biomolecules have attracted much attention [1]. However, efficient separation is not sufficient unless coupled to adequate detection. In this regard, CE presents a challenge as the nl cell volume and on-column arrangement render traditional detectors designed for high-performance liquid chromatography (HPLC) unsuitable.

The analysis of biological materials and the determination of biomolecules are of indispensable importance to the field of biotechnology. Despite the prominent role of proteins in biotechnology, the detection of proteins in CE is far from satisfactory. So far, amperometric detection [2] is hampered by electrode fouling while conductometric [3] and mass spectrometric detection [4] are unreliable and insensitive. Radiochemical detection [5], though quite sensitive, requires labeling as well as handling and disposing of hazardous materials with short shelf-lives. Although widely used and convenient, the best ultraviolet-visible absorption detectors with the cross-beam arrangement [6] offer a limit of detection (LOD) in the μM range because of the inherently short pathlength provided by the capillary tubing. This represents a major drawback for CE in the analysis of proteins in biological matrices where the demand for concentration detection sensitivity is far more stringent [7]. Besides, it excludes CE from the realm of trace analysis where HPLC is still the method of choice in spite of the poor separation efficiency of the latter.

In general, fluorescence detection in CE exhibits the best performance in sensitivity, linearity and selectivity. Even though conventional excitation sources have been used [8], the achievable sensitivity is offset by the inherent difficulty in focusing a large amount of light from a divergent light source into the nl detection region while minimizing light scattering. Utilization of the laser as a fluorescence excitation source reduces such problems and defines the state-of-the-art in CE detectors today [9]. Unfortunately, implementation of this approach to the detection of proteins is by no means straight-forward because usually it is necessary to label the analytes with fluorescent tags. Because of differences in the extent of incorporation of the tags into each protein molecule, pre-column labeling gives rise to multiple

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peaks for each type of protein in the electropherogram [10,11]. On-column labeling of proteins with laser-induced fluorescence (LIF) detection offers slightly better sensitivity than absorbance, but at the expense of separation efficiency due to the slow kinetics of inter-species conversion [11,12]. The advantages gained in the 100-fold improvement in sensitivity over absorbance afforded by a post-column labeling scheme with LIF are largely offset by instrumental complexity as well as the dependence of peak efficiency upon reagent flow rate and reaction distance [10]. A technique retaining the sensitivity of post-column labeling with LIF while avoiding derivatization is indirect fluorescence detection [13]. However, the need to work at low buffer concentrations renders practical applications difficult.

Swaile and Sepaniak [11] have demonstrated LIF detection of native, underivatized proteins in CE based on the fluorescence of tryptophan and tyrosine residues. In their study, an argon-ion laser operating at 514 nm was frequency-doubled to 257 nm with a harmonic generator. But instrumental instability and poor match between the excitation wavelength and excitation maxima of the fluorophores limited the sensitivity of the technique to no better than post-column labeling with LIF [10].

Nevertheless, the ability to detect proteins at trace levels remains attractive and, in the case of assuring the purity of biopharmaceticals, essential. According to a 'Points to Consider' draft issued by the US Food and Drug Administration (FDA) on biopharmaceuticals [14], the analytical goal for protein impurities and contaminants are in the 1 to 100 ppm range. However, it is possible that a highly immunogenic protein impurity present at the ppm level can elicit an allergic response in a high percentage of human recipients [15,16]. Hence, the current analytical goals set forth by the FDA merely reflect the sensitivity limits of existing technologies. This is supported by a more recent draft in which the term "analytical goals" was remplaced by phases such as "as free as possible" [17].On this note, the impact realized through coupling the high separation efficiency of CE to n*M*-detection sensitivity for proteins would be dramatic in the development and proliferation of biopharmaceuticals.

Despite the unparalleled performance of LIF detection in CE [9–11,13,18,19], there have only been a limited number of applications reported. The need for the separation scientist to acquire the expertise in designing, assembling, operating and/or maintaining an LIF detector may be a deterrent. Hence, there is a need for an easily constructed, user-friendly and rugged LIF detector which the non-specialist can build and use without having to expend too much effort or resources. In the present work, a highly sensitive LIF detection scheme for CE based on native protein fluorescence is reported and various aspects of it are discussed. In addition, a compact, rugged and user-friendly instrumental arrangement is described.

EXPERIMENTAL

The experimental setup used in the present work resembles the one described previously [18] with several modifications. First, a Model 2045 argon-ion laser (Spectra-Physics, Mountain View, CA) was optimized for deep UV operation. A prism was used to isolate the 275.4 nm line (utilized to excite protein fluorescence) from the total output. Secondly, 2 UG-1 band pass filters (Schott Glass Technologies, Duryea, PA, USA) were used to selectively pass the fluorescence. Thirdly, a compact, rugged LIF detector housed in a light-tight Plexiglas box (25 cm × 35 cm × 10 cm) was constructed. A diagram of the detector arrangement is shown in Fig. 1.

As depicted in Fig. 1, the device represents a simple and rugged design for LIF detection. It consists of 8 main components: a quartz 1-cm focal length lens (L), capillary holder (CH), 20× microscope objective (MO), microscope objective holder (MOH), mirror (M) and mirror mount (MM), photomultiplier tube (PMT) with filter (F), plexiglas box (PB) and 2 light shields (LS). The laser beam enters the otherwise light-tight box PB through a 3-mm hole. L is rigidly mounted to PB so that the focal point of the excitation beam is uniquely defined and used as the reference point for all the other components. The capillary with a small section of its coating removed is mounted on a 2-dimensional stage (CH) capable of 10-μm resolution. Two short pieces of quartz capillary 350 μm O.D. × 250 μm I.D. glued to CH serve to guide the separation capillary through the optical region. This can hold the common 150 μm O.D. capillary tubing. Alterna-

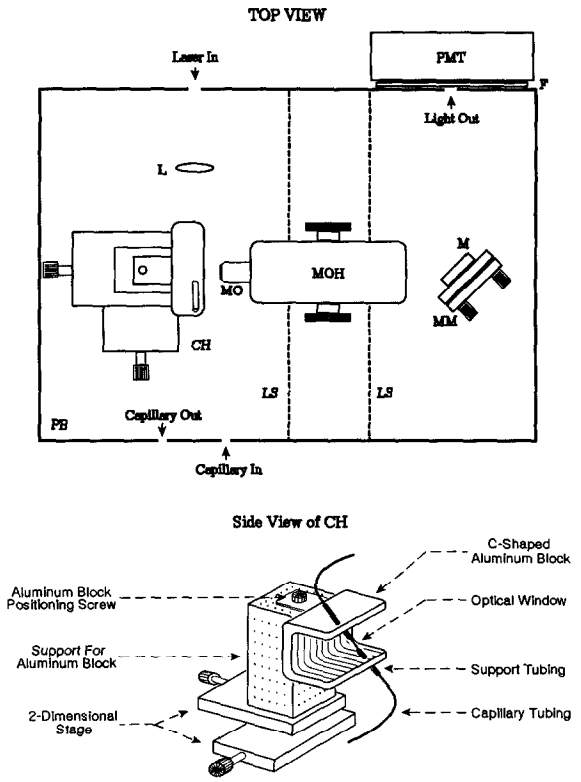

Fig. 1. Experimental arrangement of LIF detector for CE.

tively, 350 μm O.D. separation capillaries can be inserted directly into CH. The mounted capillary is at an angle of about 20° with respect to the incident laser beam to minimize scattering off the capillary walls. We find that this configuration is rigid enough with regard to noise from mechanical vibrations. With this design, capillary change can be accomplished in less than 5 min.

By examining the diffraction image of the transmitted light while translating CH, the incident light can easily be focused tightly into the center of the capillary. With a fluorophore (*e.g.* 10^{-5} *M* fluorescein solution at pH 8) running through the capillary, MO, positioned roughly such that its focal point is at the same height as the capillary window and pointing toward the excitation region, is translated with MOH so that the fluorescence image can be focused onto the exit plane of PB. By judging the sharpness of the image on the wall behind which the PMT is located, the optimal location of MO is determined. Finally, the angle of M relative to PMT and MO is adjusted with MM such that the fluorescence image clears the hole on the wall of PB that is just large enough to allow passage of the fluorescence spot while excluding light scattered off the capillary walls. F can then be used to selectively pass light of the appropriate wavelengths onto the PMT. The LSs are effective in excluding stray light from passage from the region around CH into the PMT. With this arrangement, we are able to routinely obtain detection limits within an order of magnitude of the state-of-the-art [9]. With the top of PB in place, one can generally work in moderate room light without additional shielding.

In other studies in our laboratory, an air-cooled Ar ion laser was used. The laser and the rest of the optical components can then be bolted together on a 3/4-in. Plexiglas base plate. A single 488 nm interference filter is mounted at the entrance to PB to eliminate room light. The components shown in Fig. 1 only cost a total of US$ 2000.

All separations were performed using a 5 m*M* sodium phosphate buffer at pH 10.2; the analytes were dissolved in the same buffer just before use unless specified otherwise. An electric field strength of 286 V/cm and a 77-cm long 50 μm I.D., 150 μm O.D. untreated fused-silica capillary were used throughout.

RESULTS AND DISCUSSION

The high sensitivity of LIF detection of native proteins is shown in Fig. 2 where the peak results

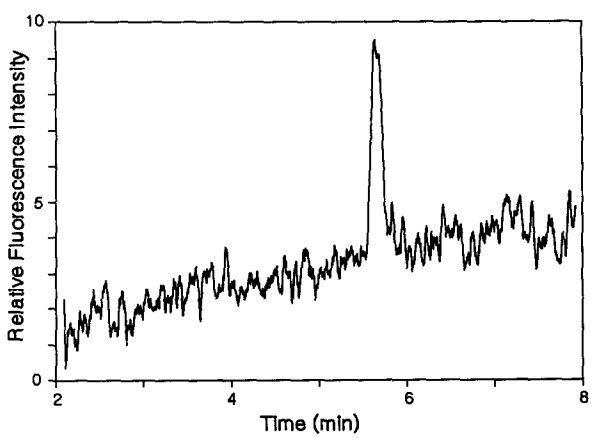

Fig. 2. Electropherogram of $5 \cdot 10^{-10}$ *M* conalbumin injected.

from the injection of conalbumin present at $5 \cdot 10^{-10}$ M in the sample. The LOD ($S/N = 2$) of $1 \cdot 10^{-10}$ M here represents a 140-fold improvement over the one reported for the same analyte previously [11]. The major reason for this is the simplicity and ruggedness of the present optical setup. Frequency-doubling is instrumentally elegant and requires a smaller argon ion laser, but produces light which is inherently noisy because of the quadratic dependence in the intensity of the frequency-doubled light on the intensity of the source. Temperature stability of the doubling crystal is also a problem. Besides, the excitation wavelength of 275.4 nm in the present system is a much better match with the fluorescence-excitation maxima of most proteins [20] than the 257 nm in ref. 11. Moreover, the high power used there prescribes long warm-up times. Since practical LIF detection is flicker noise limited, a frequency-doubled light source directly restricts the performance of the detector. However, the factors discussed above are not a concern in the present optical arrangement and, as a result of the much smaller source flicker, a drastic improvement in performance is realized. It is interesting to note that the fused-silica of the capillary tubing exhibits luminescence that can be visually discerned upon excitation at 275.4 nm. Even with 2 UG-1 band pass filters in place, this background luminescence cannot be totally eliminated, thereby providing an indication of the large spectral width of the luminescence. Consequently, the LOD of the present detector is limited by source-induced background luminescence flicker noise. Accordingly, increasing the dynamic reserve of the laser light or reducing the quantity of luminescent impurities in the fused-silica of the capillary tubing would further lower the LOD attainable with the present detector. We note that 10^{-10} M is an actual injected LOD and not one extrapolated from runs at high concentrations. This is important since adsorption of analytes onto the capillary walls can prevent one from taking advantage of the high sensitivity of LIF for studying small samples at low concentrations. The actual mass LOD (injection volume = a few nl) is around 10^{-14} g, which is quite impressive. As shown in Figs. 3 and 4, the present detector shows a linear dynamic range of at least 5 and 4 orders of magnitude for tryptophan and BSA, respectively. The correlation coefficient for each set in linear plots is better than 0.999. This is the first time that good quantitation is shown for LIF over a large concentration range. This highlights the stability of the present optical system over a series of runs. The linearity of the data in Fig. 4 also shows that adsorption of BSA is not a problem even at these low concentrations. Although the high cost of argon-ion lasers that can produce deep UV light is a disadvantage of the present detection scheme, it will eventually gain popularity as laser technology advances in the future.

Table I is a summary of the reported limits of detection for proteins in CE using various LIF techniques. Indirect fluorescence, native fluorescence excited at 257 nm, on-column and post-column labeling with LIF all offer higher LODs than native fluorescence exited at 275 nm. The LOD achievable with indirect fluorescence detection is limited by the

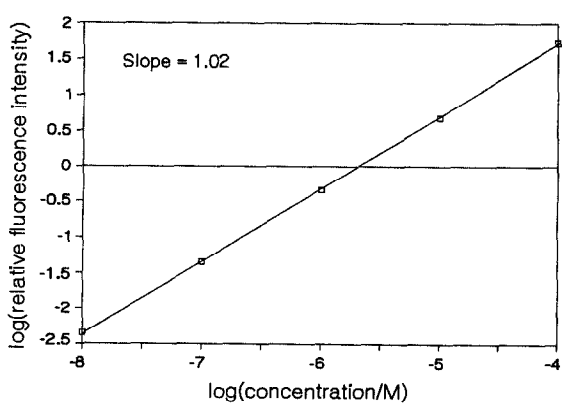

Fig. 3. Log–log plot of tryptophan calibration curve.

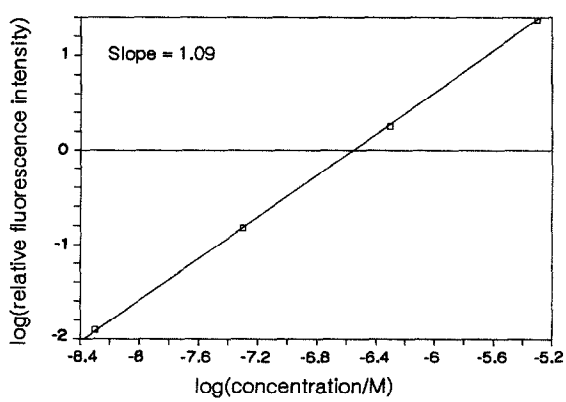

Fig. 4. Log–log plot of BSA calibration curve.

TABLE I

LIMITS OF DETECTION FOR PROTEINS IN CE BY LIF

Calculated from a signal-to-noise (S/N) ratio of 2.

Detection scheme	LOD (M)				Ref.
	Conalbumin	Horse heart myoglobin	Bovine serum albumin (BSA)	Lyoszyme	
Native fluorescence (257 nm excitation)	$1 \cdot 10^{-8}$	–	–	–	11
Indirect fluorescence	–	–	–	$5 \cdot 10^{-6}$	13
Pre-column labeling with FITC[a]	$1 \cdot 10^{-10}$	–	–	–	11
On-column labeling with TNS[b]	$3 \cdot 10^{-7}$	–	–	–	11
Post-column labeling with OPA[c]	–	$1 \cdot 10^{-8}$	–	–	10
Native fluorescence (275 nm excitation)	$1 \cdot 10^{-10}$	–	$2 \cdot 10^{-10}$	–	This work
Native fluorescence with stacking (275 nm excitation)	$3 \cdot 10^{-12}$	–	$1 \cdot 10^{-11}$	–	This work

[a] Fluorescein isothiocyanate.
[b] 2-*p*-Toluidinonapthalene-6-sulfonate.
[c] *o*-Phthalaldehyde.

dynamic reserve of the laser light [13] and the difficulty in selecting a well-behaved fluorophore with an electrophoretic mobility close to that of the analyte [21]. On-column labeling LIF detection is hampered by the large flicker noise on the fluorescence background resulting from the presence of unbound fluorophores in the eluent [11]. The performance of post-column labeling LIF is compromised by the short reaction time at the optimal reagent flow rate and flow distance [10]. Rather surprisingly, pre-column labeling LIF with FITC for conalbumin, which contains 102 side chain amine groups [22], offers an LOD no better than native fluorescence detection excited at 275 nm. This is in contrast to reports that FITC-derivatized amino acids can be detected in the 10^{-12} M range [9]. The probable reason for this is the formation of multiple peaks on the electropherogram with pre-column labeling, which increases the magnitude of background fluctuations close to the migration time of the major peak and decreases the size of the major peak [11]. Consequently, the use of native fluorescence as a detection principle for proteins in CE surrenders little in detection sensitivity. One gains in the simplicity and speed of analysis compared to fluorescence derivatization, which may not even be feasible at these low concentrations and small amounts. The sample is also preserved for further studies or use. One may even be able to implement fluorescence-detected circular dichroism [23] to study protein conformations. A drawback of the present detection scheme is that only tryptophan- or tyrosine-containing proteins are amenable to detection. Therefore, some peptides and small proteins may escape detection.

The LODs of $1 \cdot 10^{-10}$ and $2 \cdot 10^{-10}$ M for conalbumin and BSA, respectively, were obtained with the analytes dissolved in buffers identical in composition to the running buffer (5 mM sodium phosphate at pH 10.2). Interestingly, when a 5 mM sodi-

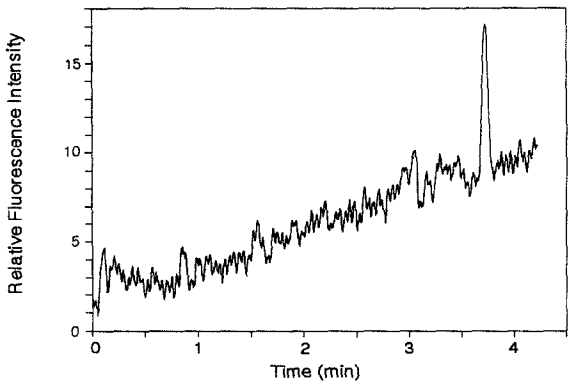

Fig. 5. Electropherogram of $1 \cdot 10^{-8}$ *M* tryptophan injected.

um phosphate solution at pH 6.68 was used as the sample buffer, the LODs for the same analytes decreased to $3 \cdot 10^{-12}$ and $1 \cdot 10^{-11}$ *M* respectively, with a small loss of separation efficiency. The phenomenon bears some similarity to electrophoretic concentration (or stacking). The lower ionic strength of the pH 6.68 sample buffer also increases the effective injection potential. Electrophoretic concentration has been applied to the analysis of dilute peptide samples with CE where the pH of the sample buffer is higher than that of the running buffer and with that, it has been claimed the LOD can be lowered by at least 5 times [24]. Therefore, electrophoretic concentration, when optimized and coupled to LIF detection of native proteins, possesses tremendous potential in protein determination at the trace level. The present enhancement should be applicable to physiological samples, which are near neutral pH. Further work on this is now under way.

Fig. 5 depicts an electropherogram showing tryptophan present at $1 \cdot 10^{-8}$ *M* in the sample. The estimated LOD (at *S/N* of 2) for tryptophan is $2 \cdot 10^{-9}$ *M*. Conalbumin, which contains 15 tryptophan and 21 tyrosine residues [22], does not show a linear increase in detection sensitivity as expected from the number of tryptophan residues. There are several explanations for this. First, the quantum yield of fluorescence of tryptophan residues in a hydrophobic microenvironment is about 3 times smaller than that for residues in a hydrophilic microenvironment [25]. Hence, it is possible that most or all of the tryptophan residues in conalbumin are located in the hydrophobic core of the protein. Secondly, a variety of functional groups such as peptide bonds [26] and protonated amine groups [27] are effective in quenching the fluorescence of tryptophan residues in proteins. Thirdly, the fluorescence and fluorescence-excitation spectra of tryptophan and proteins differ [20]. The present optical arrangement might favor tryptophan fluorescence over conalbumin fluorescence. However, the LOD of BSA ($2 \cdot 10^{-10}$ *M*) is only twice that for conalbumin, even though the former possesses only 1 tryptophan and 16 tyrosine residues [28]. The hydrophilic microenvironment of the tryptophan residue in BSA might serve as an explanation. Another reason is that energy-transfer from the tyrosine and phenylalanine residues to the tryptophan residue in BSA might increase the effective fluorescence quantum yield of the tryptophan residue [20]. Why this energy-transfer phenomenon occurs selectively in BSA but not in conalbumin is unclear.

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