Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1027 (2004) 223–229
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Native fluorescence detection of flavin derivatives by microchip capillary electrophoresis with laser-induced fluorescence intensified charge-coupled device detection

Jianhua Qin [a,b], Yingsing Fung [b,*], Derong Zhu [b], Bingcheng Lin [a]

[a] *Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China*
[b] *Department of Chemistry, University of Hong Kong, Hong Kong SAR, PR China*

Abstract

To widen the scope of laser-induced fluorescence (LIF) for detection in microchip capillary electrophoresis (CE), a microchip CE LIF–ICCD (intensified charge-coupled device) system based on a tunable wavelength dye laser pumped by a pico-second pulse nitrogen laser for excitation and a spectrograph with ICCD for detection had developed to demonstrate the enhancement in detection sensitivity by the following three approaches: direct detection of native fluorescence, improvement of signal-to-noise ratio by pulse laser excitation and time delay detection, and selective spectral acquisition by multi-channel detection. Riboflavin, flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD) have been selected as they are dietetically important and microchip CE provides a promising onsite detection method. The results indicate a strong effect of wavelength on detection sensitivity and the need to tune wavelength for direct detection. Under optimized conditions (excitation 450 nm, emission 520 nm, gate delay time 45 ns, 20 mM phosphate buffer at pH 7.1), the following results were obtained under static condition: Working ranges (0.6–350 μg/l, $r > 0.99$), detection limits (0.15–1.0 μg/l) and peak height repeatability (1.8–2.2% R.S.D.), all within the applicability range for body fluids or beverages such as human urine and cow milk. Baseline separation of three flavins was obtained under dynamic condition and the fluorescence spectra acquired assist the identification of alkaline-degraded products of riboflavin. Thus, the capability to check peak purity and identify unknown peaks has been demonstrated.

Keywords: Chip technology; Microfluidics; Detection, electrophoresis; Laser-induced fluorescence detection; Riboflavin; Flavins

1. Introduction

Increasing interest has been shown in recent years in the miniaturization of chemical analysis systems using microfabricated device [1,2]. Amongst the various techniques that can be integrated on microfabricated devices, capillary electrophoresis (CE) is the most promising one and has been applied for the analysis of various molecules of biological importance including small molecules [3–5], protein [6–9], and nucleic acid [10–13]. The advantages for developing CE-based analytical microsystems include fast speed, versatility, negligible sample/reagent consumption and high sample throughput. One of the major challenges facing the development of CE-based microsystem is a highly sensitive detection system due to the extremely small amount of analytes to be detected after separation.

Due to the very high sensitivity and capability for excitation and detection on the same side of the microfabricated chip, detection based on laser-induced fluorescence (LIF) becomes an essential system for analyte determination in microfabricated devices. To provide a general detection system, the LIF detection should ideally be flexible in selecting excitation wavelength to widen its scope of application with no constraint on choosing suitable derivative chemistries. In addition, it is preferred to record the whole fluorescence emission spectrum as it can assist peak identification and check on peak purity. This is essential for analyzing complex sample matrix. Combining capillary electrophoresis with an information-rich spectroscopic detection scheme has been demonstrated to provide very useful information on the target species as covered by several recent review papers [14–17].

However, most of the lasers currently used as excitation sources for LIF detection on microfabricated chips can only deliver one or a few excitation lines. This is due to the

* Corresponding author. Tel.: +852-28592162; fax: +852-25482132.
E-mail address: ysfung@hkucc.hku.hk (Y. Fung).

0021-9673/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2003.10.055

prohibitive high cost of acquiring solid-state lasers with tunable wavelength. The recent advance of tunable dye laser pumped by a pulse laser with a fixed wavelength provides an acceptable solution to tune the excitation wavelength for LIF, as the cost of the system is very much cheaper compared to the solid-state laser and the excitation wavelength can be tuned easily by using a suitable laser dye emitting in the region of the desired wavelength.

To acquire the full fluorescence emission spectra, a multi-channel detection system is needed. Since Mathies and coworkers introduced the use of a confocal microscope system to scan fluorescence emitted from an capillary array and the subsequent extension of the technique to microfabricated device [18,19], other groups had also performed on-channel LIF detection by launching a laser beam at the separation channel and collecting the fluorescence with a microscope mounted perpendicular to the chip [20]. The recent advance in intensified charge-couple devices (ICCDs) provides a desirable multi-channel detection system, as no mechanical scanning is needed and the whole spectrum can be recorded simultaneously. This leads to improved performance of the fluorescence spectra detection through decreased detector noise over extended period of signal averaging.

In this paper, a new development of a tunable wavelength laser-induced multi-channel fluorescence detection system for microfabricated electrophoretic devices will be described. The system employs a pico-second pulse nitrogen laser to pump a dye laser to generate tunable wavelength ranging from UV to visible region. The fluorescence light was measured by a spectrograph equipped with an ICCD detector for detection in the UV-Vis spectral range from 200 to 800 nm. The applicability of the LIF–ICCD system developed for microchip detection will be studied.

To demonstrate the applicability of LIF–ICCD for microchip detection, riboflavin and flavin derivatives are selected as a chemical system for evaluation, as they are dietetically important compounds normally present at trace levels in food and clinical samples. Flavins represent an important class of biomolecules present in most cells and tissues and the main derivatives of flavins include riboflavin (RF), flavin-adenine dinucleotide (FAD) and flavin mononucleotide (FMN). Amongst the derivatives, FAD and FMN serve as essential redox-active coenzymes associated with flavoenzymes that catalyze a variety of biochemical reactions, involving carbohydrate, protein and fat metabolism [21]. Flavins are also one of the few biomolecules that exhibit native fluorescence, and thus suitable for direct detection after separation.

Current analytical methods employed for the determination of flavin derivations include high-performance liquid chromatography (HPLC) [22,23], electroanalytical chemistry [24], and recently capillary electrophoresis-LIF method for the detection of flavins [25–27]. All existing techniques are developed for laboratory analysis, requiring special facilities and skill for analysis. The recently developed microchip CE provides a suitable method for bedside and clinical analysis, though improvement is needed to enhance the detection sensitivity. In the present work, a tunable dye laser for LIF and ICCD for detection will be used to improve the detectability of flavins. The optimization of the working conditions and factors affecting the analysis of riboflavin and flavin derivatives will be given and discussed.

2. Experimental

2.1. Chemicals and reagents

All chemicals used in this study were analytical grade. Flavin-adenine dinucleotide and flavin mononucleotide were purchased from Sigma–Aldrich (Milwaukee, WI, USA), whereas riboflavin (RF), sodium hydroxide, disodium phosphate and sodium borate from Amersham Biosciences (Hong Kong). HPLC-grade methanol was purchased from Merck (Germany). Coumarin 2 (LC4500) in methanol, used as the laser dye, was purchased from Lambda Physik (Taiwan). All buffer solutions were prepared by making up to volume in volumetric flasks with doubly distilled and de-ionized water.

2.2. Sample preparation

Stock solutions of RF, FAD and FMN were prepared by dissolving calculated quantity in deionized water to give a concentration of 600 μg/l. Sample solutions were prepared daily prior to use by serial dilution of the stock solutions to a desired concentration under dimmed light. All samples were stored in amber bottles, kept in a refrigerator at 4 °C and used immediately after opening. Borate and phosphate buffers were prepared by adjusting to desired pH by the addition of appropriate amounts of hydrochloric acid or sodium hydroxide. All buffers were filtered with 0.2 μm pore size membrane filters before introduction into the microchip.

2.3. Operation procedure for microfabricated device

A fabricated poly methyl methacrylate (PMMA) microchip was used in this study. The chip uses a single cross injector design with a separation channel of 100 μm in width at the top, and 20 μm in depth. The effective separation length is 3.0 cm. The sample solution was injected by means of the floating or gated injection method [28]. A sample volume of 5 μl was pipetted into the chip reservoir and loaded onto the injection channel by applying a potential difference of 600 V for 10 s across the injection channel with no voltage applied to separation channel. During separation, 1.2 kV was applied across the separation channel with no voltage applied to buffer channel. The buffer solution and samples were flushed through the separation channels by applying a vacuum suction at the separation waste port. The chip was washed with 0.1 M NaOH for 2 min and deionized water for

2 min between each run during experiments. Two identical high voltage suppliers (CZE 1000R, Spellman, USA) were utilized to deliver the loading and separation voltages, respectively.

2.4. Detection system

The detection system was constructed through the modification of a fluorescence-inverted microscope (PIM, USA). The pico-second (500 ps) pulsed nitrogen laser (Model MSG 803TD, LTB, Berlin, Germany) delivering an excitation wavelength at 337.1 nm was selected as the pumping source. The nitrogen laser gives a low beam divergence and hence induces a relatively small fluctuation of the fluorescence signal. The repetition rate of the laser is selected at 10 Hz. The use of a dye laser extends the wavelength to other spectral region with wavelength tunable by a suitable optical crystal. Through the use of different dye solutions, the entire visible spectrum from 400 to 900 nm could be covered and coupling with a frequency doubling units, the spectral range can be further extended to the UV region from 205 to 400 nm. Using only one dye solution, the spectral range between 40 and 70 nm are continuously tunable.

Pumped by the nitrogen laser, the dye laser using dye Coumarin 2 delivered a peak energy at 38 μJ which was monitored by a laser meter (PEM100, LTB). The wavelength is measured by an ICCD spectrograph previously calibrated against the 10 most intense lines from an Hg lamp (Oriel, Stratford, CT, USA). The laser beam was delivered through an optical fiber coupling with a focusing lens ($f = 30$ mm) and was reflected by a dichroic mirror (Omega) to focus onto the channel by a 0.65 NA, 40× microscope objective (Olympus, Japan). The emission fluorescence was collected by the same objective, and passed through the dichroic mirror again in its return path to couple with another optical fiber containing a lens to focus the fluorescence emission light onto a 30 μm entrance slit of a motorized 1/8 m spectrograph (Model 74000, 1200 grooves/mm grating, Oriel, USA). The signal was detected after dispersion by a front illuminated intensified charge fiber coupled device (ICCD, 1024 × 256 pixels, Model 77193-5, Andor Technology) positioned at the lateral port of the spectrograph.

2.5. Electronic control and data acquisition

Electronic trigger control and data acquisition were performed by the IntaSpex software (Andor Technology, Ireland). Internal trigger mode was adopted with read out from the CCD under direct and total control by the computer. Once the laser was triggered, a trigger signal was sent to the delay generator (Stanford Research System DG 535) and the pulse generator used this information to determine when the image intensifier in the CCD head was gated on (gate delay), for how long it was gated on (gated width) and the duration of each step by which the gate delay was progressively increased during the sequence of acquisitions (time intervals). The intensifier was gated on for the duration of the pulse, allowing the CCD to acquire data. When the intensifier was gated off, the acquired data were transferred from the detector head to the controller card (16-bit intelligent bus-mastering PCI card) placed in the computer. The parameters for data acquisition (gate delay, gate width and gate step) were sent to the pulse generator via a GPIB interface.

The CCD detector utilizes the read mode commonly referred to as binning. In this system, the CCD read rate is 40 Hz per frame with a serial binning of 1 to reduce the read noise and the memory required for data storage. The one-dimensional electropherogram is created from the two-dimensional data set by summing a small range of wavelengths near the emission maximum of the analyte of interest. The data were computed by numerically integrating the optimized wavelength ranges with the Origin software (Version 6.1, Micronal Software Inc.) to generate a post acquisition electropherogram. The signal-to-noise ratio (S/N) of the peak is determined by dividing the peak height calculated from the least square baseline by the noise, and the LOD is calculated by extrapolating from this value to the concentration that would yield a peak with an intensity of 3 S/N.

2.6. Background spectral analysis

Before the experiment, the background spectra are acquired while the buffer is static in order to obtain the optimized gate delay of ICCD for detection and to avoid the interference of scattered light and instrumental noise. In addition, the fluorescence lifetime of the analytes were determined by ICCD in order to optimize the gate width and to reduce noise due to stray light. The measuring procedure of the time-resolved fluorescence consists of collecting the full sample and background spectra, subtracting the background decay curve from the fluorescence decay at the wavelength of maximum fluorescence of the analyte, and fitting of the background corrected data to single exponential decays [29]. Curve fitting of the fluorescence was performed using the Origin software (Version 6.1, Micronal Software Inc.). The decay curve indicated below:

$$y = y_0 + A_1 \exp\{-(x - x_0)^{t1}\}$$

were fitted by fixing y_0 and x_0 at a value of zero. The fluorescence lifetime was derived from the decay curve.

3. Results and discussion

3.1. Microchip CE LIF–ICCD for riboflavin analysis

In order to enhance the detection sensitivity, many commercially available LIF detection systems developed for microfabricated CE are often used strong CW laser intensity at 488 nm or 532 nm for excitation and a highly sensitive

photomultiplier (PMT) for detection of the weak fluorescence light. Interference filter is often used to allow more fluorescence light to reach the PMT. However, the use of a strong CW laser for irradiation can lead to problems of sample degradation and increased amount of light scattered from the optical system. The use of a broad band detection allowed by interference filter, though increases the amount of fluorescence light to reach the PMT, also admits the entrance of unwanted radiation from scattered light and other light sources with similar wavelength. This will appear as noise in the system and limits the maximum gain that can be achieved using the photon counting PMT. Thus, there is a maximum sensitivity that can be obtained using the conventional system based on strong CW laser light excitation and highly amplified PMT detection.

As many molecules with chemical and biomedical significance do not fluoresce strongly at 488 nm or 532 nm by themselves for direct detection, strongly fluorescent dyes at one of these two wavelengths have been developed to interact with the target molecules to render them detectable by online or off-line derivatization. However, to deal with the variability introduced by the sample matrix on chemical derivatization and to ensure a complete conversion, excess dyes are often used and this can produce background fluorescence at similar wavelength that leads to noise in the detection system. In addition, many important analytes do not interact sufficiently with the dyes and thus not detected. Both of these limit the applicability of LIF for detection in microfluidic electrophoretic systems.

Three approaches have been adopted in the present work to improve the quality of the detection signal, i.e., a higher signal-to-noise ratio. This could widen the scope of application of microchip CE to areas limited by detection sensitivity. For the first approach, detection of fluorescence light directly emitted from analytes is measured to avoid problems as described in the previous paragraph. The native fluorescent riboflavin and flavin derivatives are selected in the present work to provide a suitable system to illustrate this effect. Secondly, a pico-second pulse laser excitation with a time delay detection is used. The use of a high intensity short duration pulse reduces sample photo-degradation and achieves a strong excitation of the analytes. The application of a time delay detection reduces noise due to stray light as the decay of scattered light is much quicker than molecular fluorescence. Thus, a high gain amplification can be easily performed on good quality detection signal with a low background noise. Thirdly, a multi-channel detection system is used, as the fluorescence emission spectral information acquired can assist the identification of unknown compounds and provide a check on peak purity. An intensified CCD was used in the present work, as it can provide a high gain amplification as well as to acquire the whole fluorescence spectra simultaneously during detection.

In the microchip CE LIF–ICCD system developed, a pulse laser excitation with a time delay detection is adopted. The computer commands the delay generator to start the preset electronic control for sending a pulse via the nitrogen laser, a gater signal to control the gate delay time and gate width, and a voltage trigger to fire the control timing for other I/O accessories such as laser energy meter and recording system. The instrumental parameters that are required for the optimization of a given chemical system is the gated delay time and gated width so that the detector can be programmed to stop accepting scattered light at nano-second range after laser excitation during the gate delay, and switch on again after the delay to accept the fluorescence light during the duration of the gated width. This will enhance the quality of the signal with an increased S/N ratio.

The effect of the gated delay time on the signal-to-noise ratio for measuring the fluorescence signal of riboflavin at 150 μg/l under static condition has been studied. Too short a gate delay will lead to the collection of a large amount of scattered light from the excitation laser and the channel, whereas too long a gate time will lead to the reduction in the collection of fluorescence light from analyte. Thus, a maximum S/N ratio of 18 was obtained at an optimum delay time of 45 ns. For the control of the gate width, its length should be kept as short as possible to reduce the collection of scattered light, but should be long enough to collect the fluorescent light emitted from the analytes during their fluorescence lifetime. Thus, the fluorescence lifetime of the analyte is an important parameter to measure. The measurement of the fluorescence of riboflavin and factors affecting the lifetime of the fluorescent analytes will be discussed in the next section.

3.2. Measurement of fluorescence lifetime and emission spectra of riboflavin and flavin derivatives

Useful spectral information of the fluorescence spectra of RF, FAD and FMN are within the range from 490 to 590 nm. RF and FMN show nearly identical spectra, whereas FAD exhibits a similar spectrum with a lower fluorescence in all wavelength range covered by the fluorescence spectrum at the same concentration. As ICCD is capable of measuring the full emission spectra simultaneously with predetermined wavelength range, it acts effectively as a wavelength filter with adjustable bandwidth. In this manner, the wavelength information is used in a similar manner as an interference filter to eliminate unwanted signals. The merit of this method is that the wavelengths can be adjusted dynamically so as to reduce the interfering effect of other light emitting compounds with partial spectral overlap with that of the analyte.

A trade-off can be made between sensitivity and selectivity by choosing a suitable wavelength bandwidth of the emission spectrum for integration of the fluorescence signal. This will enable the measurement of the fluorescence signal in a complex sample matrix by suitably removing the noise from non-analyte sources. The effect of integrating different ranges of emission wavelength on the signal-to-noise ratio for the analysis of riboflavin has studied using emission wavelengths centered at 520 nm. The optimal signal-to-noise

ratio was found at a bandwidth of 15 nm centered at 520 nm for riboflavin analysis. This condition is used for subsequent studies.

Under equal molar concentration (20 mM) at pH 7.1, the borate and phosphate buffer were investigated for their effect on the separation of riboflavin by microchip CE. It was found that riboflavin displayed a slightly higher fluorescence intensity in the phosphate buffer as compared to that in borate. Therefore, the phosphate buffer was selected for subsequent studies. In addition, it had been reported that the fluorescence intensity of flavin derivatives was strongly dependent on pH [30,31]. An investigation was thus made to find a suitable pH range for the running buffer during microchip separation by studying its effect on fluorescence intensity. Ranges of pH between 2 and 12 were examined using 20 mM phosphate buffer. Both RF, FMN and FAD showed a trend of increase in fluorescence intensity from pH 2 to 4, a relatively flat plateau from pH 4 to 8 and a decline in fluorescence at higher pH. The results indicate that strong acid or alkaline environment should be avoided during electrophoretic microchip separation.

As the gate width of ICCD for detection has to be set in accordance to the length of the fluorescence lifetime of the analyte in order to design an effective detection scheme, they have been measured in the present work. The fluorescence lifetime of riboflavin was found to increase rapidly from pH 2 till 6 and level off at higher pH. The same pH effect is also shown during the measurement of the actual fluorescence with the exception of a decline of fluorescence at pH higher than 8. A constant fluorescence lifetime and a decline of fluorescence at alkaline pH indicate that other factors may be in operation for the reduction of actual fluorescence and this may due to the effect of the hydroxide anion on quenching of the fluorescence or on destruction of RF upon alkaline hydrolysis. For the analysis of flavins derivatives, gate width time in the region of five to six times the lifetime of riboflavin was found to be sufficient to collect most of the flavins fluorescence light. The fluorescence lifetime of riboflavin in phosphate buffer (pH 6–10) was determined to be around 5.0 ns and the optimum gate time 30 ns.

3.3. Analysis of riboflavin and flavin derivatives

The working parameters for the analysis of riboflavin and flavin derivatives under static conditions are given in Table 1. Satisfactory linear ranges were obtained for RF (0.6–320 μg/l), FAD (4.0–350 μg/l) and FMN (3.0–320 μg/l) with LOD at 0.15, 1.0 and 0.75 μg/l respectively, and r greater than 0.99 for all three compounds. The results are within the applicability range for these compounds in beverages or body fluids such as cow milk and human urine [32,33]. Good repeatabilities for peak height measurement were obtained, ranging from 1.8 to 2.2% R.S.D. The sensitivity expressed by slopes of the linear range for measuring RF, FAD and FMN are similar, varying from 92 to 108, a fact indicating that their fluorescence mechanisms are similar and related to the same functional group.

The electropherogram obtained under dynamic condition for a mixture of RF, FAD and FMN is given in Fig. 1. The separation is strongly affected by the buffer pH. Under acidic conditions, the peaks are very small, possibly due to the reduction of the fluorescence intensity of the flavin moiety upon protonation in acidic solution. However, the migration time and elution order did not seem to be affected by pH of the solution, as similar electrophoretic patterns were obtained in both acidic and alkaline buffers. Under neutral pH (7.1) or alkaline pH (9.2), the fluorescence intensity of the three separated peaks are more or less the same, though the peak width are smaller at alkaline pH and hence a better resolution under alkaline pH. However, an additional peak (peak 4) was found to occur at alkaline pH, possibly due to the degradation of flavins upon alkaline hydrolysis. Thus, neutral pH buffer was selected to avoid potential degradation of the analyte. Compared to the results obtained under static conditions, a good linearity is obtained ($r > 0.99$) under dynamic conditions and the S/N ratio for these three

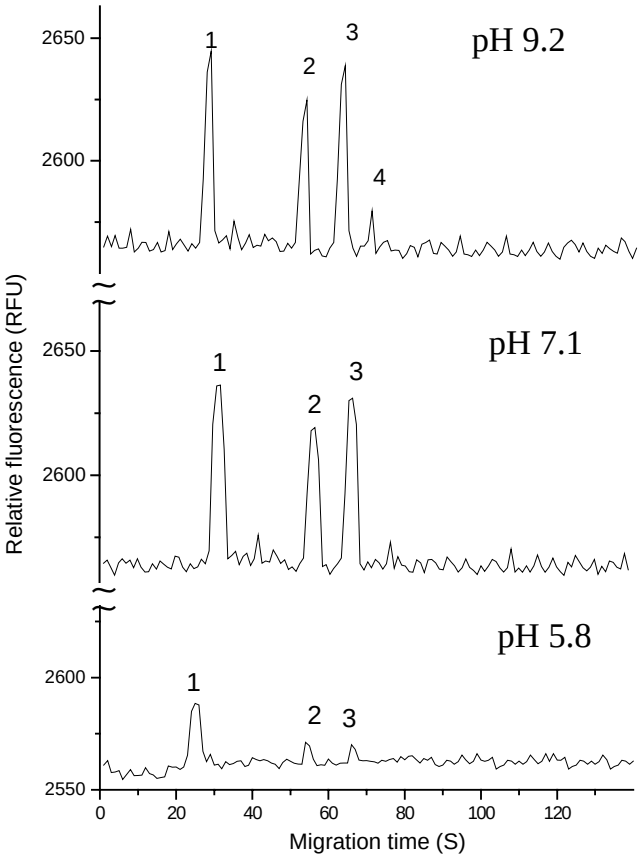

Fig. 1. Electropherograms of RF (200 μg/l), FAD and FMN (both 250 μg/l) under optimized experimental conditions. Running buffer: 20 mm phosphate solution (pH 5.8–9.2); injection time: 10 s; effective separation distance: 3.0 cm; separation voltage: 1200 V; excitation/emission wavelength: 450/(505–535) nm. delay time: 45 ns. Gate width: 30 ns. Peak: 1, RF; 2, FAD; 3, FMN; and 4, alkaline-degraded products.

Table 1
Working parameters for the analysis of flavins under static condition[a]

Compound	Slope and intercept ($Y = mx + b$)		r	Linear range (μg/l)	LOD[b] (μg/l)	Repeatability[c] R.S.D. (%)
	$b \pm$ S.D.	$m \pm$ S.D.				
RF	$(17.5 \pm 3.0) \times 10^3$	108 ± 2.6	0.9988	0.6–320	0.15	2.1
FAD	$(15.1 \pm 2.4) \times 10^3$	97.2 ± 1.4	0.9981	4.0–350	1.0	1.8
FMN	$(12.7 \pm 1.8) \times 10^3$	91.8 ± 2.3	0.9989	3.0–320	0.75	2.2

[a] Concentrations given are expressed in μg/l. Ex = 450 nm, Em = 505–535 nm.
[b] LOD: limit of detection (S/N = 3).
[c] Repeatability of peak height is expressed in percentage relative standard deviation (R.S.D.) and calculated from five replicate measurements of a 100 μg/l aqueous solution of each compound at pH 7.1.

compounds are 15 at 200–250 μg/l concentration, as compared to S/N = 18 under static condition at 150 μg/l. The additional noise is attributed to the variability of the high voltage supplies during dynamic electrophoretic separation.

The capability of using ICCD for recording the entire fluorescence emission spectrum for each of the three separated flavins have been investigated as this is useful for the identification of compounds separated and as a check on peak purity. The first three peaks, peaks 1–3 showed similar emission spectra as compared to the standard spectra of the corresponding flavins and they are also matched with the migration times, while the fourth peak is distinctly different from the rest. This shows that peaks 1–3 are compounds with flavin structure while peak 4 represents compounds from alkaline-degraded products with a slightly altered structure.

4. Conclusions

Three approaches have been adopted to increase the detection sensitivity and enhance the capability of LIF. The first approach uses native fluorescence of riboflavin and flavin derivatives for direct detection. The results indicate a strong effect of excitation wavelength on detection sensitivity and the need to tune wavelength for detection. The second approach uses a pico-second pulse laser excitation to generate a high intensity short duration excitation to minimize sample photo-degradation and a time delay detection to reduce stray light effect. High-quality detection signal has been obtained with a low background noise to enable the use of a high gain amplification. A S/N ratio of 18 was obtained at an optimum delay time of 45 ns for measuring the fluorescence signal of riboflavin at 150 μg/l. The third approach uses a multi-channel ICCD for full spectrum analysis. This allows selective integration of the fluorescence spectra to reduce spectral overlap interference and to enable identification of unknown compounds and a check on peak purity. The best-integrated bandwidth for riboflavins was found to be at 520 nm.

For buffer constituents, 20 mM phosphate with pH adjusted to 7.1 was used to avoid degradation of riboflavin. Under the optimized conditions of excitation wavelength 450 nm, emission wavelength 520 nm, spectrograph entrance slit 30 μm, and gate delay time 45 ns, the following results were obtained under static condition: RF (0.6–320 μg/l), FAD (4.0–350 μg/l) and FMN (3.0–320 μg/l) with limits of detection (S/N = 3) at 0.15, 1.0 and 0.75 μg/l respectively and $r > 0.99$ for all three compounds. The results are within the applicability range for analyses in food or bodily fluid. Good repeatabilities of peak height measurement were obtained, ranging from 1.8 to 2.2% R.S.D. The sensitivity for measuring RF, FAD and FMN are similar, a fact indicating that their fluorescence mechanism are similar and related to the same functional group.

The application of microchip CE LIF–ICCD for the analysis of riboflavin and flavin derivatives have been demonstrated under dynamic condition to give baseline separation of all three flavins. Their fluorescence spectra and fluorescence lifetimes can be measured individually. The results obtained have been used to identify a degraded product of riboflavin generated by alkaline hydrolysis to produce an emission spectrum different from the original flavin spectra. Thus, it has demonstrated the capability of the system for checking on peak purity and identification of unknown peaks.

Acknowledgements

We would like to acknowledge the financial support from the Hong Kong University Vice Chancellor Award, Seed Funding for Basic Research from the Hong Kong University Research Committee, the Research grants Council of the Hong Kong Special Administrative Region, China (HKU 7095/98P) and National Nature Science Foundation of China (NNSFC, grant number 20035010, 20299030 and 20275039) on the above work.

References

[1] D. Figeys, D. Pinto, Anal. Chem. 71 (2000) 330A.
[2] M. Freemantle, Chem. Eng. News (1999) 27.
[3] N. Chiem, D.J. Harrison, Anal. Chem. 69 (1997) 373.
[4] J. Wang, R. Polsky, B.M. Chatrathi, M.P. Tian, Anal. Chem. 72 (2000) 5285.
[5] A. Hilmi, J.H. Luong, Anal. Chem. 72 (2000) 4677.

[6] B.J. Burke, F.E. Regnier, Electrophoresis 22 (2001) 3744.
[7] V. Linder, E. Verpoorte, W. Thormann, N.F. de Rooij, H. Sigrist, Anal. Chem. 73 (2001) 4181.
[8] E. Eteshola, D. Leckband, Sens. Actuators B 72 (2001) 129.
[9] K. Sato, M. Tokeshi, T. Odake, H. Kimura, T. Ooi, M. Nakao, T. Kitamori, Anal. Chem. 72 (2000) 1144.
[10] S.C. Jacobson, J.M. Ramsey, Anal. Chem. 68 (1996) 720.
[11] P. Belgrader, W. Benett, D. Hadley, J. Richards, P. Stratton, R.J. Mariella, F. Milanovich, Science 284 (1999) 449.
[12] E.T. Lagally, P.C. Simpson, R.A. Mathies, Sens. Actuators B 63 (2000) 138.
[13] L. Mitnik, L. Carey, B. Burger, S. Desmarais, L. Koutry, O. Wernet, P. Matsudaina, D. Ehrlich, Electrophoresis 23 (2002) 719.
[14] R.R. Fuller, L.L. Moroz, R. Gillette, J.V. Sweedler, Neuron 20 (1998) 173.
[15] J.V. Sweedler, J.B. Shear, H.A. Fishman, R.N. Zare, Anal. Chem. 63 (1991) 496.
[16] A.T. Timperman, K. Khatib, J.V. Sweedler, Anal. Chem. 67 (1995) 139.
[17] A.T. Timperman, J.V. Sweedler, Analyst 121 (1996) 45R.
[18] D. Seiler, D.J. Harrison, A. Manz, Anal. Chem. 65 (1993) 1481.
[19] C.S. Effenhauser, A. Manz, H.M. Widmer, Anal. Chem. 63 (1993) 2637.
[20] S.C. Jacobson, R. Hergenroeder, A.W. Moore, J.M. Ramsey, Anal. Chem. 66 (1994) 4127.
[21] D. Leenheer, Modern chromatographic analysis of vitamins, in: Chromatographic Science Series, Marcel Dekker, New York, 2000, p. 138.
[22] A. Munoz, R. Ortiz, M.A. Murcia, Food Chem. 49 (1994) 203.
[23] L.F. Russell, J.T. Vanderslice, Food Chem. 43 (1992) 151.
[24] W. Hou, E. Wang, Analyst 115 (1990) 139.
[25] S. Hustad, P.M. Ueland, J. Schneede, Clin. Chem. 45 (1999) 862.
[26] T. Perez-Ruiz, C. Martinez-Lozano, A. Sanz, Electrophoresis 22 (2001) 1170.
[27] A.K. Su, C.H. Lin, J. Chromatogr. B 785 (2003) 39.
[28] S.C. Jacobson, J.M. Ramsey, Electrophoresis 16 (1995) 481.
[29] A.T. Bystol, A.D. Campiglia, G.D. Gillispie, Appl. Spectrosc. 54 (2000) 910.
[30] S.A. Scamsi, N.D. Danielson, I.M. Warner, J. Chromatogr. A 835 (1999) 159.
[31] P. Drossler, W. Holzer, A. Penzkofer, P. Hegemann, Chem. Phys. 282 (2002) 429.
[32] T.R.I. Cataldi, D. Nardiello, G.E. DeBenedetto, S.A. Bufo, J. Chromatogr. A 968 (2002) 229.
[33] B. Saidi, J.J. Warthesen, Int. Diary J. 5 (1995) 635.