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Short communication

Time-resolved photon-counting fluorimetry with a semiconductor laser for capillary electrophoresis

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Abstract

Effects of the gate width and the gate delay on the signal-to-noise ratio were investigated for a laser-induced fluorescence detector of capillary electrophoresis using a pulsed semiconductor laser, a photomultiplier and a time-resolved photon counting method. Separation of fluorescence and background was easier for a fluoropher with a long lifetime.

Keywords: Time-resolved photon-counting fluorimetry; Laser-induced fluorescence detector; Detectors, electrophoresis; Dyes

1. Introduction

Laser-induced fluorescence is so sensitive that even a single molecule has been detected. A laser can be strongly focused and is an ideal light source for a nanoliter to picoliter sample such as capillary electrophoresis (CE) [1–3].

Light source of a laser fluorescence detector of CE has been either an Ar laser or a He–Cd laser. They are, however, large in size and costly to maintain. A semiconductor laser (SCL) is small, inexpensive, stable and durable, although its wavelength is currently restricted to the red region. There have been a few investigations on a laser fluorescence detector of CE using a SCL [4–6].

A time-resolved photon-counting technique combined with a pulsed laser has been successful to remove laser scattering light and unwanted backgrounds from the fluorescence signal [7,8]. An application of this technique to CE was developed using a diode-pumped Nd–YLF laser, and short-lived fluorescent interferences were successfully removed from long-lived fluorophores in biological sample matrixes [9]. We have constructed a laser fluorescence detector of CE using a pulsed SCL, an avalanche photodiode and a photon counting technique [10]. In the present communication, effects of the gate width and the gate delay on the signal-to-noise ratio have been investigated, and this technique has been applied for a CE detector.

2. Experimental

The apparatus was similar to that described previously [10]. The light source was a pulsed SCL (Hamamatsu Photonics LHD-065: 655 nm). Its pulsed width was 74 ps, the peak power was 29 mW,

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and the average power was 21 μW, when operated at 10 MHz. The capillary column was made of fused silica (J & W, 75 μm I.D., 350 μm O.D., length 100 cm), and 28 kV was applied across the capillary. The sample was injected electrokinetically by regulating the voltage of the power supply to 28 kV for 2 s, and the injection volume was 10 nl [11].

The SCL light was focused by a 10 × microscope objective onto a capillary window where polyimide coating was removed. The fluorescence emission was collected at right angle by another 10 × microscope objective. After passing through an interference filter (wavelength 700 nm, bandwidth 15 nm), it was focused on a photomultiplier tube (Hamamatsu R1477, rise time 2.2 ns). The signal was amplified with a 100-MHz amplifier (Ortec 9302) and a constant fraction discriminator (Ortec 473A). The SCL light passing through the capillary was detected by a photodiode and the signal was fed to a discriminator (Ortec 436). The two signals were used as a start and a stop pulse of a time-to-amplitude converter/single channel analyzer (Ortec 567). The coincident counts in the gate window were accumulated in a personal computer. The accumulation time was 1 s.

Methylene blue, oxazine 725 perchlorate, HIDC (1,1′,3,3,3′,3′-hexamethylindodicarbocyanide iodide), and rhodamine 700 perchlorate were purchased from Lambda Physic and used as supplied. The buffer solution (20 mM) was prepared from sodium dihydrogenphosphate and was adjusted to pH 3.4. The capillary was rinsed before each experiment with 0.1 M NaOH and phosphate buffer for about 10 min.

3. Results and discussion

For a successful application of a time-resolved technique, the instrument should have a good time resolution. The time resolution of the present system was determined as 0.7 ns as a full width at half maximum by measuring a decay profile of the scattered light from phosphate buffer solution. Both the fluorescence from an impurity and the Raman scattering from the buffer solution were negligibly small in the deep-red region. Time resolution was due to those of the photomultiplier and the electronics.

The spectroscopic properties of the four dyes are summarized in Table 1. Their absorption maxima lie around the SCL wavelength. The lifetimes were obtained in the gated detection method [10]. A proper setup of the gate width (time in which photons are counted) and the gate delay (time interval between the laser light and the start of the gate) is essential for a successful application of a time-resolved technique.

The signal-to-noise ratio (S/N) was measured at various gate widths and various gate delays as shown in Fig. 1 for the two dyes; the capillary was filled with the dye solution in these measurements. S/N was calculated as $n_f/(n_b)^{1/2}$ where n_f is the fluorescence photon count and n_b the background count; they were measured as a function of the delay at each gate width. Because of a high power stability of the SCL, the background (laser scattering) was a shot noise, and its fluctuation could be represented as $(n_b)^{1/2}$. At a shorter delay, the background cannot be removed efficiently, although the fluorescence signal

Table 1
Spectroscopic properties and detection limits of the four dye molecules

Dye	Absorption maximum (nm)	Absorptivity at 655 nm (M^{-1} cm^{-1})	Fluorescence maximum (nm)	Fluorescence lifetime[a] (ns)	Detection limit (fmol)
Methylene blue	665	0.78×10^5	685	1.0	14
Oxazine 725	655	1.27×10^5	679	0.9	2
HIDC iodide	636	1.60×10^5	667	1.0	13
Rhodamine 700	642	0.50×10^5	668	1.6	28

[a]Uncorrected for the time resolution (0.7 ns) of the present experimental system.

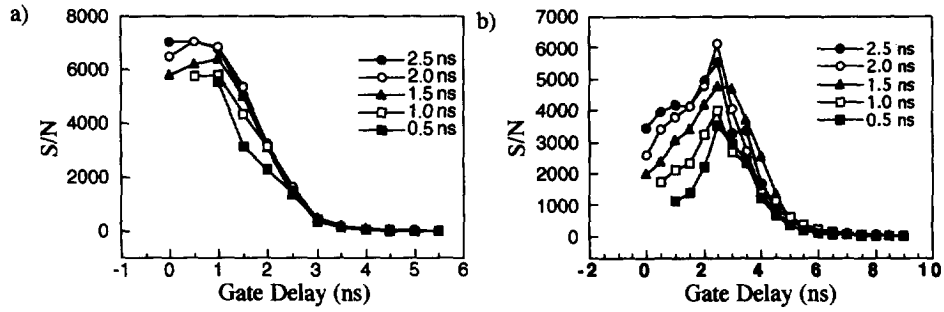

Fig. 1. Dependence of the *S/N* on the width of the gate window and the delay of the time gate. Accumulation time, 1 s; concentration 0.2 m*M*. (a) Oxazine 725, (b) rhodamine 700.

is large. Whereas at a longer delay, the background can be completely removed, although the fluorescence becomes weak, especially for a dye with a short lifetime.

The *S/N* plots exhibited a different behavior for the two dyes. The optimum gate delay and the optimum gate width of oxazine 725 were 0.5 ns and 2.0 ns, respectively. Even though the scattering light was the largest at 0.5-ns delay, the fluorescence signal also was large at 0.5-ns delay; it became too small at a longer delay due to its short lifetime. At 0.5-ns delay, the fluorescence signal increased as the width increased up to 2.0 s, and it stayed constant for an even longer width. The optimum delay and the optimum width of rhodamine 700 were 2.5 ns and 2.0 ns, respectively. Due to its longer lifetime, the scattering light could be removed by setting a longer delay (2.5 ns) without any serious loss in the fluorescence signal. A sample with a longer lifetime is more suitable for a time-resolved technique. The *S/N* ratio was, however, larger for oxazine 725 than for rhodamine 700 in Fig. 1, because the former has a larger absorptivity at 655 nm.

Effects of the gate delay were investigated on the electropherogram of three dyes (2×10^{-5} *M*) as shown in Fig. 2a,b. The peaks of rhodamine 700 and methylene blue are broad probably due to adsorption on the capillary wall. The *S/N* of oxazine 725 was 72 at 0.5-ns delay and 39 at 2.5-ns delay, and that of rhodamine 700 was 12 at 0.5-ns delay and 15 at 2.5-ns delay due to its tailing. A short delay is more preferable for a dye with a shorter lifetime, because the fluorescence signal becomes too small at a longer delay. Meanwhile, a longer delay gives better *S/N* for a dye with a longer lifetime, because the laser scattering can be removed more effectively at a longer delay as in the case shown in Fig. 1. It is essential for a small laser fluorescence detector to remove any background noise such as the scattering light and the Raman band, at a time of the laser irradiation. The time-resolved technique can solve this difficulty effectively for a dye with a long lifetime.

A typical electropherogram for the four dyes (2×10^{-5} *M*) is shown in Fig. 2c. The detection limits of the four dyes were determined on the basis of three electropherograms obtained at 0.2, 2 and 20×10^{-5}

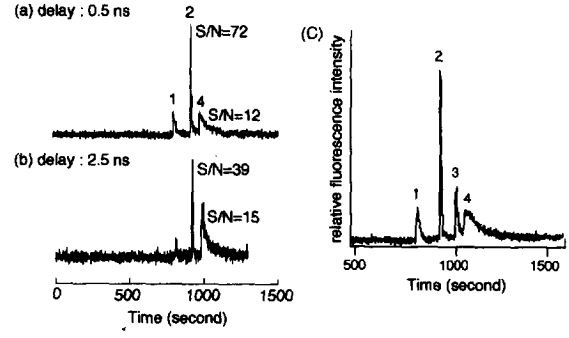

Fig. 2. Typical electropherograms of four dyes at 2×10^{-5} *M* and at pH 3.4. Peaks: 1 = Methylene blue; 2 = oxazine 725; 3 = HIDC; 4 = rhodamine 700. Gate window width, 2 ns; time gate delay, (a) 0.5 ns; (b) 2.5 ns; (c) 0.5 ns.

M, where they were smoothed by the Savitzky–Golay 15-point cubic smoothing [12]. The results are shown in Table 1. The best detection limit was 2 fmol at $S/N = 3$ for oxazine 725; this was mainly due to its intense absorption at the laser wavelength and its intense fluorescence at 700 nm. The detection limit of methylene blue was reported to be 0.4 fmol [4]; they used a CW SCL and a DC amplifier, and the power of their SCL was about 100 times larger than ours.

A time-resolved technique is capable in principle of removing any effect of the laser scattering. Because the laser scattering is the most serious problem for a miniaturized probe volume, the present technique should be improved further.

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