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Highly sensitive detection using laser two-photon excited fluorescence in capillary electrophoresis

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Abstract

The laser two-photon excited fluorescence (LTPF) has been applied to a capillary electrophoresis (CE) system using a self mode-locked titanium–sapphire laser. The analytes were coumarine 440 (7-amino-4-methyl coumarine) and DCCS (7-diethyl aminocoumarine-3-carboxylic acid succinimidyl ester). The two-photon excited fluorescence emission spectrum of DCCS was identical to the one-photon excited fluorescence emission spectrum. The LTPF quantum efficiency increased through the addition of methanol to the buffer solution, and, as a result, the detection limit decreased. The detection limit of coumarine 440 was 8.0 amol, this value was as low as that obtained by one-photon excited fluorescence.

Keywords: Detection, capillary electrophoresis; Two-photon laser-induced fluorescence; Fluorescence detection, two-photon; 7-Amino-4-methylcoumarine (Coumarine 440); 7-Diethylaminocoumarine-3-carboxylic acid succinimidyl ester (DCCS)

1. Introduction

Laser-induced fluorescence has provided highly sensitive detection for analytes separated in a capillary electrophoresis (CE) system. Recently, a detection limit of 6 molecules has been reported [1]. The first step of a fluorescence process is absorption of a photon. The absorption rate for a pulsed laser excitation is the product of the absorption cross-section (cm^2/molecule), laser intensity (photons $pulse^{-1}$ cm^{-2}) and repetition rate of the laser (s^{-1}) [2]. A pulsed laser usually has enough power to induce saturation of excitation. Accordingly, the key in the design of a high-sensitivity detection system is not how to increase the fluorescence intensity but how to reduce the laser scatter and background signal. Rayleigh or Raman scatter is faster than fluorescence, which commonly occurs at sub-picosecond–nanosecond speeds, and can be separated by the time-gated detection. Confocal microscopy can eliminate nearly all of the out-of-focus laser scattering by controlling the size and position of the pinhole properly. Thus, time-gated detection [3,4] and confocal microscopy [5] have been representatively used as an effective method to reduce the laser scattering in CE.

Laser two-photon excited fluorescence (LTPF) has been investigated little mainly because of the smaller fluorescence intensity compared with one-photon excited fluorescence. However, two-photon spectroscopy can provide analytical information not accessed by one-photon spectroscopy [6,7]. The high excitation efficiency of LTPF has recently been achieved, and its application to high-sensitivity LTPF detection

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has been carried out [7–9]. The merit of LTPF lies in its efficient rejection capability against laser scatter, because the excitation wavelength is far from the fluorescence wavelength; this merit is especially significant for a fluorescent molecule with a small stokes shift.

In this work, LTPF was applied to a CE detection system and its possibility was investigated. A few fluorescent molecules with an absorption at 400 nm with a small stokes shift were analyzed using a titanium–sapphire laser (wavelength range, 720–910 nm). The detection limit was reduced by the enhancement of the fluorescence quantum yield through the addition of methanol to the buffer solution in the CE system. The two-photon excited fluorescence spectrum measured in the CE system was compared with one-photon excited fluorescence.

2. Experimental section

2.1. Equipment.

The schematic diagram of the experimental apparatus is shown in Fig. 1. A titanium–sapphire self mode-locked laser (Coherent Mira 900) pumped by an Ar ion laser (Coherent Innova 300) was used for two-photon excitation. The titanium–sapphire laser produces a pulse with a 180 fs temporal width and 76 MHz repetition rate at 800 nm. Its average power was 0.8 W. The laser light was focused into the capillary (J&W Scientific, 50 μm×1 m, 75 cm effective length) using a 10-X microscope objective lens. The fluorescence emission was collected at a right angle by an identical lens, transmitted through a glass filter (Andover, 040FG11, 320–680 nm, transmission at 800 nm$<10^{-5}$), and then focused onto a photomultiplier tube (PMT) (Hamamatsu, R5600U). The quantum efficiency of this PMT at 800 nm is as low as 0.01, and the PMT is insensitive to the laser scattering light. The photocurrent from the PMT was amplified by a current amplifier (EG&G, ORTEC 9302, 100 MHz) and conditioned by a constant fraction discriminator (EG&G, ORTEC 473 A) and then analyzed with a computer (NEC PC-9801RX).

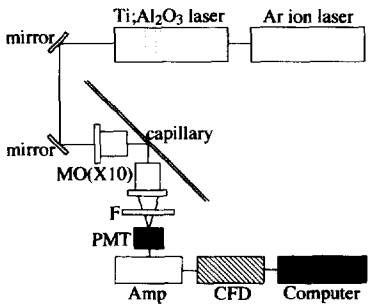

Fig. 1. Schematic diagram of experimental apparatus. MO = microscope objective; F = glass filter; Amp = amplifier; CFD = constant fraction discriminator; PMT = photomultiplier tube.

A high-voltage power supply (Matsusada Precision, HCZE-30PN0.25) was used to generate the electric field across the capillary. The injection volume was 2 nl, controlled electrokinetically [10]. The running buffer consisted of 20 m*M* sodium carbonate adjusted to pH 11. In order to clean the capillary, vacuum-flushing using a 0.1 *M* NaOH solution was performed for about 10 min before each measurement.

2.2. Chemicals

Coumarine 440 (7-amino-4-methyl coumarine) and 2-amino-anthracence were obtained from Tokyo Kasei. DCCS (7-diethyl aminocoumarine-3-carboxylic acid succinimidyl ester) was obtained from Molecular Probes. Sodium hydroxide, ethanol and sodium carbonate were purchased from Kishida, and used without further purification.

3. Results and discussion

Analyte molecules used in the present work have no absorption at 800 nm. Accordingly, the two-photon excitation should proceed in a simultaneous absorption. In this case, the fluorescence intensity P_f can be expressed as follows [8]:

$$P_f = KP_{av}[\delta l(P_{peak}/A)]C(\phi/2) \quad (1)$$

where ϕ is the fluorescence quantum efficiency, K is the collection efficiency of the detection system, δ is the two-photon absorption cross-section, C is the analyte concentration, l is the path length, A is the cross-sectional area of the focused laser beam, P_{av} is the average laser power and P_{peak} is the peak power.

Thus, both the laser average power and the peak power is proportional to the LTPF intensity. Accordingly, the titanium–sapphire laser (0.8 W average power, 0.58 MW peak power) used in the present work can be used efficiently for the two-photon excitation.

Fluorescein isothiocyanate has been widely used as a fluorescence-labelling reagent of amino acid. It has an absorption band at around 490 nm, and is suitable for fluorescence analysis, using an Ar-ion laser [11]. DCCS is another fluorescence-labelling reagent for amino acids with an absorption band at around 419 nm, and analysis of amino acids using DCCS in second-harmonic generation (415 nm) of near-infrared semiconductor lasers has been reported [12]. Two-photon excited fluorescence emission spectra of 1×10^{-5} *M* DCCS excited at 800 nm by the titanium–sapphire laser was measured by a spectrograph (Thermo Jarrell Ash, Monospec 18) with a CCD detector (SBIG, ST-6), as shown in Fig. 2; DCCS was flowed through the capillary under the 20 kV applied voltage in the CE system. One-photon excited fluorescence spectra of 2×10^{-6} *M* DCCS excited at 400 nm was measured by a fluorescence spectrophotometer (Hitachi F-4010) using the identical carbonate buffer solution. They were normalized at the peak. The two-photon excited fluorescence spectrum of DCCS is virtually identical to the one-photon excited fluorescence spectrum within the resolution of the experiment. Thus, there would be no difference in the emission process even if excited by one-photon or two-photon process. Two-photon excited and one-photon excited fluorescence emission spectra are indistinguishable in most cases [13,14].

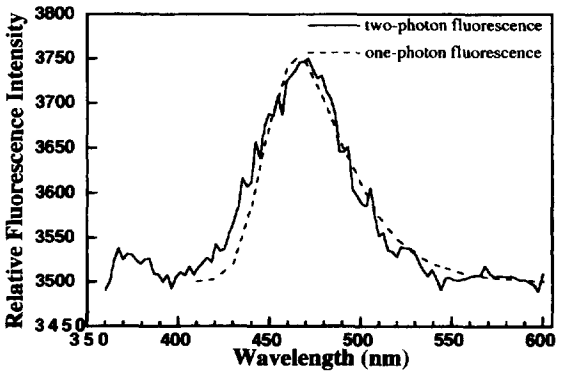

Fig. 2. Comparison of two-photon excited fluorescence spectra and one-photon excited fluorescence spectra; solid line = two-photon excited fluorescence spectrum, dashed line = one-photon excited fluorescence spectrum.

Detection efficiency of LTPF depends on ϕ, which may depend on the solvent. The detection efficiency increased through the addition of methanol to the buffer solution in the constructed CE detection system. The electropherograms of the DCCS obtained in pH 11, 20 m*M* carbonate buffer solution and in 30% methanol (v/v) 20 m*M* carbonate buffer solution of pH 7 are shown in Fig. 3a and b, respectively. The applied voltage (25 kV) and the injection amount (4 fmol) were the same. The detection limit was determined from an analytical

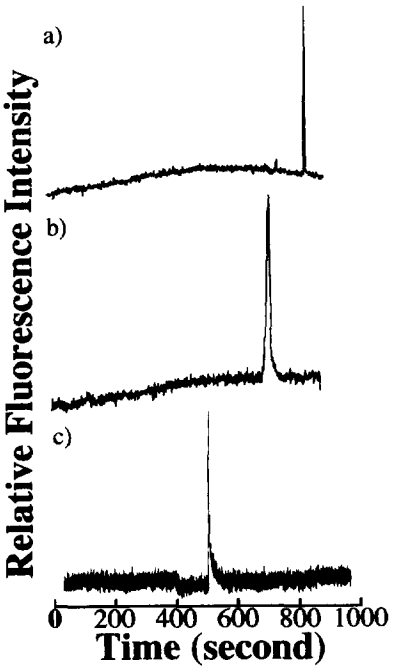

Fig. 3. Electropherograms of DCCS obtained in different buffer composition and electropherogram of 2-amino-anthracene by two-photon excited fluorescence. Applied voltage was 25 kV. (a) Electropherogram of DCCS obtained in 20 m*M* carbonate and NaOH buffer solution of pH 11; electrolyte concentration was higher due to the presence of NaOH. Injection amount was 4 fmol and detection limit was 81 amol. (b) Electropherogram of DCCS obtained in 30% methanol (v/v) 20 m*M* carbonate buffer solution of pH 7; electrolyte concentration is lower. Injection amount was same as in (a) and detection limit was 33 amol. (c) Electropherogram of 2-amino-anthracene obtained in same buffer solution as in (a). Injection amount was 20 fmol and detection limit was 0.4 fmol.

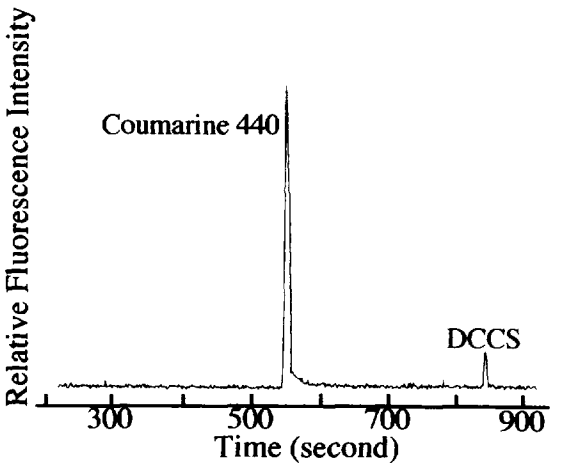

Fig. 4. Electropherogram of dyes mixture by two-photon excited fluorescence. Applied voltage was 25 kV. Injection amount of coumarine 440 and DCCS were all 4 fmol. Buffer solution was pH 11 and 20 m*M* carbonate solution.

curve of peak height as 33 amol for Fig. 3b and 81 amol for Fig. 3a at $S/N=3$. The lower detection limit for Fig. 3b would be mainly due to the increase of ϕ, because the intensity ratio of Fig. 3a to Fig. 3b is approximately equal to the fluorescence intensity ratio measured by the conventional fluorescence spectrophotometer, and, thus, the effect of photostability at a higher excitation intensity is negligible.

As a result of the change in the buffer composition, the migration velocity was apparently different in Fig. 3a and Fig. 3b. The increase in the migration velocity of DCCS in the case of Fig. 3b is mainly due to the neutralization of DCCS at pH 7 and the resulting disappearance of the electrophoretic migration. The electroosmotic flow in Fig. 3b was smaller than that obtained in Fig. 3a, due to the addition of methanol [15], the change in pH and the electrolyte concentration [16]. This was confirmed by the electropherogram of the neutral molecule as 2-amino-anthracene measured using an identical buffer solution with Fig. 3a, as shown in Fig. 3c.

The electropherogram of coumarine 440 and DCCS is shown in Fig. 4. These two dyes have a small stokes shift. They are good target molecules for highly sensitive detection by LTPF. The detection limits of dyes were determined from an analytical curve of peak height using a least-square method and the S/N was 3. The analytical curves were linear for more than 2 orders of magnitude above the LOD. Electropherograms of coumarine 440 obtained at three injection amounts are shown for demonstration in Fig. 5. The lowest detection limit of coumarine 440 was 8.0 amol (1.4 fg, 4.5 n*M*) which is as small as a typical on-column detection sensitivity (10^{-9} M–10^{-10} M) [17,18] of one-photon excited fluorescence for which confocal microscopy was not used. The similar detection limit indicates that the much smaller laser scattering in LTPF offsets the lower excitation efficiency in LTPF. The large laser scattering in one-photon excited fluorescence has been effectively removed by confocal microscopy, and an excellent detection sensitivity of 6 molecules has been reported [1]. The above result also proves the usefulness of the

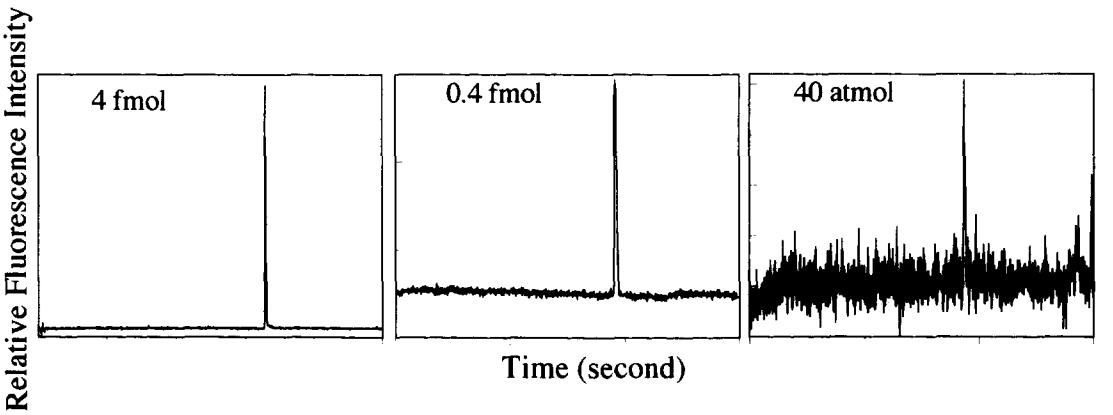

Fig. 5. Electropherograms of coumarine 440 obtained at different injection amounts: 4 fmol, 0.4 fmol, 40 amol in 2 nl injection volume.

application of LTPF to high-sensitivity detection in CE. This detection limit is 2 orders of magnitude better than the other result (250 fg, 1 nM) [7] obtained using LTPF in liquid chromatography in terms of the absolute value, but an equal order in terms of concentration. The excitation efficiency ($P_{av} \times P_{peak}$, 4.7×10^4) of the titanium–sapphire laser in the present work is nearly the same as that (6×10^4) of the Cu vapour laser in Ref. [7]. The photon counting system suitable for the mode locked laser with a high repetition rate would contribute to the detection limit. The two-photon absorption cross-section, fluorescence collection efficiency and quantum efficiency should also be considered; however, these factors can not explain the large difference (2 orders in the detection limit). In particular, it should be noted that the detection limits in the concentration (mol/l) are in the same order for the two investigations. This can be explained mainly by the compatibility of CE with LTPF. The LTPF intensity increases with decreases in the cross-sectional area of the laser beam, as shown in Eq. (1), and the tight focus of the excitation laser is effective. Thus, LTPF is very suitable for a small-volume measurement, such as in CE.

The background signal (~200 cps) was unexpectedly large, even at the two-photon excitation, as confirmed by a fluctuation of the background signal in the electropherogram. There were two main sources for the background. The first one is the second harmonic generation (400 nm) from the capillary wall due to the nonlinear optical process (~100 cps). The other is laser scattering (800 nm) at the capillary wall; the expansion of the capillary by thermal heating deviated the laser focal point to cause an increased laser scatter. These backgrounds were detected owing to the rather broad wavelength path range of the filter (320–680 nm). The dark current signal of the PMT was small. The detection limit in the present work can be lowered further, down to a sub-amol level, if the large background can be removed using a narrow-bandpath optical filter. Enhancement of the fluorescence collection efficiency, K, in Eq. (1) by using a lens with a higher numerical aperture would also contribute to a higher detection sensitivity.

In this work we showed that highly sensitive detection using a LTPF merit of large difference between the excitation and fluorescence wavelengths could be successfully achieved in a CE system. We confirmed that LTPF can be efficiently used as an alternative to the one-photon excited fluorescence method.

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