Application of an avalanche photodiode in a near-Geiger operation as a fluorescence detector for capillary electrophoresis

Hirofumi Kawazumi[a,*], Joon Myong Song[b], Takanori Inoue[b], Teiichiro Ogawa[b]

[a]*Department of Industrial Chemistry, Kinki University in Kyushu, Iizuka 820, Japan*
[b]*Department of Molecular Science and Technology, Kyushu University, Kasuga 816, Japan*

Abstract

A laser-induced fluorescence detection system for capillary electrophoresis has been developed by use of a semiconductor laser (655 nm) as an excitation light source and an avalanche photodiode (APD) as a fluorescence detector. The APD may be used in a near-Geiger condition by adjusting its bias voltage, and its internal gain to photoelectrons increases substantially. Subnanosecond time-resolved photon counting using the APD in the near-Geiger operation has provided very high sensitivity due to its well-defined photoelectron pulse shape and an enhancement of the counting efficiency. The detection limit of Oxazine 725 was 47 amol, which was about 100 times better than that using a photomultiplier.

Keywords: Detection, electrophoresis; Laser-induced fluorescence detection; Photodiode, avalanche; Oxazine; Methylene blue; Hexamethylindodicarbocyanine iodide

1. Introduction

Laser-induced fluorescence (LIF) is a very powerful detection technique in capillary electrophoresis (CE) because the coherence of the laser light can produce a high photon flux in a small capillary. Chen and Dovichi have recently reported a detection limit as low as 30 molecules for a rhodamine dye in CE separations [1]. In developments in the CE instrumentation, designs regarding reliabilities and dimensions are very important. In most previous studies with the LIF detection, a gas laser, such as an Ar ion or He-Cd laser and a photomultiplier tube (PMT), was used as the light source and detector. However, the gas laser and PMT are relatively poor in respect of the reliabilities and dimensions, respectively, compared to the latest electronic and optic components.

As a light source of a fluorescence detector, the power stability is important rather than its power because the scattering light becomes dominant in backgrounds. A semiconductor laser (SCL) is an alternative to a gas laser [2–5]. The stability of the former is better by 1–2 orders than that of the latter due to power controls with simple current feedback. Furthermore, the SCL is a small and reliable light source, having advantages of high lasing efficiency, long lifetime and low cost compared with gas lasers. These advantages offer a promising prospect as the light source in the instrumental analysis [2]. There are some studies on LIF detection in CE using the SCL [6–10]. They have reported detection limits below the femtomol level for dyes, dye-labeled amino acids and polycyclic aromatic hydrocarbons. Indirect photometry in CE has also been successful utilizing the power stability of the SCL [11,12].

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*Corresponding author.

Recently an avalanche photodiode (APD) has been used as an alternative to a PMT in LIF detection [9,13–16]. This solid-state device has an internal electron amplification action, and provides superior sensitivity and fast response compared to an ordinary photodiode [17]. However, the internal amplification in a normal operation (ca. 300) is much lower than that of the PMT (ca. 10^6). The advantages of the APD are a high quantum efficiency of photoelectron production, a fast response and the robustness against mechanical damage and an over light exposure. If the photoelectron pulses could be counted selectively in excess dark current, the APD has potential as a highly sensitive detector. When the bias voltage applying to the APD is increased, its internal amplification is boosted and a photon induces a Geiger discharge pulse. Such an APD especially prepared for single-photon counting has been successfully used for an ultrasensitive detection of near-IR dye-labeled amino acids [13,15] and a single molecule detection combined with a confocal technique [14,16].

Our previous works presented fast time-resolved photon counting detection for CE by combining the SCL as the light source and the APD or PMT as the detector [9,10]. In this work we evaluate the performance of the APD that has operated in normal or near-Geiger modes. The near-Geiger discharge condition has allowed a very sensitive photon counting with a wide dynamic range. Such an operation is suitable for a practical fluorescence detector.

2. Experimental

Fig. 1 presents a block diagram of the LIF detection system using the SCL and APD for CE. A scheme of the subnanosecond time-resolved photon counting is similar to that described previously [9,10]. The light source was a pulse-operated SCL (Hamamatsu Photonics, Shizuoka, Japan; LDH-065/ PLP-01); its emission wavelength was 655 nm, peak power 29 mW, and pulse width 74 ps. The laser light was delivered through an optical fiber and focused to a capillary with a 10 × microscope objective (Edmund Scientific, Barrington, NJ, USA). The emission was collected with another 10 × microscope objec-
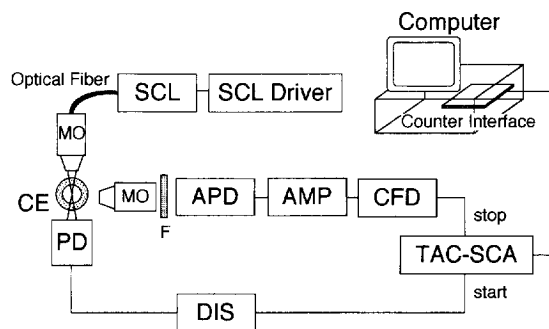

Fig. 1. Block diagram of the time-resolved LIF detection system using semiconductor laser (SCL) and avalanche photodiode (APD). MO, microscope objective; F, interference filter; AMP, preamplifier; CFD, constant fraction discriminator; TAC-SCA, time-to-amplitude converter/single-channel analyzer; PD, photodiode; DIS, discriminator.

tive, filtered optically with an interference filter (center wavelength of 700 nm, 15 nm band width, Andover, Salem, NH, USA), and focused on an APD (Hamamatsu Photonics; C5331-03). A red-sensitive PMT (Hamamatsu Photonics; R1477) with a multialkali photocathode replaced the APD as a reference. Photocurrents from the APD were amplified with a wide-band current amplifier ($2 \cdot 10^4$ V/A) which was preassembled by the manufacture. Photocurrents from the PMT were converted voltages with a 50 Ω resistance. Outputs from the APD or PMT were conditioned using an 100 MHz amplifier (Ortec, Oakridge, TN, USA; 9302) and a constant-fraction discriminator (Ortec; 473A). The laser light passing through the capillary was detected by a photodiode (Hamamatsu Photonics; S1336-5BQ) and its output was conditioned to a discriminator (Ortec; 436). The two pulses of the laser light and the emission from the sample were, respectively, fed to the start and stop inputs of a time-to-amplitude converter/single-channel analyzer (Ortec, 567). The personal computer (NEC, Japan; PC9801RX) accumulated only coincident events in the gate window. The repetition rate of the gate was 140 kHz. Pulse-height distributions were measured with a multi-channel analyzer (Laboratory Equipment, Ibaraki, Japan; MCA/ PC98BX) after the outputs from the APD or PMT were conditioned by a filter amplifier (NF, Yokohama, Japan; P-61/P-82).

The CE system was constructed in our laboratory. The full length of the fused-silica capillary (J & W, Folsom, CA, USA; 75 μm I.D., 350 μm O.D.) was 1 m and the effective length was 0.75 m at a detection window. A high-voltage power supply (Matsusada Precision, Shiga, Japan; HCZE-30PN0.25) was used to apply a positive voltage of 28 kV across the capillary. Samples were injected electrokinetically and the injection volume was 10 nl.

Methylene Blue, Oxazine 725 perchlorate, and HIDC (1,1′,3,3,3′,3′-hexamethylindodicarbocyanine iodide) were obtained from Lambda Physik (Acton, MA, USA) and used as supplied. The running buffer consisted of $2.0 \cdot 10^{-2}$ mol/l sodium phosphate adjusted to pH 3.4. The capillary was pressure-flushed with a 0.1 mol/l sodium hydroxide solution for 10 min in each experiment. This was critical to avoid any adsorption of the dyes by the capillary inner wall.

3. Results and discussion

In order to achieve an efficient and sensitive photon counting even with the low internal amplification, pulse heights of photoelectrons and dark pulses should be clearly separated. The pulse-height distributions of the PMT and APD were measured to reveal the character of the near-Geiger mode, as shown in Fig. 2. The light source used was the same SCL in the continuous wave operation. The results of the PMT at applied voltages of 700 and 900 V are presented in (a) and (b). In (c), the normal mode of the APD, the internal gain was set to be about 300. In (d), the near-Geiger mode, the bias voltage was increased till the Geiger discharge pulses sometimes appeared. This adjustment of the bias voltage was essential for the highly sensitive detection. The total counts in the light-on and off (dark) conditions were normalized at $6 \cdot 10^5$ and $3 \cdot 10^5$. The normal mode of the APD cannot discriminate the photon and dark pulses due to the similar traces of the light-on and off conditions. For the APD with the near-Geiger mode and the PMT applied voltage of 900 V, the pulse-height distributions clearly show thresholds that offer efficient photon counting. If the dark current was excluded in the APD detection, the APD
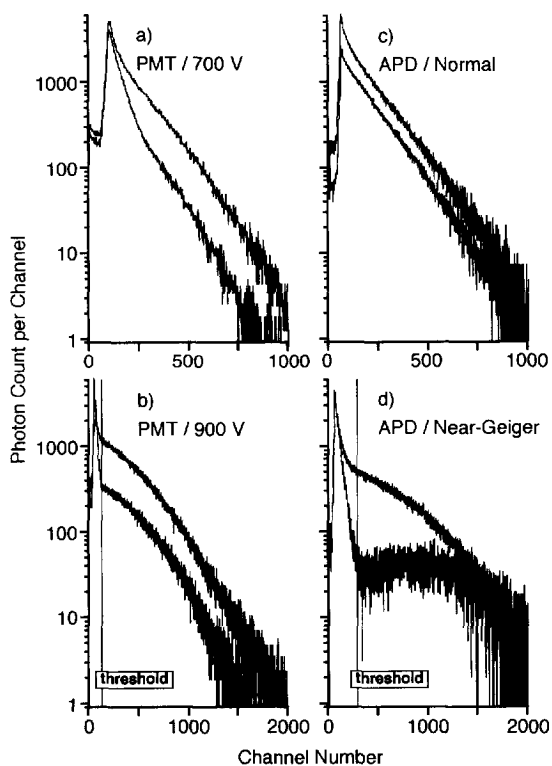

Fig. 2. Pulse-height distributions of outputs from PMT and APD. The upper and lower traces correspond to light-on and off (dark) conditions, respectively, in all layouts. Channel numbers are nominal resulting from different gains in each layout.

could be advantageous in regard to the quantum efficiency.

The time-resolved photon counting technique has been successfully used to remove laser scattering light and unwanted backgrounds from the fluorescence signal [9,10,13,18]. The pulse-operated SCL easily provides subnanosecond resolution. Fig. 3 shows electropherograms with a 0.5 ns gated measurement by using the PMT (above) and APD (below). The accumulation time was 1 s. The APD operated in the normal mode. Peaks corresponded to Methylene Blue ($1 \cdot 10^{-4}$ mol/l), Oxazine 725 ($1 \cdot 10^{-5}$ mol/l), and HIDC($1 \cdot 10^{-4}$ mol/l) in the migration order. Detectability seems to be identical for these two electropherograms. The baseline was flat due to high power-stability of the SCL. We define the S/N as

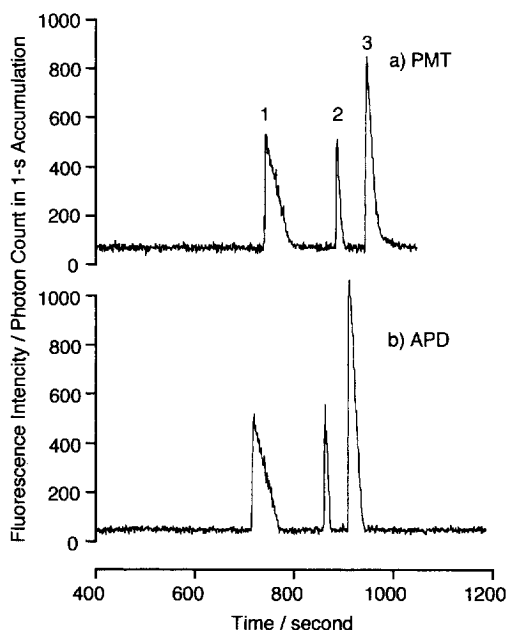$$S/N = \frac{\text{Peak height of Oxazine 725}}{2\sigma}$$

Fig. 3. Electropherograms with the 0.5 ns gated measurement by using PMT (a) and APD (b). Peaks: Methylene Blue (1), $1.0 \cdot 10^{-4}$ mol/l; Oxazine 725 (2), $1.0 \cdot 10^{-5}$ mol/l; HIDC (3), $1.0 \cdot 10^{-4}$ mol/l.

where σ is a standard deviation for 200 s baseline fluctuation in the electropherogram. The S/N using the PMT and APD was 29 and 39, respectively, in Fig. 3. In spite of the low internal amplification of the APD, the S/N was slightly better than that of the PMT in the subnanosecond time-resolved measurement. This may be explained as resulting from the fact that the quantum efficiency of the APD is superior to that of the PMT at the detecting wavelength; typical quantum efficiencies of the APD and PMT at 700 nm are 0.75 and 0.04, respectively [17,19].

Table 1 shows an improvement of S/N in various measurement modes with APD. The value of S/N in the first column is converted to 1 for $1 \cdot 10^{-4}$ mol/l Oxazine 725. The S/N of the 0.5 ns gated measurement was 35 times higher than that of no-gated one in which a counter continuously accumulated the pulses. The 2 ns gated mode offered better S/N than the 0.5 ns one because of optimizing the gate width to the fluorescence decay; the fluorescence lifetime of Oxazine 725 was 0.9 ns [10]. The S/N significantly increased in the near-Geiger mode.

Table 1
Signal-to-noise ratios (S/N) in various measurement modes with APD

Mode	S/N[a]	Improvement factor[b]	
		Observed	Calculated
No-Gate	11	1	1
Gate (0.5)	390	35	44
Gate (2 ns)	540	48	56
Gate + Near-Geiger	43 000	3900	–

[a] Sample: $1 \cdot 10^{-4}$ mol/l Oxazine 725; accumulation: 1 s.
[b] Values relative to the no-gate mode.

The last two columns in Table 1 present observed and calculated improvement factors; these are relative values to the no-gate and normal operation mode. Calculated factors were obtained as follows. If the noise, that is, the baseline fluctuation is decided by the dark current that produces Poisson-distributed pulses, the noise counts could be reduced down to that corresponding to an effective counting time [18,20]. For the shorter gate to the fluorescence decay, a correction is necessary for a signal collection efficiency. An overall relationship is given by

$$S/N = k \frac{f_c}{\sqrt{t_g r}} \qquad (1)$$

where f_c is the fluorescence collection efficiency, t_g the gate width, r the repetition rate and k proportional constant. The f_c is 0.37 and 0.94, respectively, for the gate width of 0.5 ns and 2 ns; these are estimated from time profiles of emission in [9]. Good agreements are found in changes of the observed and calculated improvement factors. This indicates that the dark current governs the noise and the laser scattering light is not dominant in the noise under the APD detection. Since the APD was not cooled in the present experimental conditions, this result seems to be reasonable. The time-resolved technique is especially useful for the APD detection in which it is difficult to discriminate between photons and dark current pulses due to the low internal amplification

We observed significant enhancement of the S/N in the near-Geiger operation, as shown in Table 1. The APD seems to work properly with the mechanism that we expected as the well-defined photoelectron pulses are distinguished from the dark pulses.

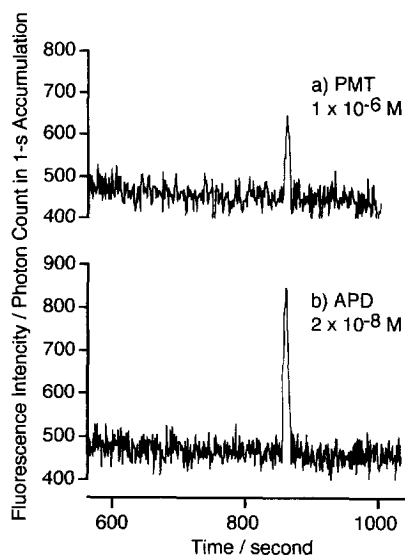For a comparison of the detectability, the elec-

Fig. 4. Electropherograms near detection limits by using PMT (a) and APD (b). Sample: Oxazine 725. The APD was operated in near-Geiger mode.

Table 2
Characteristics of APD and PMT in photon counting mode

	APD[a]	PMT[b]
Quantum efficency (Q)	0.75	0.04
Count efficency for photosignal (f_p)	0.65	0.70
Count efficiency for dark current (f_d)	0.23	0.44
Dark current (D)	12 nA	38 nA

[a] Hamamatsu, C5331-03; near-Geiger operation.
[b] Hamamatsu, R-1477; applied voltage of 900 V

tropherograms near detection limits of Oxazine 725 were measured by using PMT (above) and APD (below), as shown in Fig. 4. The scheme of the time-resolved photon counting was the same as in Fig. 3 except for the expansion of the gate window to 2 ns and the near-Geiger operation of the APD. Therefore, in Fig. 3 and Fig. 4a the limits of detection using the PMT were identical and $4.5 \cdot 10^{-7}$ mol/l at $S/N = 2$. The S/N of the electropherogram with the APD was 98 times higher than that with the PMT; note the concentration of the sample $(2 \cdot 10^{-8}$ mol/l) in Fig. 4b. The limit of detection using the APD was $4.7 \cdot 10^{-9}$ mol/l and the mass detectability was 47 amol. No long-term fluctuation in the baseline of the electropherogram indicates the stability of the near-Geiger operation of the APD used without cooling.

Characteristics of the APD and PMT in the photon counting operation are summarized in Table 2. In the single photon counting method, the S/N is represented as follows [20];

$$S/N = k \frac{Q f_p}{\sqrt{D f_d}} \qquad (2)$$

where Q is the quantum efficiency of the photo-

cathode, D the dark current, f_p and f_d the count efficiency for the photocurrent and the dark current, respectively. These efficiencies were estimated from sums of the counts in the channels before and after the threshold in Fig. 2. From calculations of Eq. (2) based on Table 2, the S/N of the APD was 43 times higher than that of the PMT. This value is consistent with the observed one (98 times) within the factor of 2.

The present study demonstrated the LIF detection system for CE by using the subnanosecond time-resolved measurement with all solid-state devices of the SCL and APD. This system provided a comparable detection limit in the normal operation of the APD to that of the PMT. In the near-Geiger mode the S/N increased by about 2 orders and presented amol level detectability. The SCL is now making progress in extending its emitting wavelength and will straightforwardly replace the gas laser as the light source in the CE detector. The reliability and compactness of the solid-state components used in this study provide potentials for instrumental design of CE.

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