

Study on a Highly Sensitive On-Column Detector
for Capillary Electrophoresis
Using Laser-Induced Capillary Vibration Effect

レーザー誘起キャピラリー振動効果を用いた
キャピラリー電気泳動の高感度オンカラム検出器の研究

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Study on a Highly Sensitive On-Column Detector
for Capillary Electrophoresis
Using Laser-Induced Capillary Vibration Effect

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CHAPTER 1 Backgrounds and Purposes of This Study

1.1 Introduction

--Importance of Highly Sensitive Detectors for Ultramicroanalysis--

In recent years, ultramicroanalysis has been becoming to play an important role in the scientific technologies, such as medical, pharmaceutical, physiological, or biochemical field. In the material sciences, for instance, information obtained from the micro volume, or micro region are essential to probe the small defects, which may become decisive to the material. In such cases, the representative value obtained from bulk has no meaning. While, in the biological sciences, researches on, for example, gene analysis depend largely on the techniques of ultramicro analysis. Analytical technique involves a series of operation: sampling, pretreatment including separation and concentration, and detection. The last process, detection, is a key process in the ultramicroanalysis. To find out physiologically important materials, many plants and animals are sacrificed, because there are not sensitive detectors for them. In gene analysis, cloning process to scale up enough to be detectable takes a deal time, and polymerase chain reaction (PCR) amplification faces danger of suffering from by-products. Laser-induced fluorometric detection is very sensitive, but it needs process of fluorescent derivatization of analyte. Such chemical

handling in ultramicro volume becomes very difficult.

Thus, in the ultramicroanalysis, to detect small quantity of low concentration involved in small volume, highly sensitive detectors which need no precedures such as fluorescent derivatization are desired. They will give us new information about the very small fluctuations of materials inside the small volumes, such as single cells.

1.2 Capillary Electrophoresis

Capillary electrophoresis (CE) is a separation method, which is said to be first proposed and demonstrated by Jorgenson around in 1980. The sample volume needed in CE is nanoliters or less that CE technique was considered to be effective in ultramicroanalysis. In this section, the basic principle of CE separation and detection limit are introduced.

The typical arrangement of CE system is shown in Figure 1-1. Capillary tube used is, in most case, fused silica. The inner diameter is 50 to 100 μm , and the outer diameter is 150 to 500 μm . The outer is usually covered with polymer (polyimide) to give flexibility. A part of this polymer coating, where photometric or fluorometric detection is performed, is removed usually using gentle flame. The capillary length is usually 50 to 100 cm. When the capillary is filled with electrolyte buffer

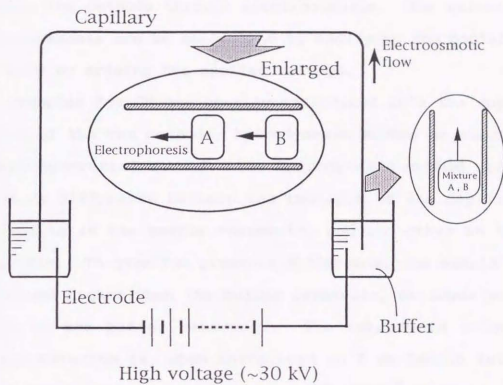
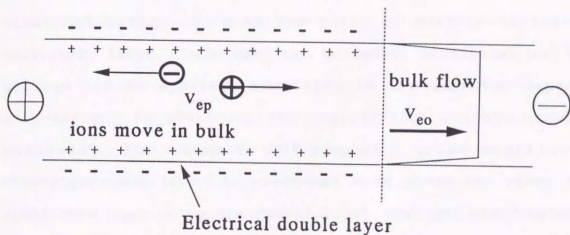


Figure 1-1. Typical arrangement of CE system.

solution, electrical double layer is formed inside the capillary as shown in Figure 1-2. The ζ potential becomes the pumping source of flow, and when the high voltage is applied between both ends of the capillary, the fluid inside the capillary moves towards the cathode through electroosmosis. The velocity of the electroosmosis can be controlled by modifying the capillary wall, not only by arising the applied voltage.

Samples for CE are usually introduced into the capillary by either of the two methods, hydrodynamic method or electrokinetic (electrophoretic) method. The hydrodynamic method utilizes the pressure difference between the two ends of the capillary. The one end is in the sample reservoir, and the other in the buffer reservoir. To give the pressure difference, the sample reservoir is placed higher than the buffer reservoir, or lower pressure is given to the buffer reservoir. The introduced volume of the sample solution is, when introduced in 1 mm length into a 50 μm i. d. capillary, calculated to be 2×10^{-9} l (2 nl). In the electrokinetic introducing method, a voltage is applied between the two ends, one is in the sample reservoir. The composition of introduced solution is different from that in the sample reservoir, because the electrophoretically faster material is introduced more than the electrophoretically slower material.

After the introduction of the sample, the sample end of the capillary is put in the buffer reservoir again, and a high voltage up to 30 kV is applied for separation. The separation of analytes is performed according to the difference of electrophoretic mobilities, which is dependent on the net charge,



$$V_x = V_{eo} + V_{ep}$$

Figure 1-2. Schematic illustration of electric double layer formed near the capillary wall.

eo: electroosmosis

ep: electrophoresis

size, shape of molecules. The net charge depends largely on the separation buffer component. Therefore, it is very important to select the best buffer system for separation.

The separation performance of CE is wonderful. The plate number reaches $10^5 - 10^6$, which is about 2 orders of magnitude superior to the other separation techniques such as high performance liquid chromatography (HPLC) and slab gel electrophoresis. Because the ratio of surface-to-volume is relatively large, Joule heat can be easily dissipated and higher voltage can be applied, resulting in the superior separation efficiency. In addition, the applied high voltage accelerate analysis. For example, DNA sequence using capillary gel electrophoresis (CGE) is performed with about ten times higher speed than that using the conventional slab gel electrophoresis. (Drossman et al., 1990; Swerdlow and Gesteland, 1990)

Thus, the CE technique has excellent characteristics as a separation technique. It has another merit that it needs only ultramicro amount (less than nl) of samples, which is suited for ultramicroanalysis of small volumes. However, this merit has not fully been utilized because there are not sensitive detectors for it. Because analytes in low concentration have to be previously concentrated, a sufficient amount of sample solution is still needed. therefore, the development of a sensitive detector is a key to further advance in ultramicroanalysis.

As can be easily imagined, the detection limit is largely dependent on the detector introduced. In the ultramicroanalysis,

the word "limit" usually implies the "lower limit". So far as CE is used only as a separation technique with high efficiency, the lower detection limit would be of no importance. But once the CE technique is applied as an ultramicroanalyzer, the detection limit becomes important.

The most commonly used detector, which is equipped with the CE instrument, is UV-Vis absorbance detector. The absorbance is given by the following equation.

$$A = \epsilon cl$$

where A is dimensionless absorbance, ϵ is molar absorptivity, c is concentration, and l is optical path length. The detection limit of absorbance is at most 10^{-4} . Because the molar absorptivity is characteristic to the substance, the detection limit of concentration varies with the substance. The optical path length can be taken as the inner diameter of the capillary. Using the 50 μm capillary, the detection limit of concentration can be calculated as 10^{-4} with the molar absorptivity 2×10^2 . Considering the detection volume, which can be defined by the volume where the incident light passes and calculated to be about 2×10^{-9} l (2 nanoliter, 2 nl), the detection limit of absolute amount becomes 2×10^{-13} mole (20 picomoles). In the present ultramicroanalysis, picomole amount of detection limit is no far sufficient. The reason why the conventional absorbance detector is not sensitive is that the optical path length is so short (in micrometer size). To improve the detection sensitivity, several researches have been made including multiple reflection cells (Wang et al., 1991), rectangular capillaries (Tsuda et al.,

1990), and so on. In the next section, sensitive detection methods are introduced, which is NOT the conventional absorbance detection.

1.3 Laser-Induced Capillary Vibration Method as a Sensitive Detection Method for Capillary Electrophoresis

Sensitive detectors using laser-induced fluorometry (Amankwa et al., 1990; Cheng and Dovichi, 1988; Christensen and Yeung, 1989; Cobb and Novotny, 1989; Lee et al., 1991; Lee and Yeung, 1992), photothermal spectrometry (Bornhop and Dovichi, 1986 and 1987; Bruno et al., 1991; Chen et al., 1989; Earle and Dovichi, 1989; Peck and Morris, 1988; Waldron and Dovichi, 1992; Yu and Dovichi, 1989), electrochemical detection (Huang et al., 1991; Wallingford and Ewing, 1987), and mass spectrometry (Moseley et al., 1991; Olivares et al., 1987; Smith et al., 1988) have been developed for CE to improve sensitivity. Comparison of these techniques is summarized in Table 1-1. The outline of each detection method is described in this section.

As to the sensitivity, laser-Induced Fluorometry (LIF) can be said to be the most sensitive detection method. To perform LIF detection, however, most materials, which do not fluoresce, must be derivatized with fluorophore. When the analyte is excited from the ground state to the excited state,

Table 1-1. Comparison of sensitive detectors for CE (I).

	LIF	PTS (TO)	ECD	MS
LOD Absorbance	—	$10^{-7} \sim 10^{-6}$	—	—
Amount	sub-amol	~amol	~amol	~amol
Applicable I. D.	$<10 \mu\text{m}$	$50 \mu\text{m}$	$<10 \mu\text{m}$	$<10 \mu\text{m}$
Modification	Yes	No	No	Yes
On-column	Yes	Yes	No	No
Affect of medium	Yes	Yes	Yes	No

LIF : Laser induced fluorometry

PTS : Photothermal spectrometry

TO : Thermo-optical detection

ECD : Electrochemical detection

MS : Mass spectrometry

radiative relaxation occurs emitting fluorescence. The fluorescence intensity is dependent on the fluorescence quantum yield. Detection limit of zeptomole (10^{-21} mol) level was achieved using highly fluorescent reagent.

Disadvantage of LIF detection is that only fluorescent analyte can be detected with high sensitivity. Because most of biological materials are not fluorescent, analytes must be fluorescent derivatized before detection. However, when the sample volume becomes smaller, less than nanoliter (10^{-9} l), such chemical handling as fluorescent derivatization becomes difficult. Therefore, the LIF technique is not desirable to analyze samples of small volume. Another disadvantage of LIF is that scattered light of the excitation laser from the capillary wall becomes large back ground signal and prevent sensitive detection. To avoid this affection, sheath flow technique has been developed.

Electrochemical detection (ECD) has been shown to allow the sensitive detection of many biologically important molecules. The main advantage of electrochemical detection is that it is highly selective and useful for very sensitive detection of many electroactive species without prior sample derivatization. The major difficulty in combining electrochemical detection with CE is in the electrical isolation of the working electrode from the high-potential field applied across the electrophoresis capillary. This difficulty can be overcome by using a segmented capillary and introducing the concept of off-column detection.

(Wallingford and Ewing, 1987) A major concern when coupling capillaries with off-column detector is band broadening, which is the common concern in the off-column technique.

Electrochemical detection is only applicable to electroactive analytes, and easy to be affected by the electrophoretic buffer, which is usually electrolytic. Therefore, separation buffer system available is limited in sensitive detections.

Mass spectrometry (MS) is useful to structurally identificate the very small quantities of analytes eluting from an electrophoresis capillary. Detection limit is in the femtomole or attomole range. The main difficulty in CE-MS is in coupling the two systems and in the removal of the electrolyte system. Because CE is operated with aqueous buffer whereas the MS at high vacuum, it is necessary to use an interface that effectively transfers the analytes from solution to the vapor phase without thermal degradation with ionization of the analytes.

Photothermal spectrometric (PTS) detection techniques have been developed in various kinds of sensitive analysis as photoacoustic spectroscopy, photothermal beam deflection spectroscopy, etc. Photothermal techniques are based on light absorption by sample and heat generation from the sample as illustrated in Figure 1-3. When the sample absorbs light energy and excited, relaxation process next comes. Most materials relax

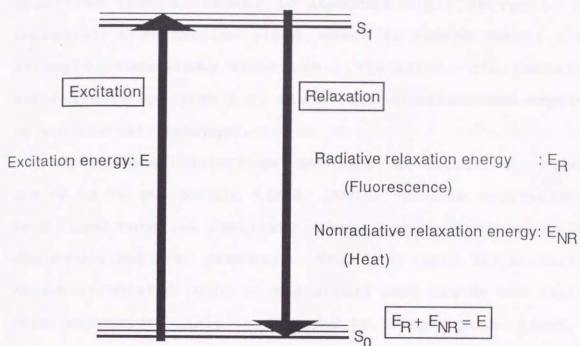


Figure 1-3 Schematic illustration of energy conversion.

accompanying heat generation. Therefore, most of the absorbed light energy is considered to be converted to heat. The ratio of generated thermal energy to absorbed light energy is called nonradiative relaxation yield, which is almost nearly 1 except strongly fluorescent materials. Therefore, the photothermal technique is applicable to almost all materials and expected to be a universal technique.

Photothermal detection technique was applied as a detector for CE by Yu and Dovichi (1988, 1989). When an excitation laser is focused onto the capillary, there occurs light absorption by the sample and heat generates. This heat makes temperature field at the irradiated point of the liquid part inside the capillary. This temperature field is detected by using a probe laser, which is passed through inside of the capillary tube, the liquid part. They called this detection method as thermooptical detection method. The lower detection limit of absorbance reaches around 10^{-6} absorbance, which is about 2 orders of magnitude superior to the conventional absorbance detector.

The difficulties of the thermooptical detection method lie in the optical alignment. To detect the temperature field formed inside the tube, the probe beam has to be passed inside of the tube, and it becomes more difficult when the inner diameter of the capillary becomes smaller, less than 50 μm . In addition, this detection scheme is so easy to be affected by optical properties (e. g., refractive index) of separation medium that microadjustment is necessary when using different buffer system, and during the application of high voltage, and it is difficult

to obtain a stable baseline.

Wu et al. (1990) found out capillary vibration induced by laser (CVL) effect and proposed a novel highly sensitive detection method using the CVL effect. The CVL detection method is also utilizing the photothermal phenomena. The details are described in the next chapter. The sensitivity of the CVL method is as good as the thermooptical method. In the CVL detection, the probe beam does not have to pass inside of the capillary tube, and is applicable to small i. d. capillaries and NOT easy to be affected by the separation medium inside the capillary. Therefore, the CVL method is suitable to detect ultramicro volumes.

Comparison of the sensitive detectors including the CVL technique is represented in Table 1-2.

1.4 Purposes of This Study

CE is an excellent separation technique with high separation efficiency and speed, and it has a potential feasibility to be applied for ultramicroanalysis. The problem is lack of a detector highly sensitive, widely applicable, and easy to operate.

The CVL effect was first found out in Sawada laboratory and reported in 1990. Using the CVL effect, highly sensitive detection of analyte inside the capillary tube was confirmed.

Table 1-2 Comparison of sensitive detectors for CE (II).

	LIF	PTS		ECD	MS
		TO	CVL		
LOD Absorbance	—	$10^{-7} \sim 10^{-8}$		—	—
Amount	sub-amol	~amol		~amol	~amol
Applicable I. D.	$<10 \mu\text{m}$	$50 \mu\text{m}$	$<10 \mu\text{m}$	$<10 \mu\text{m}$	$<10 \mu\text{m}$
Modification	Yes	No		No	Yes
On-column	Yes	Yes		No	No
Affect of medium	Yes	Yes	No	Yes	No

LIF : Laser induced fluorometry

PTS : Photothermal spectrometry

ECD : Electrochemical detection

MS : Mass spectrometry

TO : Thermo-optical detection

CVL: Capillary vibration induced by laser detection

The purposes of this study is a development of CE-CVL system as a detector highly sensitive, widely applicable, and easy to operate. It was performed through the experimental and theoretical considerations. In the next chapter, Chapter 2, the confirmation of CVL effect is described. The feasibility as a sensitive detector for CE is also described. In Chapter 3, the CVL technique combined with CE is described and its detection performance is discussed. In Chapter 4, several detection schemes for the capillary vibration is considered to pursue detection stability and reproducibility. In Chapter 5, development of pulse excitation CVL is described. It is to widen the range of applicable analyte, because the CVL can be applied to light absorbing species and the excitation wavelength has to be chosen to match the absorption band of the analyte. Therefore, it is necessary to excite CVL by various lasers including continuous wave (CW) lasers and pulsed lasers. In Chapter 6, the application of CE/CVL to ultramicroanalysis of immunoprotein is described. In Chapter 7, the combination of CVL and capillary gel electrophoresis (CGE) is discussed. CGE is one of the separation modes of CE, and is especially efficient to separate biopolymers such as proteins and polynucleotides. In the last chapter, Chapter 8, the whole of this study is summarized and future development is described.

CHAPTER 2 Experimental Verification of Laser-Induced Capillary Vibration

In this chapter, laser-induced capillary vibration (CVL) is experimentally verified. The CVL was first found out by chance and reported in 1990. (Wu et al.) From the first of its discovery, the CVL effect was expected for ultramicroanalysis because of its high sensitivity. The CVL was expected to become an effective detection method for capillary LC and CE.

2.1 Introduction

--Discovery of Laser-Induced Capillary Vibration Effect--

The laser-induced photothermal beam deflection (PBD) method has been proved to be one of the most promising methods for determination of liquid samples in a capillary tube. A detecting volume of about 10^2 pL has been achieved and an ability to measure weak optical absorbance reached to 10^{-7} order according to the evaluation criterion of the CE system in short-term stability. However, in the PBD method, the deflection of the probe beam is affected by the temperature and composition of the mobile phase, and the curvature of the interface between the sample and capillary tube. Therefore, the PBD method stability is insufficient and the fluctuating base-line drift makes quantitative analysis difficult. In addition, the PBD method is

difficult to use in gradient mobile phase liquid chromatography due to the composition change of the mobile phase, and the optical alignment has to be changed as mobile phases have different refractive indexes.

Thus, the PBD method has the above difficulties. During the PBD experiment, J. Wu and his co-workers discovered capillary vibration, which was induced by the intensity-modulated excitation laser beam. They placed a capillary on a stage and signal was suddenly reduced as soon as the capillary was broken. If signal was from the PBD effect, the probe beam should be deflected even though the capillary was broken. Therefore, they considered that the capillary was vibrating and that the vibration made the probe beam deflected.

2.2 Generation Principle of Capillary Vibration

To clarify the mechanism of signal generation, the following experiments was performed.

Apparatus. A block diagram of the experimental arrangement is shown in Figure 2-1. A 50 μm i. d. fused silica capillary tube was used. It was coated by polyimide, and a portion of the coating was removed for irradiation by the focused excitation beam. The tube was mounted on a two-axis stage. It was subjected to constant stress by hanging a weight at one end, and both ends of the tube were fixed. The liquid sample was pumped into the capillary tube and kept flowing during the measurement.

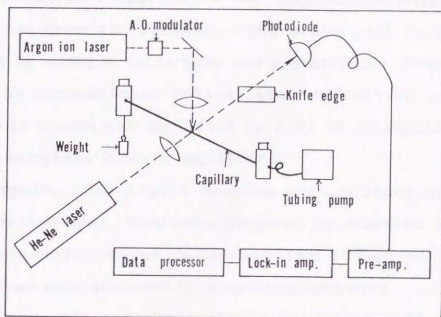


Figure 2-1. Block diagram of the experimental setup.

The excitation beam was an argon ion laser beam of 488 nm wavelength and 70 mW output power. The beam intensity was modulated by an acoustooptic modulator. The excitation beam was focused on the capillary tube by a 50 mm focal length lens which was mounted on a one-axis stage. As shown in Figure 2-1, a He-Ne laser was used as the probe beam, and it was focused by a 50 mm focal length lens just above the tube at the point where the excitation beam was focused. The probe beam deflection was measured by using a knife edge and a photodiode detection system as in the conventional optical beam deflection method. The photodiode output was amplified by a 20 dB preamplifier and fed into an autophase lock-in amplifier.

Reagent. The liquid samples were aqueous solutions of sunset yellow dye. They were prepared by stepwise dilutions of the stock solution of concentration 100 $\mu\text{g/mL}$, and their absorbances were measured by a spectrophotometer.

Confirmation of absorption measurement. The probe beam deflection due to the optical absorption of the sample was confirmed to be detectable with the present methodology. Under the conditions that the power of the excitation beam was 70 mW and the modulation frequency was 1.2 kHz, when distilled water was flowing in the capillary tube, the blank signal level was 0.75 μV . The signal level from sample of $1.0 \times 10^{-2} \text{ cm}^{-1}$ absorption coefficient, which corresponded to 5.0×10^{-5} absorbance for a 50 μm optical path, was 1.25 μV . The difference between the sample and blank (distilled water) levels, 0.50 μV , could be identified in comparison with the base-line noise level,

0.07 μV . Hence it was confirmed that the beam deflection signal of the probe beam from the sample of weak optical absorbance could be detected.

Mechanism for signal generation. The distance between the capillary tube and the probe beam is about 40 μm , and there are two possible mechanisms for signal generation. One is beam deflection by a thermal wave which is the main source in the ordinary PBD method, and the other is acoustic wave due to mechanical vibrations of the capillary tube. In the former mechanism, the optical energy of the intensity modulated excitation beam absorbed by the sample creates a periodic heat flow from the sample to the capillary tube. This periodic heat flow becomes a thermal wave which has the same frequency as that of the excitation beam modulation. The thermal wave passes through the capillary wall, and the temperature field is formed in the surrounding air, which produces a spatial distribution of the refractive index. When the probe beam passes through the temperature field, it is deflected periodically due to this spatial distribution and a beam deflection signal is produced. On the other hand, in the capillary vibration mechanism, periodic heat generation in the sample leads to a temperature fluctuation in the capillary tube near and at the irradiated area. The temperature fluctuation induces a local tension fluctuation in the capillary, which then mechanically vibrates. The capillary vibrations emit an acoustic wave in the surrounding air which accompanies the density and refractive index fluctuation. The probe beam can also be deflected by the acoustic wave.

Tension dependences. To clarify the mechanism of the signal generation, the capillary tension dependences and the frequency characteristics of the signals were measured. The absorption coefficient of the sample was 5 cm^{-1} , which corresponded to a 2.5×10^{-2} absorbance for a $50 \text{ }\mu\text{m}$ optical path. The capillary tube was subjected to different tensions by hanging different weights at its end. The tension dependences of the signal magnitudes at a modulation frequency of 700 Hz are shown in Figure 2-2. The magnitudes decrease with the applied tensions of $10\text{--}150 \text{ g}$. If the mechanism of the signal generation is the thermal wave, the signal magnitude will be unaffected by the tube tension. However the tube tension affects the mechanical vibration motion, and it causes the signal tension dependence. The results shown in Figure 2-2 suggest that the signal generation is not dominated by the thermal wave but by the mechanical vibrations.

Frequency characteristics. Figure 2-3 shows the frequency characteristics of the signal magnitudes for a capillary of 95 mm length. The resonance peaks appear at 462 and 775 Hz for 35.8 and 108.5 g capillary tensions, respectively. By assuming that the motion of the capillary is a string vibration, the theoretical resonance frequency f_r is given as

$$f_r = (T/\gamma)^{1/2} n/2L \quad n = 1, 2, 3, \dots$$

where T is the tension, γ is the mass of the unit length, and L is the length of the capillary. The calculated resonance frequency for the first mode ($n = 1$) are 490 and 851 Hz for the respective tensions and material constants of the capillary. The theoretical results agree well with the experimental ones. The

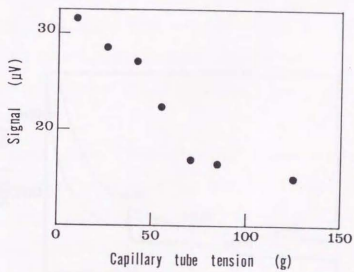


Figure 2-2. Tension dependence of the signal magnitude.

The modulation frequency was 700 Hz.

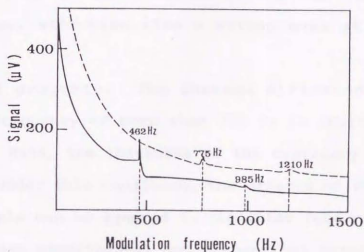


Figure 2-3. Frequency characteristics of the signal magnitudes with tensions of 35.8 g (solid line) and 108.5 g (dashed line).

slight differences between them are considered to attribute to the coating material and liquid sample in the capillary.

Capillary length dependence. The capillary length dependence of the signal magnitude was also measured. At the modulation frequency of 700 Hz, when the capillary length varied from 65 to 200 mm, the signal amplitude changed from 161 to 473 μV . This length dependence of the signal at the off-resonant frequency suggests that the signal generation mechanism is dominated not by the local motion of the capillary, but by the bulk capillary vibration like a string even at off-resonance frequencies.

Thermal property. The thermal diffusion length for a modulation frequency of more than 700 Hz is shorter than 13 μm . On the other hand, the thickness of the capillary tube wall used was 50 μm . Under this condition, the effects of the thermal wave from the sample can be ignored in the outer tube region.

From these experimental and theoretical results, the signal generation mechanism is confirmed as dominated by the vibration motion of the capillary in this frequency region.

Modulation frequency. To choose the modulation frequency giving the best signal-to-noise ratio, the frequency dependence of the signal-to-noise ratio was measured by using a sample of 5 cm^{-1} absorption coefficient (2.5×10^{-2} absorbance for a 50 μm optical path). The tension of the capillary tube was 35.8 g. As shown in Figure 2-4, the frequencies giving the best signal-to-noise ratio appear in the range of 1-1.5 kHz, although the signal magnitudes are larger in the lower frequency range than in the

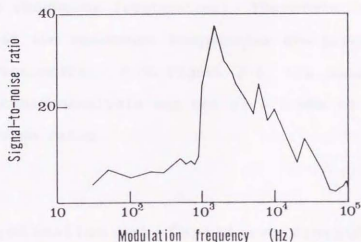


Figure 2-4. Frequency dependences of the signal-to-noise ratio.

higher range. This is because most of the mechanical environment noises are in the low-frequency range. The signal-to-noise ratio at the resonance frequencies is worse than that at adjacent frequencies. This can be explained as follows. The string vibration is apt to be driven at the system natural frequencies by an external force. The natural frequencies consist of resonance frequencies, and the main component is the first Fourier component. Hence frequencies of external noise are the same as the resonance frequencies. Therefore, the noise signal magnitudes at the resonance frequencies are larger than those at adjacent frequencies. From Figure 2-4, the modulation frequency used in ultramicroanalysis was set at 1.2 kHz to achieve the best signal-to-noise ratio.

2.3 Application of Capillary Vibration Effect to Ultramicroanalysis as a Highly Sensitive Detector

The Ultramicroanalytical ability of the CVL method was examined by using sunset yellow dye solutions as samples. The signal magnitude was found to be proportional to the sample absorbance. The linear range of the calibration curve for the absorption coefficient is from 3.0×10^{-3} to 5 cm^{-1} . The detection limit, taken to be double the noise, corresponds to an absorption coefficient of $3.0 \times 10^{-3} \text{ cm}^{-1}$, which also corresponds to an 1.5×10^{-5} absorbance for the $50 \text{ }\mu\text{m}$ optical path. The sampling volume is defined as the capillary tube volume inside

the focal area of the excitation beam, which is given by the inner diameter of the capillary and the focal length of the lens. In the present experiment, the focal length of the lens used for focusing the excitation beam was 50 mm. Under these conditions, the sampling volume is calculated to be 1.0×10^2 pL with the 50 μm tube inner diameter and 50 μm spot size of the focused excitation beam. Hence the absolute amount of the detection limit for sunset yellow dye becomes 6.0 fg, corresponding to 13 amol. The value is 2-3 orders lower than those of the conventional fluorometric and spectrophotometric methods, which have a sampling volume of about 1 μL , and the same order as that of the ordinary PBD method.

Under the present experimental conditions, drifts of the signal level in 100 min from distilled water are within 10 % in the 10^{-4} absorbance range, which is about 1 order smaller than the base-line drifts observed in the ordinary PBD method. This is because the probe beam does not touch the capillary tube in the CVL method, and deflection of the beam is not affected by the refractive indexes of either sample or the capillary tube.

This CVL method is a noncontact and remote technique, and it requires only a small part of the long capillary tube as a detection area. In addition, the CVL method also has a favorable absolute amount for the detection limit. Therefore, it is expected to become an effective detection method for capillary LC and CE.

CHAPTER 3 Instrumentation of Capillary Electrophoresis/

Capillary Vibration induced by Laser System

In this chapter, a detector for CE using the CVL effect is constructed. The performance of the CVL detector was investigated using visible excitation with riboflavin (vitamin B₂) as a sample. The lower detection limit of absorbance was 1.8×10^{-7} for a 30 μm capillary, and the corresponding absolute amount was 80 amol, when the 476 nm, 40 mW lasing line of an argon laser was the visible excitation beam. These results were at least 3 orders superior to conventional absorption detectors and are of the same order as laser-induced fluorometry for riboflavin. Nonderivatized amino acids separated by CE were also detected with the second harmonic of the argon laser (257.2 nm, 5 mW) for excitation, and 15 fmol of tryptophan and 110 fmol of phenylalanine were detected without derivatization.

3.1 Introduction

Capillary electrophoresis (CE) has been developed as a powerful method for separation of samples in ultramicro quantities (Ewing et al., 1989). Its significant advantages are fast and high efficient separation, and the extremely small sample volume ($< 1 \text{ nL}$) required (Wallingford and Ewing, 1989; Olefirowicz and Ewing, 1990). The latter merit is expected to

provide an effective analytical tool for ultramicro sciences such as cell and molecular biology, medicine, and biological chemistry. However, this merit has been less popular because of lack of a suitable ultrasensitive detection method for ultramicro analytes separated in the capillary (Pang and Morris, 1985).

A novel photothermal spectroscopic method, capillary vibration induced by laser (CVL) was proposed in the previous chapter. In this chapter, the feasibility of combining the CVL detector with CE was confirmed (Wu et al., 1991). Riboflavin was chosen as the sample, and the excitation beam was in the visible region, allowing the results to be compared with both conventional absorption spectrometry and laser-induced fluorometry. Next, femtomole amounts of an amino acid mixture were separated and determined by this laboratory-constructed CE/CVL system without derivatization. In this experiment, an ultraviolet (UV) laser beam was used for excitation.

3.2 Experimental

The laboratory-constructed CE/CVL system arrangement is shown in Figure 3-1. The CE system was constructed following the basic format, which includes a 0-30 kV dc power supply, two Plexiglass boxes that isolate the two electrodes, and a microampere meter. A capillary having an inner diameter of 30 μm , outer diameter of 150 μm , and length of 70 cm was used. Details of the CVL detector was described previously. The

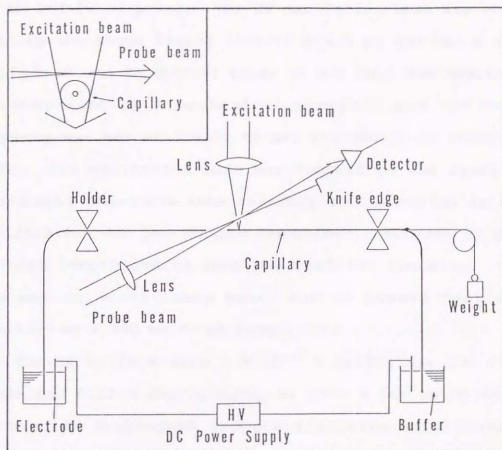


Figure 3-1. Experimental arrangement of the CE/CVL system.

Insert: vertical arrangement of the capillary,
probe beam, and the excitation beam.

capillary was fixed by two holders, and tension of 16 g was applied by using a weight before the capillary was fixed in place. The visible excitation beam was an argon laser beam of 476 nm and 40 mW power. The UV excitation beam was obtained by doubling the argon lasing line of 514.5 nm and had a wavelength of 257.2 nm and an optical power of 5-8 mW. The excitation beam was modulated by a mechanical chopper, and the modulation frequency was set at 710 Hz to get the desirable signal-to-noise ratio. The excitation beam was focused on the capillary by a microscopic objective lens assembly that resulted in a 20 fold magnification for the visible laser beam. For the UV beam, a 30 mm focal length quartz lens was used for focusing. The probe beam was an He-Ne laser beam, and it passed just above the capillary by a 100 mm focal length lens.

The CE buffers were 2×10^{-3} M NaHCO_3 for the riboflavin sample and 0.02 M $\text{KH}_2\text{PO}_4/\text{H}_3\text{PO}_4$ at pH = 3 for amino acids. The amino acids tryptophan and phenylalanine were chosen as the samples, since they have absorption bands around the UV excitation wavelength. The samples were dissolved in the buffers. Although isoelectric points of these amino acids lie around pH = 5, the buffer was prepared at pH = 3 to reduce the electroosmotic flow and to get sufficient separation. The samples were hydrodynamically introduced into the capillary, and the sample volume was controlled by the introduction time. The absolute amounts of the analytes were determined by changing the sample volume or sample concentration. The molar absorptivity of these samples was measured by a spectrophotometer.

3.3 Results and Discussion

To confirm the CVL detection of the CE separated material and to discuss technique performance, riboflavin and the 476 nm argon lasing line were used as sample and visible excitation, respectively. The CE separation voltage was 28.5 kV. Two example electropherograms are shown in Figure 3-2. The peak heights show good linearity for the sample concentration of 2×10^{-6} - 2×10^{-3} M, and the retention time is consistent with reported results under the almost same separation conditions. Therefore, feasibility of the CVL/CE combination is demonstrated.

The lower detection limit of absorbance and that of absolute amounts for riboflavin can be calculated from these results, and comparisons with those obtained by using other spectroscopic methods can be made. The measured molar absorptivity of the riboflavin at wavelength 476 nm is $8.0 \times 10^3 \text{ cm}^{-1} \text{ M}^{-1}$. Hence, considering the dilution factor, the absorbance corresponding to the peak in Figure 3-2a is calculated as 1.8×10^{-6} . The detection limit, taken to be double the signal-to-noise ratio in Figure 3-2a, is estimated to be 1.8×10^{-7} . As the introduced volume is calculated to be $4.3 \times 10^2 \text{ pL}$, the detection limit of the absolute amount of the riboflavin is determined to be 80 amol. This absorbance detection limit is at least 3 orders superior to conventional absorption detectors and is the same as for the PBD method, though the excitation beam power of the latter was 130 mW, which is higher than that used in the present experiment. Furthermore, it is surprising to find that the

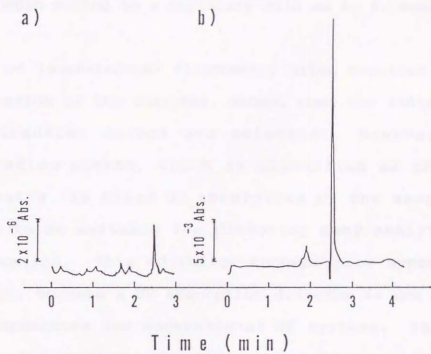


Figure 3-2. Electropherograms of riboflavin:

(a) 2×10^{-6} M; (b) 2×10^{-3} M.

detection limit of the absolute amounts for riboflavin in this CVL method is of the same order as that of the laser-induced fluorometric method, even though riboflavin is a strong fluorescent substance. In addition to the ultrasensitivity, this is the first report of the application of the photothermal spectroscopic method to a capillary with an i. d. smaller than 50 μm .

Use of laser-induced fluorometry often requires fluorescent derivatization of the analytes, except when the indirect and the energy transfer method are selected. However, the CVL determination method, which is classified as photothermal spectrometry, is based on absorption of the samples and is expected to be suitable for detecting many analytes without derivatization. This advantage becomes more apparent for UV excitation, because a UV absorption detector is one of the most popular detectors for conventional CE systems. In comparison with the PBD method, the CVL method has a stabler signal baseline, as shown in Figure 3-2, which is an essential characteristic for quantitative detection of separated materials. To demonstrate these favorable properties, UV excitation was applied to detect nonderivatized amino acids.

The electropherograms of different amounts of tryptophan and phenylalanine, separated in the $\text{KH}_2\text{PO}_4/\text{H}_3\text{PO}_4$ buffer and detected by the UV excitation CVL method, are shown in Figure 3-3. The separation voltage was 15.0 kV. The quantities of the sample were controlled by the hydrodynamic introducing time of the sample solution. These electropherograms show separation and

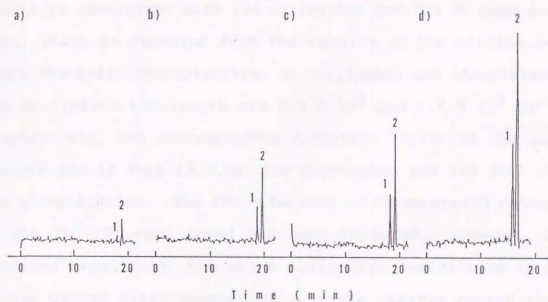


Figure 3-3. Electropherograms of tryptophan (peak 1) and phenylalanine (peak 2).

Introduction amounts:

(a) Trp 100 fmol, Phe 1 pmol;

(b) Trp 300 fmol, Phe 3 pmol;

(c) Trp 600 fmol, Phe 6 pmol;

(d) Trp 1.2 pmol, Phe 12 pmol.

detection of ultramicro amounts of the amino acids without derivatization. The peak heights show a good linearity for the amounts of the samples as in Figure 3-4, and a stable base line can be seen. As the absorbance at peak 2 in Figure 3-3b is 1.8×10^{-4} , by doubling the signal-to-noise ratio, the lower detection limit of absorbance is calculated to be about 8.0×10^{-6} . This result is consistent with the estimation for the UV beam power of 5 mW, which is expected from the results in the visible region. Since the molar absorptivities of tryptophan and phenylalanine at the excitation wavelength are 2.2×10^3 and $1.6 \times 10^2 \text{ cm}^{-1} \text{ M}^{-1}$, respectively, the corresponding detection limits of the absolute amounts are 15 fmol (3.0 pg) for tryptophan and 110 fmol (18 pg) for phenylalanine. The PBD detection of CE separated amino acids at the 10^1 - 10^2 amol level has been reported. However, in the reported experiment, the amino acids were derivatized to give 2 orders larger molar absorptivity in the visible region than our nonderivatized ones in the UV region, and a 130 mW visible excitation power was applied, which is 26 times higher than that in the CVL system. The present results are the first to report detection of femtomole levels of amino acids without derivatization based on direct optical absorption.

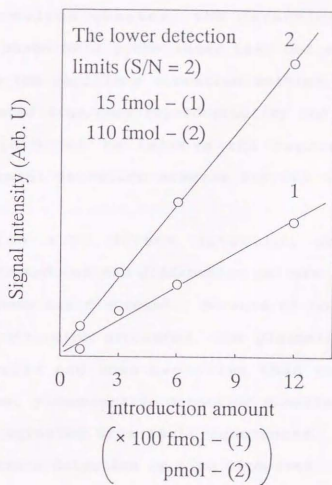


Figure 3-4. Signal intensities vs. introduction amounts of tryptophan and (1) and phenylalanine (2).

In the previous chapter, the detection of capillary vibration was based on a probe laser beam deflection due to an acoustic which the capillary vibration emitted. However, this detection suffered from poor reproducibility and lacked detector stability. In order to improve the reproducibility and stability, several detection schemes for CVL is considered in this chapter.

In Section 4.1, direct detection scheme using a piezoelectric transducer and diffraction pattern detection scheme using a probe beam are discussed. Because of no probe laser and fewer energy conversion processes, the piezoelectric detection scheme is simpler and more sensitive than the previous OBD method. However, piezoelectric detection sometimes suffered from ghost peaks originated from ionic substances. Therefore, the diffraction pattern detection is also discussed. In Section 4.2, the optimum detection conditions of the detection scheme using a diffraction pattern of a probe beam is discussed. This detection scheme is superior in reproducibility, and 4 kinds of amino acids are successfully separated and sensitively detected using this method.

4.1 Development of Capillary Vibration Detection

4.1.1 Direct Detection Method Using Piezoelectric Transducer

For its extremely high separation efficiency, with the theoretical plate number presently reaching nearly 10^6 , capillary zone electrophoresis (CZE) is a powerful separation method, especially for biological materials (Cobb and Novotny, 1989; Kennedy et al., 1989; Drossman et al., 1990; Cohen et al., 1987; Ewing et al., 1989; McCormick, 1988). The method has another merit that sampling volume can be reduced to less than a nano liter. This merit can be taken an advantage especially in ultramicro analysis. However, conventional absorbance detectors are not sensitive enough for such small sampling volumes due to the short optical path. Several highly sensitive spectrometric detection methods have been proposed as on-column methods, including direct (Cheng and Dovichi, 1988; Amankwa et al., 1990; Sweedler et al., 1991; Swerdlow et al., 1991; Jorgenson and Lukacs, 1981) and indirect laser induced fluorometry (Kuhr and Yeung, 1988 and 1989; Garner and Yeung, 1990), and a laser induced photothermal beam deflection method (Yu and Dovichi, 1989; Bruno et al., 1991; Bornhop and Dovichi, 1987). Capillary vibration induced by laser (CVL) method is another highly sensitive detection method, and details of the signal generation and detection mechanisms have been described in the previous chapter.

In the previous chapter, the capillary vibration was caused by local tension fluctuation of the capillary irradiated by an

intensity-modulated excitation laser beam. Analytes in the capillary tube absorbed this laser beam, and generated heat periodically according to the photothermal effect, which made the capillary tension fluctuate locally. This vibration emitted an acoustic wave, which was detected by an optical beam deflection (OBD) method. In this case, the probe laser beam passed just above the vibrating capillary and was deflected by the acoustic wave.

As described in this section, the capillary vibration is directly detected by a piezoelectric transducer (PZT). The energy conversion process in this direct detection method does not involve energy transfer from mechanical vibration energy to acoustic wave energy as illustrated in Figure 4-1-1, so this method is expected to be more sensitive than the OBD detection. Furthermore, for PZT detection, alignment of the probe beam is not required. Therefore, the instrumentation of PZT detector is much simpler than that of OBD detector.

The experimental arrangement was almost the same as in the previous chapter. One of the holders which supported the capillary tube (50 μm i. d., 150 μm o. d.) was replaced by a PZT disc (20 mm in diameter, 2 mm thick) as shown in Figure 4-1-2. The excitation beam was an argon ion laser beam of 488 nm wavelength and 100 mW output power. The beam intensity was modulated by a light chopper. The beam was focused with a 50 mm focal length lens on the capillary tube, which was tensioned by a hanging weight. The generation mechanism of the capillary

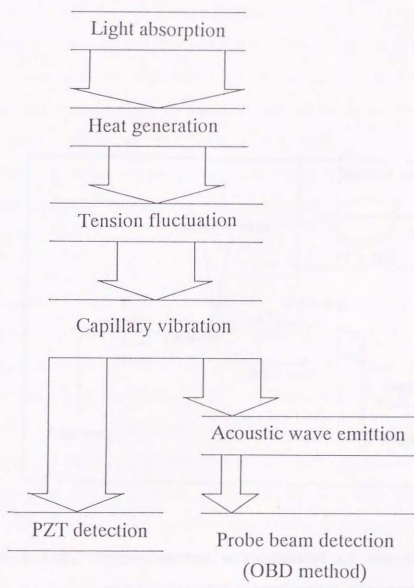


Figure 4-1-1. Flow sheet of energy conversion.

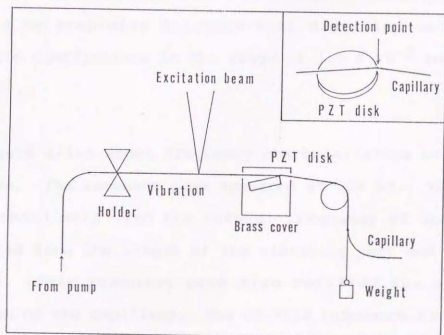


Figure 4-1-2. Experimental arrangement of the direct detection system for CVL with a PZT detector.

Insert: enlargement of the detector.

vibration was the same as before and the capillary vibrated like a string between the two supports, one of which was the PZT detector. Mechanical vibration of the capillary was directly detected by the PZT detector. The PZT output was amplified by a 40 dB preamplifier and fed into an auto phase lock-in amplifier. The liquid samples were aqueous solutions of sunset yellow dye (absorption peak at 482 nm). The sample concentrations were adjusted by step-wise dilution with distilled water to have absorption coefficients in the range of $1.7 \times 10^{-3} \text{ cm}^{-1}$ to $1.7 \times 10^{-2} \text{ cm}^{-1}$.

Figure 4-1-3 shows frequency characteristics of the signal magnitude. The resonant peak appeared at 604 Hz. The resonance corresponded well with the natural frequency of the capillary calculated from the length of the vibrating part and the applied tension. This resonant peak also verified the string-like vibration of the capillary. Use of this resonance for analytical applications could not be recommended for the following reasons. First, as pointed out previously, the signal-to-noise ratio was not satisfactory at this resonant frequency, because noise induced by mechanical factors occurred at the natural frequencies of the vibration system. Second, the peak was too sharp (the full width of the half maximum was 7.5 Hz), and it was difficult to keep the resonating conditions. Therefore, a nonresonant frequency at 710 Hz was used in the following experiments.

Typical examples of vibration signals obtained from the PZT detector are shown in Figure 4-1-4. The samples were distilled

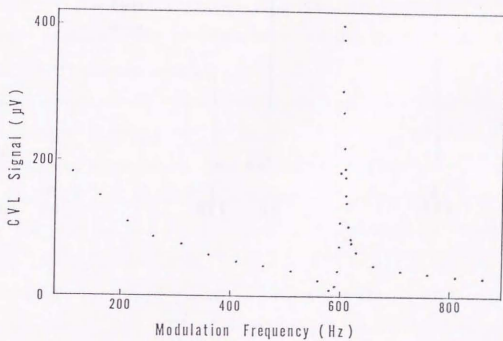


Figure 4-1-3. Frequency characteristics of the signal magnitudes.

(a)

(b)

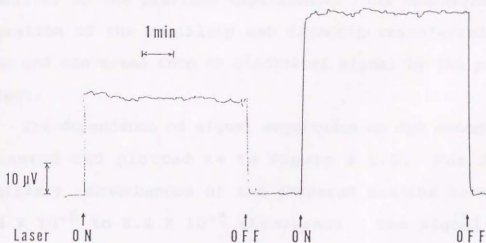


Figure 4-1-4. Typical examples of signals.

a) Distilled water

b) sunset yellow dye solution of

2.5×10^{-5} absorbance.

water and sunset yellow dye solution of $5.0 \times 10^{-3} \text{ cm}^{-1}$ absorption coefficient, which corresponded to absorbance of 2.5×10^{-5} for 50 μm i. d. capillary tubes. Stable signals were acquired for both samples. These results confirmed the principle of this photothermal detection method of optical absorption, as described in the previous experiments. The mechanical energy of vibration of the capillary was directly transferred to the PZT disc and converted into an electrical signal by the piezoelectric effect.

The dependence of signal magnitudes on dye concentration was measured and plotted as in Figure 4-1-5. For 50 μm i. d. capillary, absorbances of the prepared samples corresponded to 8.4×10^{-6} to 8.4×10^{-5} absorbance. The signal magnitudes showed linearities for absorbances of the sample as often occurs with the photothermal effect (correlation coefficient was 0.99), because absorbances of the samples were sufficiently low. The detection limit ($S/N = 2$) of absorbance was calculated as 8.5×10^{-7} for the 50 μm capillary. For sunset yellow dye solution, the lower limit of detection for concentration was calculated to be $6.8 \times 10^{-9} \text{ M}$. Considering the optical sampling volume estimated to be $1.0 \times 10^{-10} \text{ L}$, the detection limit of absolute amount was $6.8 \times 10^{-20} \text{ mol}$. Considering that the detection limit reported previously in the OBD method was 1.5×10^{-5} for the same capillary, the PZT detection was proved to be at least one order of magnitude more sensitive than the OBD method.

Comparing the energy conversion process of the two detection methods of CVL, energy loss in the PZT detection process is

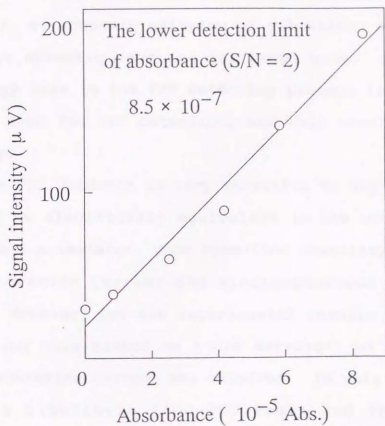


Figure 4-1-5. Dependence of the signal intensity on the absorbance of sunset yellow dye solutions.

smaller. Energy conversions from optical to thermal, and to mechanic energy of vibration by the photothermal effect are common for both methods. However, the mechanical energy is directly converted to electric energy in the PZT detection, while there are two processes before the final conversion for the OBD detector, i. e. acoustic emission of vibrations and interaction between the acoustic wave and the probe beam. Therefore, the total energy loss in the PZT detecting process is considered to be smaller than for OBD detection, and this results in superior sensitivity.

As the PZT detector is very sensitive to any electric field because it is electrically equivalent to the combination of a capacitor and a resistor, poor base-line stability and noise due to the separation current and electrophoresis potential are possible. However, for the experimental results (Figure 4-1-6) from applying this method as a CZE detector, no serious effect from the separation current was observed. In this experiment the sample was riboflavin (200-300 fmol) and the separation conditions were the same as in the previous chapter. In addition to the superior sensitivity, the direct detection method does not need optical alignment for probing. This results in favorable reproducibility and simpler instrumentation.

As conclusions, the two detection schemes, OBD method and PZT method, are compared with the conventional absorbance detection method as in Table 4-1-1.

The only disadvantage of the PZT detection is that ionic substances give signals caused by the pyroelectric effect of the

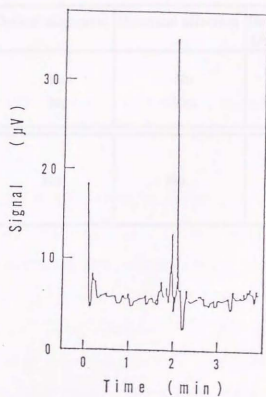

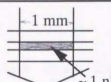


Figure 4-1-6. Electropherogram of riboflavin obtained with the CVL method.

Separation voltage was 20kV.

Table 4-1-1. Comparison of the CVL detection methods and the conventional absorbance detection method (I).

Detection method	Optical alignment	Electrical affection	Detection limit (Absorbance)	Detection volume
OBD PZT	Yes No	No Yes	$\sim 10^{-6}$ $10^{-7} \sim 10^{-6}$	
Conventional absorbance detection	No	No	$10^{-5} \sim 10^{-4}$	

pZT disc, which is independent on the light absorption by the sample. This disadvantage may be easily overcome by using micellar electrokinetic capillary chromatography technique, in which neutral molecules are separated. In the next section, another detection technique of the capillary vibration is investigated.

4.1.2 Diffraction Pattern Detection Method

As in Figure 4-1-7, diffraction pattern generates when the probe beam passes the capillary. The capillary vibration can be detected by monitoring the movement of the diffraction pattern, which synchronously moves with the vibration. This diffraction pattern method is very similar to the previous OBD detection method on the point that it uses a probe beam. The difference is that in the OBD detection, the probe beam is mainly deflected by the acoustic wave emitted from the mechanical vibration, while in the diffraction pattern detection, the probe beam is mainly deflected by the different angle of refraction caused by the displacement (vibration) of the capillary. Comparing the other detection schemes of capillary vibration, the diffraction pattern detection scheme has some superior advantages. First, in spite of using the probe beam, microadjustment of the probe beam is much easier than the OBD detection, because one can easily find out the precise position on the capillary by watching the change of the pattern. Second, the diffraction pattern detection is

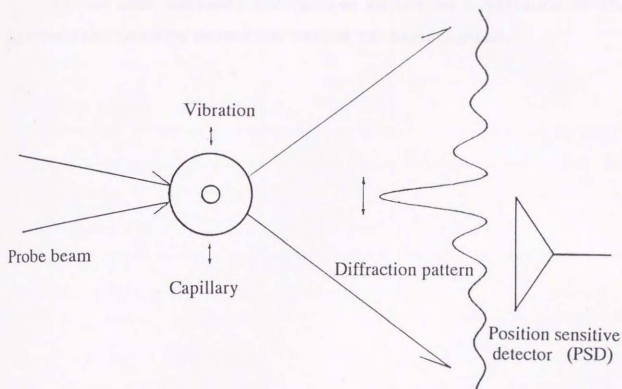


Figure 4-1-7. Diffraction pattern of the probe beam and its detection using a position sensitive detector.

sure to be unaffected by ionic substances. Comparison of the three detection techniques including the diffraction pattern detection with the conventional absorbance detection is summarized in Table 4-1-2.

In the next section, the optimal detection conditions of the diffraction pattern detection method is investigated.

Table 4-1-2	Comparison of detection techniques	Conventional absorbance detection	Diffraction pattern detection	Comparison of detection techniques

Table 4-1-2	Comparison of detection techniques	Conventional absorbance detection	Diffraction pattern detection	Comparison of detection techniques

Table 4-1-2. Comparison of the CVL detection methods and the conventional absorbance detection method (II).

Detection method	Optical alignment	Electrical affection	Detection limit (Absorbance)	Detection volume
OBD	Yes	No	$\sim 10^{-6}$	~ amol
PZT	No	Yes	$10^{-7} \sim 10^{-6}$	
Diffraction pattern	No	No	$\sim 10^{-6}$	

Conventional absorbance detection	No	No	$10^{-5} \sim 10^{-4}$	~ fmol
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4.2 Optimum Detection Conditions of Diffraction Pattern Detection

In this section, the optimal detection conditions of the diffraction pattern detection is investigated. The problem is, however, other photothermal effects, such as the thermooptical effect and photothermal beam deflection (PBD) effect, were also induced. In certain geometrical configurations, these effects caused a very high background and prevented quantitative detection. The dominant effect and the optimum configuration were determined by scanning a probe beam along the capillary. To avoid the influence of the heat generated from the glass part of the capillary tube which cannot be neglected in such a weak absorbance detection, it was found that the probe beam should pass outside of the thermal diffusion region of the glass, where the capillary vibration induced by laser (CVL) effect was dominant. Under this condition, 8 amol of four DABSYL-amino acids were detected.

4.2.1 Introduction

The reputation of capillary electrophoresis (CE) as a separation method with high resolution and speed is spreading (Jorgenson and Lukacs, 1981; Ewing et al, 1989). Another merit of CE is that only a small quantity of a sample is required. For

example, a single cell quantity, sub-nL, is enough. Ewing et al. (1988) removed cytoplasmic samples from single nerve cells of *planorbis corneus* directly with the tip of a capillary, and the CE separation was successively carried on. As the inner diameter of the capillary becomes smaller, this advantage can be better utilized.

But when the inner diameter of the capillary becomes smaller, a serious problem is faced that an ultramicro amount of sample in the capillary must be detected. Conventional absorbance detectors are not sensitive enough. Therefore, detection using photothermal effects is suggested to be much more sensitive than conventional absorbance detectors. In actual applications, a lower detection limit of absorbance ranging from 10^{-7} to 10^{-6} has been attained (Bornhop and Dovichi, 1987; Yu and Dovichi, 1989; Waldron and Dovichi, 1992; Bruno et al., 1991; Wu et al., 1990 and 1991; Otake et al., 1992). Photothermal effects are caused by the light absorption of the sample. Most of the absorbed light energy is then nonradiatively converted to heat. This heat causes the refractive index of the surrounding materials to change; i. e., both the fluid in the capillary and the glass part of the capillary. This change is detected optically by a probe laser beam passing perpendicularly to both the capillary and the excitation laser beam. The refractive index change occurs synchronously with the light chopping, which modulates the intensity of the excitation laser. Therefore, the part of the probe beam passing through the capillary is deflected with this period by the thermally induced refractive index

change. This effect is called the photothermal beam deflection (PBD) effect. Dovichi's group and Bruno et al. demonstrated a detection system for this known as the thermooptical method. Focusing the probe beam within the capillary channel, they detected the PBD effect induced by the refractive index change of the liquid sample in the capillary.

The CVL effect is another photothermal effect. This effect takes place only when the capillary is tension applied and it vibrates synchronously with the light modulation by the thermally induced tension fluctuation. When the probe beam passes near this vibrating part of the capillary, the part of the probe beam which has passed the capillary is deflected with the same period because the spatial vibration of the capillary changes the direction of the passing probe beam synchronously. The amplitude is proportional to the absorbance of sample in the capillary. In the previous chapter, this CVL effect was applied to the ultrasensitive detector for CE and femtomole amounts of amino acids were detected without derivatization. In comparison with the thermooptical or other PBD detections, the CVL detection is more advantageous for a smaller i. d. capillary and a signal baseline is stabler, because the probe beam of the CVL method does not pass through the liquid part and it is insensitive to the optical properties of the separation medium. The quantitative CVL signal is, however, hindered by enormous background signals under certain conditions, just like the thermooptical method is. These conditions are considered to depend on the spatial arrangement of the excitation and probe

beams, and the capillary, and it is rather troublesome to find suitable quantitative conditions.

In this section, two photothermal effects in the capillary is investigated by changing the distance of the two laser beams perpendicular to each other. The most sensitive conditions for absorbance of samples inside the capillary is found. Next, based on the investigation results for the optimum distance conditions of the two laser beams, a detection system is constructed and applied to an absorbance detector for CE.

4.2.2 Experimental

The experimental arrangement is shown in Figure 4-2-1. A fused silica capillary tube of 30 μm i. d., 150 μm o. d. was tension applied by moving, in opposite directions, two X-stages on which the capillary was wound around. A part of this tension applied capillary was then supported by two wedge-shaped supports, and this supported part was the vibration part. The excitation laser was an argon ion laser of 488 nm and it was irradiated on the middle of the capillary between the two supports. The polyimide coating of the irradiated part of the capillary was removed along a distance of 3 to 10 mm. The capillary vibration was directly detected using a probe beam. The probe laser was a He-Ne laser of 633 nm, irradiated perpendicularly to both the capillary and the excitation laser beam. When the probe beam was focused onto the capillary, a

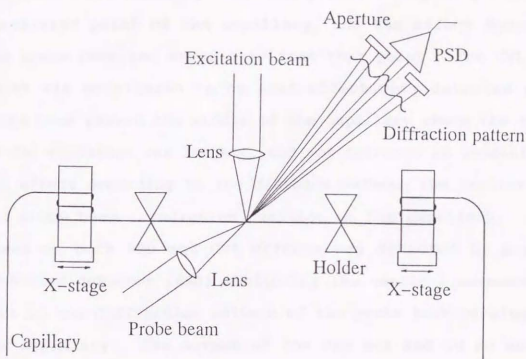


Figure 4-2-1. Experimental arrangement. One of the spots in the diffraction pattern generated when the probe beam was irradiated on the glass part of the capillary was projected on the detection plane of the position sensitive detector. The vertical movement of the probe beam according to the refractive index change induced by the heat from the fluid or the glass part, or the mechanical the mechanical movement of the capillary was monitored with this spot.

diffraction pattern was generated. The capillary displacement was most effectively detected when the probe beam was irradiated not in, but off, the center of the capillary as shown in Figure 4-2-2. However, in this alignment of optical detection, both PBD and CVL effects were detected at the same time. The PBD effect was seen most strongly when the probe beam just passed on the irradiated point of the capillary, and the effect decreased as the probe beam was moved away from that point. The CVL effect, which was considered to be most effectively detected when the probe beam passed the middle of the capillary where the amplitude of the vibration was largest, did not decrease as suddenly as the PBD effect according to the distance between the excitation beam and probe beam irradiation position on the capillary. A signal based on both PBD and CVL effects was detected by a position sensitive detector (PSD) monitoring the vertical movement of one spot in the diffraction pattern of the probe beam passing through the capillary. The output of the PSD was fed to an auto-phase lock-in amplifier after passing through an electrical isolation amplifier with a hundred-fold gain and a band pass filter.

To change the relative distance of the two irradiated points on the capillary, the probe laser beam was fixed and the excitation laser beam was moved along the capillary by using a 30 mm focal length lens. The X axis was in the direction of the length of the capillary as shown in Figure 4-2-2. In reality, the excitation beam was scanned along the capillary, but what was important was the relative distance between the excitation beam and probe beam. Therefore, the positions of the two laser beams

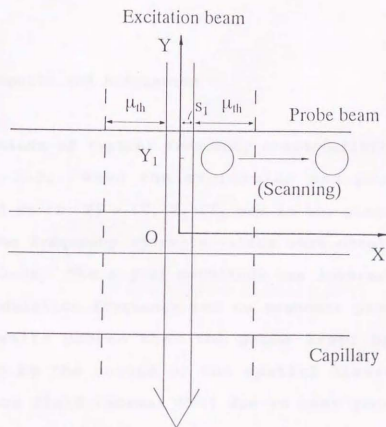


Figure 4-2-2. Longitudinal cross-section of the capillary, and schematic representation of the coordinates used in the experiments and discussion. The X axis was set in the direction of the length of the capillary, and the Y, that of the excitation beam.

irradiated on the capillary could be illustrated as shown in Figure 4-2-2 as if the probe beam had been scanned.

DABSYL-amino acids (Gly, Ala, Met and Pro) were used as samples and dissolved in pH 7 phosphate buffer. The concentration of each DABSYL-amino acid was 5×10^{-5} M. A mixture of four DABSYL-amino acids in equal quantities (1×10^{-5} M) was electrophoretically injected (20 kV, 5 s) into the capillary. Separation voltage was 20 kV.

4.2.3 Results and Discussion

Two kinds of typical frequency characteristics are shown in Figure 4-2-3. When the excitation and probe beams were overlapped at $(X, Y) = (0, Y_1)$ (Y_1 was in the glass part in Figure 4-2-2), the frequency characteristics were obtained as given in Figure 4-2-3a. The signal magnitude was inversely proportional to the modulation frequency and no resonant peak was observed. These results proved that the probe laser beam was mainly deflected by the change of the spatial distribution of the temperature field (normal PBD) due to heat generated from the irradiated part of the capillary including the glass part. When the distance between the excitation and probe laser beams was long enough ($X > \mu_{th}$: thermal diffusion length), the frequency characteristics were obtained as in Figure 4-2-3b. A peak appeared at 1077 Hz corresponding to the calculated natural frequency of the string vibration of the capillary and it was the

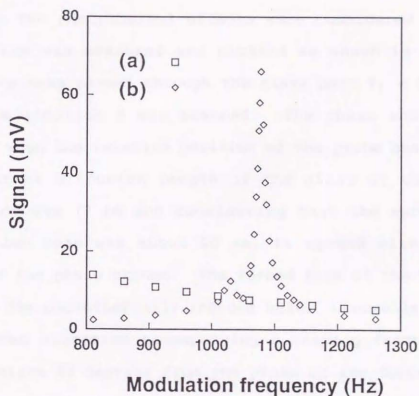


Figure 4-2-3. Typical frequency characteristics in two different relative positions of the excitation and probe laser beams. (a) $X = 0$. (b) $X > \mu_{th}$: thermal diffusion length of the glass part of the capillary.

resonant peak of the capillary vibration system, which indicated that the capillary was tension applied with a weight of 30.7 g in this vibration system. Thus, according to the distance between the two laser beams, two different photothermal effects dominated the signal.

The two photothermal effects were considered and the phase difference was measured and plotted as shown in Figure 4-2-4. The probe beam passed through the glass part $Y_1 = 50 \mu\text{m}$, and the relative location X was scanned. The phase shifted about 90 degrees when the relative position of the probe beam X was $40 \mu\text{m}$. The thermal diffusion length of the glass at this modulation frequency was $17 \mu\text{m}$ and considering that the spot size of the excitation beam was about $20 \mu\text{m}$, it agreed with the critical point of the phase change. The forced term of the CVL effect was clearly the photothermally induced heat. Generally, the phase of the forced vibration accompanying a damping factor in a steady state shifts 90 degrees from the phase of the forced term at the resonant frequency. Therefore, the observed 90 degree phase shift at the thermal diffusion length proved that the normal PBD effect dominated the signal generation when the probe beam was within the thermal diffusion region, while the CVL effect was more important outside the region. This result also suggested that according to the distance between the excitation and probe beams, the probe beam detected different phenomena; PBD and CVL, although they occurred in the same capillary. In short, the PBD effect dominated the signal at $X < \mu_{th}$, while the CVL effect did at $X > \mu_{th}$.

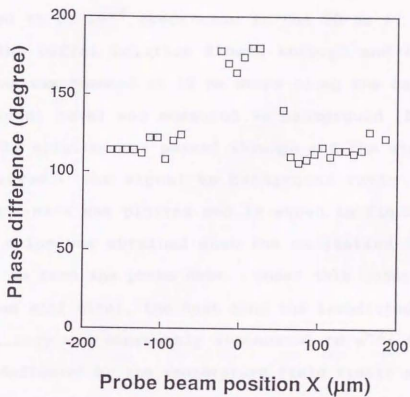


Figure 4-2-4. Phase differences plotted when the excitation laser beam was scanned.

The absolute signal magnitude was really a maximum at $X = 0$, but it was not clear if this signal magnitude was quantitative for the absorbance at this condition. To investigate this, we passed two solutions, phosphate buffer and DABSYL-Gly solution, through the capillary. The absorbance of DABSYL-Gly was estimated to be 10^{-4} absorbance in the $30\text{ }\mu\text{m}$ i. d. capillary. First, the buffer solution flowed through and the excitation laser beam was scanned at $10\text{ }\mu\text{m}$ steps along the capillary. This blank signal level was measured as background (B). Then, the DABSYL-Gly solution was passed through and the signal level (S) was measured. The signal to background ratio (S/B) at each location X-axis was plotted and is shown in Figure 4-2-5. The maximum value was obtained when the excitation laser beam was about $70\text{ }\mu\text{m}$ from the probe beam. Under this condition, $X = 2\mu_{th} + s_1$ (beam spot size), the heat from the irradiated glass part of the capillary was thoroughly attenuated to e^{-2} ; the probe beam was not deflected by the temperature field itself anymore and the CVL effect was dominant. Therefore, the CVL effect had much more sensitivity to the absorbance of sample in the capillary than the PBD effect had.

Within the thermal diffusion region, the probe beam was sure to be deflected by the temperature field, but the temperature field in the capillary was generated not only by the heat from the fluid, but also by the heat from the irradiated glass part. The effect of heat from the latter cannot be neglected in such a weak absorption measurement, because it hinders quantitative signals. Dovichi's group passed the probe beam through the

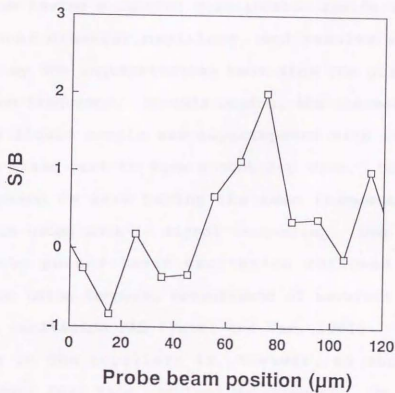


Figure 4-2-5. The S/B dependence on the relative distance of the two laser beams. The signal was from the DABSYL-Gly solution of 10^{-4} absorbance in the 30 μm i. d. capillary and the background was from pH = 7 phosphate buffer.

channel of the capillary within the thermal diffusion region ($(X, Y) = (0, 0)$). This was the only quantitative condition for passing the probe beam within the thermal diffusion region. They detected the refractive index fluctuation of the liquid in the capillary channel. However, it would be difficult to pass the probe beam having a spatial distribution inside the channel of a small inner diameter capillary, and results would be easily affected by the unquantitative heat from the glass with the low modulation frequency. In this region, the thermal wave generated from the liquid sample was superimposed with the thermal wave from the glass part to form a combined wave. Separation of the superimposed CW wave having the same frequency is basically impossible using lock-in signal recovering. One possible method may be the pulsed laser excitation followed by the signal separation using temporal measurement of waveform as demonstrated in pulse excitation PAS (Patel and Tam, 1981). The propagation distance in the capillary is, however, so short that it may require very fast time resolved measurement. On the other hand, when the excitation beam was separated by more than $2\mu_{th} + s_1$ from the probe beam, and so sufficiently outside of the thermal diffusion region, the gradient of the temperature field was small enough, and the obtained signal was much more dependent on the absorbance of the fluid in the capillary. Under this condition, the CVL effect was dominant. In order to detect the capillary vibration induced by the heat, the probe beam should not pass through the channel. Therefore, the CVL effect detection would be more convenient for quantitative detection than the ordinary

quantitative PBD effect detection or thermo-optical detection. Furthermore, considering the sample heat transmitted to the capillary wall, only the heat near the wall would be efficiently converted to the vibration energy. Therefore, the smaller inner diameter capillary was considered to generate vibration energy more effectively, corresponding to the sample heat.

Based on the above considerations, an electropherogram of the mixture of four DABSYL-amino acids (Gly, Ala, Met and Pro) was obtained under the quantitative conditions in which the CVL effect was dominant for the signal (Figure 4-2-6). The inner diameter of the capillary was 30 μm . Taking into account the dilution factor, we detected an absolute amount of each DABSYL-amino acid of 8 amol.

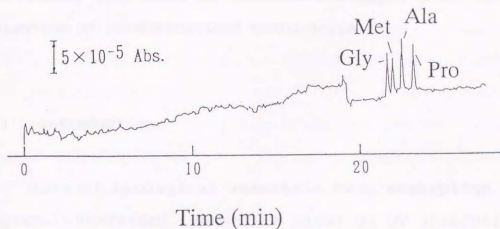


Figure 4-2-6. Electropherogram of the mixture of four kinds of DABSIL-amino acids (Gly, Ala, Met and Pro). Injection: 20 kV, 5 s. Separation: 20 kV. The absolute amount of each DABSIL-amino acid detected was 8 amol.

To widen applicability of CVL detector to various kinds of excitation lasers, pulsed laser excitation is discussed in this chapter. Using the pulse repetition rate equal to the natural frequency of the vibrating system and the heat released time, stationary CVL could be induced and applied to the sensitive detection of nonderivatized amino acids.

5.1 Introduction

Most of biological materials have absorption band in UV region. Therefore, detection based on UV absorption is most widely applicable and universal. However, the UV absorbance detector equipped with commercially available CE systems does not have sufficient sensitivity. One approach is using laser as a light source. High intensity laser induces heat from sample which has absorbed the light, proportionally to the absorbed light quantity. Therefore, because sensitivity can be easily improved using larger light intensity, detection of the heat is more advantageous than that of absorbance, which related to the ratio of incident light and transmitted light intensity. Quantitative heat analysis can be performed by measuring changes of refractive index, or capillary displacement. The CVL detection method is proposed as a highly sensitive on-column

absorbance detection method for CE. In the previous chapter, the detection conditions for CVL was optimized, which was induced by intensity-modulated continuous wave (CW) laser, and detection limit of dabsylized amino acids reached attomole amount. However, in order to eliminate such derivatization process as dabsylation, introducing the excitation laser which emits in UV region becomes necessary.

In this chapter, an excimer laser is introduced as a UV light source. Because the excimer laser is a pulsed laser, CVL by forced vibration cannot be induced and pulse repetition rate have to match not only with the heat release time but also with the natural frequency of the vibrating system, resulting in a stable standing wave CVL. The development of pulsed UV laser-induced standing wave CVL system and its application to CE is described.

5.2 Experimental

Apparatus. The experimental setup for CVL detection system is described in the previous chapters in detail. The CVL excitation laser used this time was a waveguide excimer laser (Potomac photonics, SGX-1000) with 248 nm lasing line, 60 ns pulse duration, and the output nearly 10 μ J per pulse at 1 kHz repetition rate. The CVL was detected using a position sensitive detector (PSD) by monitoring the change of propagation direction of a probe laser beam after passing through the wall of the

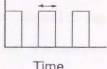

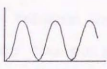
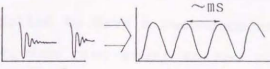
vibrating capillary. The output from the PSD was first fed into an isolation amplifier with one hundred fold, passed through an electrical band pass filter, and a lock-in amplifier. The reference signal for the lock-in amplifier was obtained from a function generator triggered by a reference signal output from the excimer laser. Fused silica capillaries used here were 20 μm i. d. or 50 μm i. d., and 150 μm o. d.. Overall capillary length was 75 cm and effective length was 40 cm.

Reagents. Tryptophan and phenylalanine were used to evaluate the pulsed UV laser-induced CVL detection scheme. These amino acids were resolved with phosphate buffer as described in Chapter 3.

5.3 Results and Discussion

CW laser-induced CVL and pulsed laser-induced CVL. First, differences between CW laser-induced CVL and pulsed laser-induced CVL should be discussed. Comparison of the two schemes are represented in Table 5-1. CW laser was intensity-modulated mechanically by a light chopper. Induced vibration was a forced vibration and the amplitude was inversely proportional to the modulation frequency, which is characteristic to photothermal phenomena. In addition, resonant peak appears at the natural frequency of the vibration system. While, when a pulsed laser was used to induce CVL, the capillary vibrated with the period of its natural frequency in the condition that the pulse repetition

Table 5-1. Comparison of CW laser-induced CVL and pulsed laser-induced CVL.

	CW-CVL	Pulsed-CVL
Excitation	$\sim \text{ms} (10^2 \sim 10^3 \text{ Hz})$  Light intensity Time	$\sim 10 \text{ ns}$  Light intensity Time
Vibration	 Signal magnitude Time	 Signal magnitude Time
Conditions	Resonance Nonresonance (Forced vibration)	Resonance Heat released time $\sim \text{ms} \rightarrow$ Pulse repetition rate $\sim 10^3 \text{ Hz}$

rate did not exceed the natural frequency. The amplitude was attenuated within 20 ms with a single pulse excitation. Each peak intensity in waveforms depended on the absorbed light quantity, but fluctuation of each pulse intensity so affected baseline stability that sensitive detection could not be performed. To make the best use of heat generated from sample liquid, heat released time should be considered. From the thermal properties of glass, heat released time was calculated around 1 ms, which corresponded well with the experimental data in the literature. The capillary tension was controlled to have a resonance around 1 kHz. By using the pulse repetition rate equal to the resonant frequency, a stationary CVL signal was obtained as shown in Figure 5-1. This stationary CVL signal was fed into the lock-in amplifier to recover the amplitude component of the same period as the excitation laser pulse. In this signal recovering method using the lock-in amplifier, stable baseline could be obtained, because it was uneasy to be affected by the intensity fluctuation of each excitation laser pulse. Standing wave CVL detection scheme was adopted in the following experiments.

Stationary CVL detector. First, to evaluate sensitivity, phenylalanine solution was flowed, by reducing pressure of outlet reservoir, through the capillary filled with phosphate buffer. When the liquid phase changed from buffer solution to sample solution at the detection point, baseline elevation was measured as the net signal from the phenylalanine molecules as observed in Figure 5-2. Considering the signal to noise level, CVL detection was at least one order of magnitude more sensitive than the

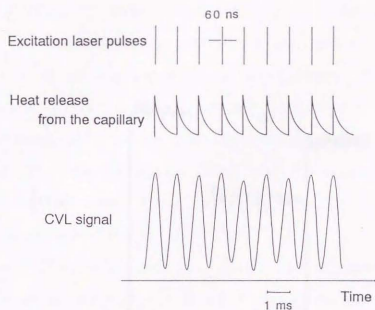


Figure 5-1. Stationary CVL signal.

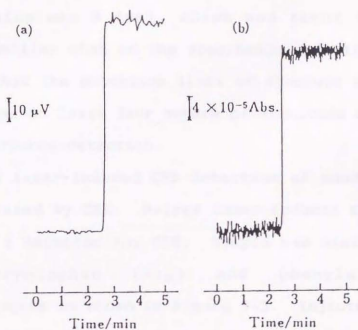


Figure 5-2. Signal level for Phe solution.

(a) pulsed CVL detection

(b) conventional absorbance detection

conventional absorbance detection. In the conventional absorbance detection, incident light width was about 1 mm, and detection volume was 2 nL for 50 μ m i. d. capillary. Therefore, detected amount of phenylalanine was calculated to be 2 pmol. While, in the CVL detection, the spot size of the excitation laser on the capillary was about 10 μ m, and detection volume was estimated as 3 pL for 30 μ m i. d. capillary. Therefore, detected phenylalanine was 3 fmol, which was about three orders of magnitude smaller than in the absorbance detection. It could be concluded that the detection limit of absolute amount in the CVL detection was at least four orders of magnitude smaller than that in the absorbance detection.

Pulsed laser-induced CVL detection of nonderivatized amino acids separated by CZE. Pulsed laser-induced CVL detection was applied as a detector for CZE. Sample was mixture of two amino acids, tryptophan (Trp) and phenylalanine (Phe). Electropherogram is shown in Figure 5-3. Injected amounts of Trp and Phe were 0.3 pmol and 3.0 pmol, respectively. Because the molar absorptivity of Trp in buffer solution was about one order of magnitude larger than that of Phe in buffer solution, the ratio of these two peak intensities were reasonable. Considering the excitation beam spot size, detection volume was estimated as around 1 pL. Therefore, detected amounts of Trp and Phe were 0.5 fmol and 5.0 fmol, respectively, at each time. Taken to be double the noise, the detection limits of absolute amount was calculated to be 10 amol in Trp and 100 amol in Phe. Comparing with the previous results obtained by using the SHG of an argon

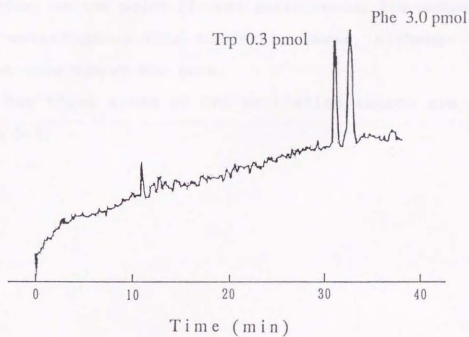


Figure 5-3. Electropherogram of Trp and Phe obtained by pulsed CVL detection.

laser ($\lambda = 257.2 \text{ nm}$, 10 mW), the sensitivity of pulsed laser-induced CVL was almost comparable to CW laser-induced CVL. Taking into account of the large space and capacity needed for pumping the SHG, the excimer laser was considered to be more portable and convenient than the high-output argon ion laser. In addition, on the point of cost performance, the excimer laser was more advantageous than the argon laser, although the average output were almost the same.

The three kinds of CVL excitation source are compared in Table 5-2.

Table 5-2. Comparison of CVL excitation sources.

Excitation laser	Wavelength	Output	Power supply	Detection limit
Argon ion laser	Visible	100 mW	1 kW	0.3 amol
Argon ion laser (SHG)	UV	5 mW (pump: 10 W)	60 kW	10~10 ² amol
Excimer laser	UV	15 mJ/pulse	0.4 kW	

CHAPTER 6 Application to Ultramicroanalysis of Immunoproteins

Ultramicro amount of immunoproteins plays an important role in medical, and biological aspects. Enzyme immunoassay (EIA) and radioimmunoassay (RIA) techniques were developed. However, a highly sensitive, simple, and rapid immunoassay technique is still desired. Photothermal immunoassay (PIA) using gold ultrafine particles was developed as a sensitive, simple, and rapid method to determine immunoproteins (Kitamori et al., 1987; Matsuzawa et al., 1993; Tu et al., 1993). However, the PIA technique have a separation process of immunologically bound (B) and free (F) particles, which is still troublesome. In this chapter, the immunological reaction solution was introduced into the capillary without any conventional B/F separation procedure. A novel, highly sensitive, simple, and rapid immunoassay technique, combining high speed B/F separation using the superior separation efficiency of CE technique and highly sensitive detection using the CVL technique, is developed.

6.1 Introduction

A sensitive, simple, rapid immunoassay method would be desirable for medical and biochemical studies. Our PIA is one choice, in which antigen-antibody reaction products in turbid solutions were detected and determined ultrasensitively using

laser-induced photoacoustic spectroscopy as a photothermal technique. PIA was already demonstrated as at least three orders of magnitude more sensitive than the conventional immunoassay with turbidimetry. However, in laser spectrometry, background reduction and stabilization are still serious problems, and B/F separation was desired for ultrasensitive detection.

The CE technique has been known as a superior separation technique due to high applied voltage. The CE separation is performed according to the difference of electrophoretic mobility, which is decided not only by net charge of substance, but also by size, formation of substance, its interaction with capillary wall, etc. In this chapter, CE separation was developed to perform the B/F separation with high speed, which was followed by a highly sensitive detection using the CVL technique. In our PIA technique, gold ultrafine particles were used as a labeling reagent to enhance the photothermal signal, and polystyrene latex particles were used as a reaction solid phase of immunoreaction. Gold ultrafine particles are still effective in the CVL detection, which is a kind of photothermal spectrometry. In most cases, proteins are apt to adsorb electrostatically on the capillary wall, but by introducing polystyrene latex particles as a reaction solid phase, this adsorption could be avoided. The immunological reaction solution was introduced into the capillary without conventional B/F separation procedure. High speed B/F separation using CE technique and sensitive detection using CVL technique is demonstrated, which we are proposing as a highly sensitive,

simple, and rapid immunoassay technique. Using the above immunoassay technique, attomole level of antigen IgG was selectively and sensitively detected.

6.2 Experimental

Sample procedure. Schematic illustrations of immunological sample procedure is shown in Figure 6-1. First, antibody molecules are adsorbed on polystyrene microparticles, which are introduced as reaction solid-phase. Then, the analyte antigen molecules are adsorbed on the polystyrene microspheres through antigen-antibody binding. Next, the antibody molecules labeled with colloidal gold ultrafine particles are fixed on the solid-phase also through antigen-antibody binding (Chakraborty et al., 1990). These reaction solutions were introduced electrophoretically into the capillary and CE separation was performed.

Reagents. The radius of polystyrene microparticles was 0.38 μm . By addition of 4 % antibody solution into 1 % polystyrene latex solution with stirring, the polystyrene latex particles were coated with the antibodies. The colloidal gold ultrafine particles were prepared according to the literature (Frens, 1973). The particle size ranged from 10 - 30 nm. The colloidal gold were also coated with antibodies. The concentration of the analyte antigen, IgG, was 1mg/mL. The immunological reaction solution was diluted with phosphate buffer solution, which was

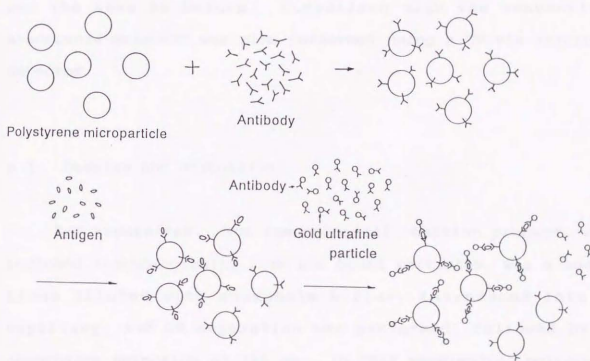


Figure 6-1. Schematic illustrations of preparation procedure of immunological reaction product.

used as electrophoretic buffer, before introduced into the separation capillary.

Apparatus. The experimental setup was the same as before. The CVL excitation laser was an argon laser of 514.5 nm, because the gold ultrafine particles, which were the labeling material, have absorption in this wavelength. The detection scheme of CVL was the same as before. Comparison with the conventional absorbance detector was also performed using a UV-Vis absorbance detector.

6.3 Results and Discussion

B/F separation. The immunological reaction product, which included immunologically free and bound particles, was a hundred times diluted with phosphate buffer, introduced into the capillary, and CE separation was performed, followed by the absorbance detection at 254 nm. In this wavelength, polystyrene latex particles and colloidal gold particles were detected as shown in Figure 6-2. Immunologically bound particles and free particles were successively separated with the concentration of IgG 1 mg/mL. In this wavelength, not only immunologically bound particles (IgG), but also immunologically free particles (gold ultrafine particles, and excess polystyrene particles) were detected.

Sensitive and selective detection by CVL. So, if 514 nm of wavelength was used for detection, only immunologically bound

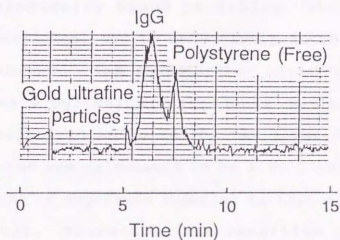


Figure 6-2. Electropherogram of the immunological reaction product detected by the absorbance detector. Introduction amounts of antigen IgG: 4 pg, and detection wavelength: 254 nm.

particles would be selectively detected. At the detection wavelength 514 nm, these particles could not be detected in the same dilution condition as shown in Figure 6-3b. So, the CVL detection was performed using 514.5 nm wavelength as a CVL excitation, and electropherogram was obtained as in Figure 6-3a. Only the immunologically bound particles were detected. This selectivity was considered to be caused by absorption signal from the colloidal gold particles. Therefore, using this wavelength, the immunologically bound particles, which adsorbed gold ultrafine particles, can be selectively detected in homogeneous reaction solution. The introduction amount of antigen IgG was estimated as 4 pg, which corresponds to 21 amol. Taken the double of signal to noise ratio, the lower detection limit of absolute amount was calculated to be 1 to 2 amol. This result is nearly 1 order of magnitude superior to that of PIA technique (Tut et al., 1993). Therefore, the sensitive and selective CVL detection combined with CE is at least 1 order of magnitude superior in sensitivity to those of EIA and RIA. Comparing with those conventional immunoassay techniques, this technique has another important advantage that by using the CE separation technique, conventional B/F separation is not necessary. Furthermore, the sensitive and selective detection can be performed within 10 minutes. Therefore, the proposed CE/CVL system can be applied as a superior immunoassay technique with simplicity and rapidity as well as with high sensitivity. Furthermore, introducing another size of polystyrene latex particles which are coated with another kind of antibodies,

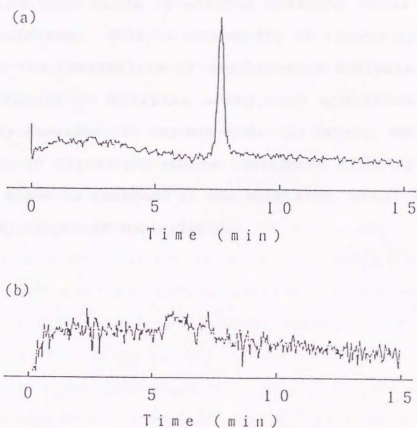


Figure 6-3. Electropherograms of the immunological reaction product detected by (a) CVL detection, and (b) conventional absorbance detection. Injection amount of antigen IgG was 4 pg.

another kind of analyte antigen can be analyzed at the same time, by utilizing the different size of polystyrene latex particles separated by the CE technique. Therefore, simultaneous analysis of two or more kinds of analyte antigens would be feasible by this technique. This is noteworthy in immunological diagnosis, because the feasibility of simultaneous analysis, which used to be difficult in analysis using such specificity as antigen-antibody reaction, is represented. In future, many immunological analytes in ultramicro volume introduced directly from the blood vessel might be analyzed at the same time, which really makes the clinical diagnosis much simpler.

CHAPTER 7 Application to Capillary Gel Electrophoresis

Capillary gel electrophoresis (CGE) is one separation mode in CE separation, which uses gel-filled capillary instead of open tubular capillary. The separation principle of CGE can be considered as an analogy to slab gel electrophoresis. In CE, the CGE technique is effective to separate biological polymers, such as proteins, or polynucleotides. In this chapter, the CVL technique is applied as a highly sensitive detector for CGE. This highly sensitive detection technique combined with CGE has many merits that it require only very small sample volume and amount, and does not need derivatization, and may not need the procedure such as polymerase chain reaction (PCR) amplification, which is troublesome and uses very expensive reagents. Therefore, these advantages will give a significant contribution to gene sciences, biological and medical research, and clinical techniques through ultramicroanalysis of biopolymers.

7.1 Introduction

Capillary gel electrophoresis (CGE) is a hybrid of traditional slab gel and free solution capillary open tub electrophoresis technology. The first spectacular electropherograms, published in the late 1980s, demonstrated an opportunity for significant advances in the practice of

separation science (Cohen and Karger, 1987; Cohen et al., 1987). The promise of a powerful new separation tool has turned into reality with improvement of techniques for generating gels in capillaries. Single base separations of polynucleotides have been achieved (Karger et al., 1989). DNA sequencing runs resolving 350 bases have been completed in 1 hour (Drossman et al., 1990). Accurate molecular weight sizing of proteins have been achieved (Cohen and Karger, 1987). Gel filled capillaries are thus effective to separate proteins, peptides, nucleotides, double-stranded DNA, and chiral molecules. It is said that it takes one century to resolve all human genome using conventional slab gel electrophoresis. The speed of CGE separation is about 10 times as faster as the slab gel electrophoresis. If combined with a highly sensitive detector, analysis of all genome will be completed within 10 years. As a sensitive detection, LIF is one choice but fluorescent labeling reagents are always expensive. If CVL detection with UV laser is performed, there needs no labeling reagents and this makes DNA analysis very simple and fast.

Thus, if the CVL technique is introduced as a sensitive detector for CGE, the sample size can be reduced to less than nanogram, which is 2 - 3 orders of magnitude less than the conventional detection technique (UV absorbance). Furthermore, because any light-absorbing species can be detected in the CVL detection, extracted DNA fragments, for example, can be analyzed directly without such derivatization as fluorescence, still retaining the sensitivity.

In this chapter, application of the CVL detection technique as a sensitive detection method for CGE is investigated. An excimer laser was introduced as a UV light source to excite biological molecules without derivatization. Because the excimer laser is a pulsed laser, CVL by forced vibration cannot be induced and pulse repetition rate have to match not only with the heat release time but also with the natural frequency of the vibrating system, resulting in a stable stationary CVL. The development of pulsed UV laser-induced stationary CVL system is already described in Chapter 5. Gel durability against laser irradiation and sensitivity compared with LIF are also discussed.

7.2 Experimental

Apparatus. The experimental setup for CVL detection system is described in detail in Chapter 4. The CVL excitation laser used this time was a waveguide excimer laser (Potomac photonics, SGX-1000) with 248 nm lasing line, 60 ns pulse duration, and the output nearly 10 μ J per pulse at 1 kHz repetition rate. The CVL was detected using a position sensitive detector (PSD) by monitoring the change of propagation direction of a probe laser beam after passing through the wall of the vibrating capillary. The output from the PSD was first fed into an isolation amplifier with one hundred fold, passed through an electrical band pass filter, and a lock-in amplifier. The reference signal for the lock-in amplifier was obtained from a function generator

triggered by a reference signal output from the excimer laser. Fused silica capillaries used here were 20 μm i. d. or 50 μm i. d., and 150 μm o. d.. Overall capillary length was 75 cm and effective length was 40 cm.

Preparation of Gel Filled Capillaries. Polyacrylamide gel was used in CGE experiment. The preparation of gel matrix inside the capillary was preceded according to the literature (Baba et al., 1992). Polyacrylamide gel filled capillaries were prepared as follows. The capillaries were cut into the appropriate length and 3 to 10 mm of polyimide coating was burned off at the detecting position. The capillary was rinsed by successively passing 1N NaOH, distilled water, acetonitrile by using a simple vacuum injection system (Baba et al., 1992). The inner surface of the capillary was treated with the solution of 0.4 % 3-methacryloxypropyltrimethoxysilane in acetonitrile for 1 hour at 40 C. The capillary was then rinsed with acetonitrile and distilled water. A stock solution of acrylamide was prepared by dissolving the appropriate amount of acrylamide and N,N'-methylenebis(acrylamide)(BIS) as a cross-linking agent in distilled water. The total concentration of monomer and the concentration of the cross-linking agent for the polyacrylamide gel are generally expressed as % T and % C, respectively. The stock solution of acrylamide (40 % T and 5 % C, or 40 % T and 0 % C) was diluted with the running buffer solution, which was the mixture of 0.1 M tris(hydroxymethyl)aminomethane, 0.1 M boric acid, and 7 M urea (pH 8.6), giving a final acrylamide solution (5 % T and 5 % C, 3 % T and 5 % C, 7 % T and 0 % C, 5 % T and 0 %

C). The diluted acrylamide solutions were degassed in an ultrasonic bath. Polymerization was initiated by the addition of 20 μ L of 10 % (w/v) ammonium peroxydisulfate solution and 80 μ L of 10 % (v/v) N,N,N',N'-tetramethylethylenediamine (TEMED) solution into 5 mL of the diluted solution of acrylamide. The polymerizing solution was quickly passed through the capillary. Polymerization in the capillary was completed at room temperature within a night.

Samples. Samples for CGE were polyadenylic acid (Poly (A)) digested by nuclease P1 and C-termination sequencing reaction product, both of which was given by Dr. Baba of Kobe Pharmaceutical University. Oligoadenylate fragments from poly (A) were prepared by enzymatic hydrolysis of poly (A) with nuclease P1. Enzymatic digestion of poly (A) gave a mixture of polynucleotides containing a 5' terminal phosphate in the chain length range from monomer to 300mer (Baba et al., 1991).

The DNA sequencing product was obtained using commercially available kit of cycle sequencing reaction for the template of M13mp18 single-stranded DNA. The concentration of the sequencing reaction product was estimated as 10^{-9} M. Only for the purpose of comparing the sensitivity with LIF, the sequencing product was labeled with fluorescent tag. As for C-terminated DNA fragments, 6-carboxy-fluorescein (FAM) was used as a labeling reagent (Swerdlow et al., 1991).

7.3 Results and Discussion

Gel composition. CGE technique has remarkable performance in separation of biological polymers such as protein, nucleic acid, and so on. Nucleic acid has the maximum absorption at ca. 260 nm, and about 80 % of that at 248 nm. Therefore, the lasing line of the excimer laser at 248 nm was still effective to excite the nucleic acid. However, different from the free solution capillary electrophoresis, irradiation of the excitation laser at the same point (gel) in the capillary sometimes causes a fatal destruction of gel. To avoid the affect of gel destruction, polyacrylamide gel solutions of different concentrations of monomer and cross-linking reagent were investigated on endurance to the laser irradiation. The results are summarized in Table 7-1. Gels containing no cross-linking reagent (7 % T and 0 % C, 5 % T and 0 % C) represented sufficient endurance to laser radiation, in comparison with gels containing cross-linking reagent (5 % T and 5 % C, 3 % T and 5 % C). So, the following experiments were undertaken using the gel composition of 7 % T and 0 % C. Next, poly (A) digested by nuclease P1 was used as a sample, which had different number of nucleotide chains by one. Electropherogram of Poly (A) is shown in Figure 7-1. Each peak represented the nucleotide chain separated by one difference of the chain length. Though the polyacrylamide gel contained no cross-linker such as BIS, sufficient separation was achieved as represented in Figure 7-1. This gel composition (7 %T, 0 %C) was thus decided to perform the CGE separation of polynucleotides. Another merit of the gel in this component was that it had

Table 7-1. Gel conditions and endurance to laser radiation.

Gel condition	Endurance to Laser Radiation
7 %T, 0 %C	Good
5 %T, 0 %C	Good
5 %T, 5 %C	Not good
3 %T, 5 %C	Not good

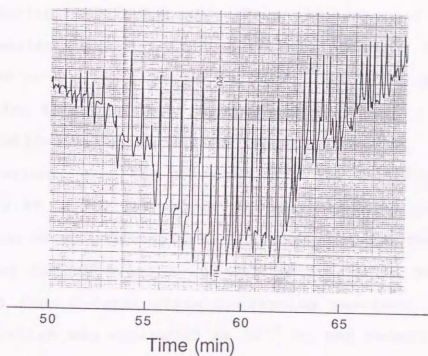


Figure 7-1. Sensitive detection of polyadenylic acid
digested product by CGE/CVL.

fluidity and was easy to be refilled in the case of destruction by excess focus of intense laser pulses. The signal intensities were largely dependent on the baseline, which was slowly and periodically fluctuating. This fluctuation seemed to be attributed to the instrumental matter. For example, small wall thickness resulted in affection from the channel heat, which varied during electrophoresis. This problem would be solved by using smaller i. d. capillaries (e. g., 30 μm). Sample amount injected could be reduced to 36 %, but more skillful gel fabrication technique might be required.

Sensitive detection of DNA fragments by CVL. In the above considerations, even the polyacrylamide gel containing no cross-linker (7 %T, 0 %C) was proved to have sufficient performance in separation of polynucleotides. Figure 7-2 shows the preliminary result of CGE separation and CVL detection of DNA fragments obtained from C-termination sequencing reaction. The sample concentration was estimated as 10^{-9} M, and cannot be detected using the conventional absorbance detection. This result using the CVL technique was almost as sensitive as the LIF detection. Because the CVL detection needs no derivatization process, it is free of by-products derived from chemical reaction, which confused analysis and has advantage of acceleration of the sequencing process. The sensitivity and property of the CVL detection suggests the feasibility of ultramicroanalysis without derivatization nor (PCR) amplification. The PCR amplification has been essential in gene analysis, but this amplification method is available only when a part of the sequence is known and

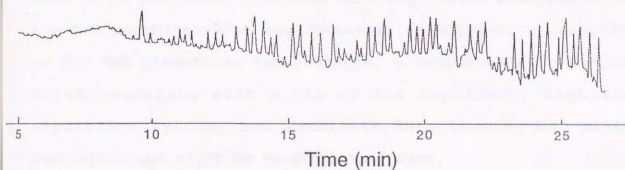


Figure 7-2. Sensitive detection of sequencing reaction product (C-termination) by CGE/CVL.

a suitable primer is available. Therefore, highly sensitive detection methods are desired and the CVL detection is more advantageous than the LIF detection that it does not need derivatization. Only sensitive detection methods can approach analysis of DNA fragments, the sequence of which is utterly unknown, or when there is no primer available. The most advantageous merit of the CVL detection is that it can analyze the sample as it is, which may enable the detection of ultramicro quantity of important (in some sense) analytes which used to be excluded. Combined with high speed analysis of CGE, the CVL technique also has advantage in clinical applications. As for DNA diagnosis, for instance, a series of such analyses, direct sampling with a tip of the capillary, high speed separation by CGE, and sensitive detection by CVL without derivatization might be feasible in future.

In this chapter, this study is summarized as conclusion and future development is considered.

Recent advances in ultramicroanalysis is remarkable. Especially, the efficiency of capillary electrophoretic separation, which needs sample of small quantity less than nanoliter, has been proved to be extremely high. The CE technique has another potential of sensitive detection of ultramicro volumes, if combined with highly sensitive detector. In reality, a single cell itself or cytoplasm was directly introduced into the capillary followed by separation and sensitive detection using the LIF technique. But the LIF detection has disadvantage that analyte must be fluorophore. Except using indirect technique or native fluorescence, fluorescent derivatization process is essential. Photothermal spectrometry has no such disadvantage. The CVL effect is a kind of photothermal effect. The authors found out the CVL effect and this doctoral dissertation begins with coupling the CE separation and the CVL detection. The CVL detection was first performed by OBD technique, because the mechanical vibration of the capillary emits an acoustic wave, which deflects the probe beam. However, the OBD technique had difficulty in reproducibility and stability. So, considering the characteristics of the CVL effect, two CVL detection schemes, PZT detection and diffraction pattern detection, were developed. The PZT detection is simple

and easy in instrumentation that it does not use a probe beam, but when ionic substances passed the touching point of the capillary and the PZT disc, a peak sometimes appeared by pyroelectric effect of the piezoelectric transducer. The other detection technique is the diffraction pattern detection, and though it uses a probe beam, the optical alignment is much easier than the OBD detection. Considering the photothermal effects induced in the capillary, the diffraction pattern detection was optimized and through such investigations, sufficient reproducibility and stability of the CVL detection could be attained. In addition, by the development of pulse CVL detection, pulsed UV laser has become also applicable as a CVL excitation source. Because many biological materials have absorption band in UV region, introduction of pulsed UV laser like an excimer enables detection without derivatization. The excimer laser is more convenient and inexpensive than the SHG of an argon ion laser. Now, the CVL detector has become widely applicable to any light absorbing species, because it has become possible to be excited not only by CW laser but also by pulsed laser. In this dissertation, there are two applications of the CVL detection as a sensitive detection of biological analysis. One is immunoassay and the other is CGE. The highly sensitive, simple, and rapid immunoassay technique combined with CE separation and CVL detection was developed, in which B/F separation, and highly sensitive and selective detection could be performed. The CGE technique is one separation mode of CE, and biopolymers are effectively separated. The CVL detection was applied as a

sensitive detector for CGE, and gel compositions suitable for CVL were investigated. The feasibility of ultramicroanalysis of polynucleotides was also confirmed.

The major merit of the CVL detection is high sensitivity even in ultramicro volumes and wide applicability. To make the advantage of ultramicro volume detection, single cell analysis is also applicable. Coupling with the CGE technique, gene analysis including DNA sequencing, gene diagnosis, etc., can be performed with high sensitivity. This technique will give a large impact especially in DNA analysis, or gene analysis, because this sensitive detection will make amplification of DNA fragments such as PCR, which is troublesome and expensive, unnecessary.

Furthermore, the superior characteristics of the CVL detection will contribute to ultramicroanalysis, with high sensitivity, wide applicability, needless of derivatization, easy to be performed, because the CVL detection fulfills all three factors, sensitivity, selectivity, and speed, which are desired in biological analysis. The CVL detection may face to practical problems derived from using laser and detecting vibration, but these problems are solvable with technical approach.

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