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ARTICLE *in* ANALYTICAL CHEMISTRY · MAY 2006

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Anal. Chem. **2006,** *78,* 2646−2650

Circular Dichroism Thermal Lens Microscope for Sensitive Chiral Analysis on Microchip

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A novel chiral detector, a circular-dichroism thermal lens microscope (CD-TLM), was developed to realize sensitive and selective detection of small volume chiral samples on a microchip. To realize chiral recognition on TLM, an excitation beam was phase-modulated at a frequency of 1.2 kHz, and left-circularly polarized light (LCPL) and right-circularly polarized light (RCPL) were generated. Then, the differential light absorption between LCPL and RCPL, which is the CD effect, was detected as thermal lens signal intensity and phase. As a standard sample, optically active tris(ethylenediamine)cobalt(III) [Co-(en)$_3$]$^{3+}$I$_3^-$ aqueous solutions were used for performance evaluations. First, we verified the basic principle for selective chiral analysis by comparing the signals in intensity-modulation and phase-modulation modes of the excitation beam. Also, we found that the g-factor, which is significant for determining enantiomeric excess, agreed well with the value obtained by the CD spectrometer. The limit of detection (LOD) for enantiopure [Co-(en)$_3$]$^{3+}$I$_3^-$ was 6.3×10^{-5} M (1.9×10^{-7} abs) for (−)-Co(en)$_3^{3+}$, and the sensitivity in absorbance units was more than 250 times higher than that in a CD spectrophotometer. Finally, we demonstrated enantiomeric excess determination on a microchip. The LOD was 1.7% (8.5×10^{-7} abs) for (−)-Co(en)$_3^{3+}$ and at least one order superior to the LOD of a CD spectrometer. The applicability of CD-TLM for sensitive chiral analysis on a microchip was verified, and CD-TLM is expected to be promising for microchip-based chiral synthesis and analysis systems.

In recent years, there has been great interest in miniaturized chemical systems on microchips, and integration of various chemical processes (mixing, chemical reaction, separation, etc.) has progressed rapidly.[1,2] The benefits of miniaturization and integration include smaller sample and reagent volumes, more effective reaction due to the large surface-to-volume ratio, smaller space requirement, and lower cost. These advantages have led to microchip technology applications in various analytical procedures and chemical syntheses. As one of these applications, microchip chiral synthesis and analysis systems are of particular interest to the pharmaceutical industry, where the goals are integration of multiple functions such as synthesis, screening, detection, and biological evaluation on a single integrated microchip to realize high-throughput systems.[3−8]

However, a detection method for chiral analysis has been a stumbling block, because accurate and precise determination of enantiomeric content is necessary because of the difference in bioactivity of each enantiomer and the resultant strict guidelines for the products.[9] One candidate detection method is high-performance liquid chromatography (HPLC) or electrophoresis separations combined with a UV−vis spectrophotometric method.[10,11] A large variety of enantioselective stationary phases are now commercially available, and the UV−vis absorption method has wide applicability to nonfluorescent samples. Despite the success of enantioselective separations, however, many enantiomeric separation problems remain intractable.[12] An alternative approach is circular dichroism (CD) spectrometry, because it responds directly to the chirality of a given molecule even without separations.[13−15] CD spectrometry is based on the difference in absorption (ΔA) between left-circularly polarized light (LCPL) and right-circularly polarized light (RCPL). In principle,

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10.1021/ac0519920 CCC: $33.50
Published on Web 03/10/2006

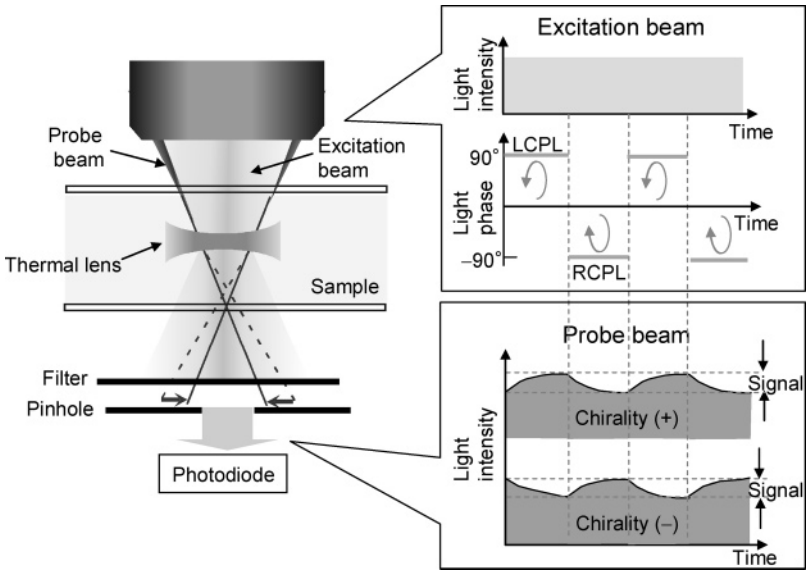

Figure 1. Principle of CD-TLM.

the sensitivity is linearly dependent on the optical path length. However, the optical path length in a microchannel, which is usually the depth of the microchannel (~1−100 μm scale), is 2 or 3 orders shorter than in conventional cuvettes (mm to cm). Therefore, 2 or 3 orders of higher sensitivity with high spatial resolution are necessary for the detection method on a microchip.

Thermal lens spectrometry (TLS) is a kind of photothermal spectrometry which holds promise for overcoming the low sensitivity of absorption-based detection methods.[16] TLS can sensitively detect heat generated by absorption of light, and it has been applied for trace analysis in liquids on a macroscopic scale since the end of the 1970s.[17−22] Our group has developed a first coaxial dual-beam thermal lens microscope (TLM) that utilized an objective lens with chromatic aberration,[23,24] and we achieved sensitive thermal lens detections on microchips with single molecule concentration.[25,26] Since then, TLM has been successfully applied to various integrated analytical systems on microchips, such as environmental analysis, clinical diagnosis, food analysis, and single-cell analysis.[27−30]

However, conventional photothermal spectrometry has no selectivity for chiral recognition. For this purpose, several groups reported novel methods by combining CD spectrometry. For example, Tran's group has realized CD-TLS combined with HPLC by modulating an excitation beam with a Pockels cell and reported excellent sensitivity of $\Delta A = 1.9 \times 10^{-6}$ abs compared with $\Delta A = \sim 10^{-5}$ abs in CD spectrometry.[31−33] However, the detection size was 10 mm scale, and sensitive detection on a microchip was not achieved. Tong's group has realized CD spectrometry combined with a nonlinear degenerate four-wave mixing method, which is a kind of photothermal spectrometry, and reported excellent sensitivity of $\Delta A = 5.5 \times 10^{-7}$ abs in a 0.1 mm optical path length cell.[34] The detection volume was 98 pL, which was still large compared with the typical detection volume (<1 pL) on a microchip. In addition, CD spectrometry has a feature of direct enantiomeric excess (ee) determination by measuring $\Delta A/A$. However, the previous works were done with pure enantiomers or racemic mixtures. Therefore, there are no reports on sensitive ee determination without separations.

In this study, we developed a circular-dichroism thermal lens microscope (CD-TLM) to realize sensitive and selective detection of small-volume chiral samples on a microchip without separations. An excitation beam was phase-modulated with a Pockels cell, and the generated LCPL or RCPL was tightly focused onto the sample in a microchannel. The sign and intensity of ΔA corresponding to the CD effect was measured as a thermal lens signal phase and intensity with a lock-in amplifier. In addition, direct ee determination was demonstrated. The detection limits for the concentrations and ee values were evaluated in a 100 μm path length microchip. The CD-TLM sensitivity was more than 2 orders higher than that of a CD spectrometer. The applicability of CD-TLM for sensitive chiral analysis on a microchip was verified.

EXPERIMENTAL SECTION

Principle of CD-TLM. The principle of CD-TLM is illustrated in Figure 1. In normal TLMs, excitation beams were intensity-modulated, and the signal intensity was proportional to the

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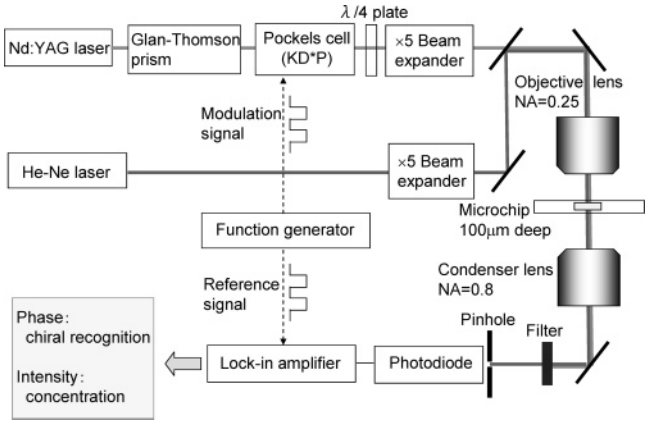

Figure 2. Schematic diagram of CD-TLM.

absorbance (A) of the sample. In CD-TLM, however, a linearly polarized excitation beam is phase-modulated by an electrooptic modulator, and LCPL or RCPL is generated periodically at a certain frequency (typically ~kHz). The LCPL or RCPL is alternately introduced to the objective lens and tightly focused onto a sample in the microchannel. The sample absorbs the LCPL or RCPL, and the thermal lens effect is induced by photothermal conversion. The strength of the thermal lens for LCPL or RCPL irradiation is determined by the absorbance (A_{LCPL} or A_{RCPL}), which depends on the CD effect of the sample. For detection of the thermal lens effect, a probe beam is coaxially introduced into the objective lens and focused onto a sample with a longer focal length than that of the excitation beam for sensitivity enhancement.[23] The probe beam is converged by the thermal lens effect, and the change of the optical path is detected by measuring the change of transmittance ($\Delta I = I_{LCPL} - I_{RCPL}$) through a pinhole with a photodiode. The intensity change (ΔI) is linearly dependent on the difference of the thermal lens effect, which is also proportional to the differential absorbance ($\Delta A = A_{LCPL} - A_{RCPL}$) of the sample. Therefore, the sign of the intensity change (ΔI) for LCPL or RCPL irradiation, that is, the phase of the corresponding waveform in Figure 1, determines the chirality of the sample, and the amplitude determines the concentration. When the sample is a mixture of enantiomers, the phase and amplitude correspond to the chirality of the excess enantiomer and the excess concentration, respectively. Thus, we can sensitively determine the chirality and the concentration by the thermal lens effect.

CD-TLM System. Figure 2 shows a schematic diagram of our CD-TLM system. A 532 nm Nd:YAG laser (100 mW) was used for an excitation beam, and a 633 nm He−Ne laser (15 mW) was used for a probe beam. The excitation beam from the laser source was linearly polarized, and the extinction ratio was 10^2:1. The extinction ratio became 10^5:1 with a Glan−Thomson prism. Then, the excitation beam was introduced into a Pockels cell (Inrad Inc., PKC21SG09). We selected a KD*P crystal because it showed higher stability for the output beam intensity than an ADP crystal had. The excitation beam was phase-modulated by applying a zero- and a half-wave voltage (3.3 kV) periodically (1.2 kHz of square-wave with 50% duty ratio) to the Pockels cell using a driver (Inrad Inc., 2-021) and a function generator (NF Corp., DF1905). As a result, the polarization direction of the linearly polarized excitation beam was periodically changed by 90° with the frequency of 1.2 kHz. After passing through the Pockels cell, the linearly polarized excitation beam was converted to LCPL or RCPL by a quarter-wave plate. Both the excitation and probe beams were made coaxial with a dichroic mirror and focused onto a sample with an objective lens (numerical aperture, NA: 0.25), though the focal length of the excitation beam was made longer by ~15 μm than that of the probe beam for sensitivity enhancement.[23] The spot size of the excitation beam was calculated as 3 μm by Gaussian propagation theory.[16] The extinction ratio under the objective lens was measured by converting the LCPL or RCPL to linearly polarized beams with a quarter-wave plate and measuring the ratio of the transmittance for each beam through a polarizing filter. The value was approximately 500:1, and the loss of sensitivity by degradation of the extinction ratio was negligible. As a sample cell, we used a quartz demountable cell with an optical path length of 100 μm. In the sample, the thermal lens effect was induced and the probe beam was converged by the effect. After passing through the sample, the beams were again collimated with a condenser lens (NA 0.9). Then, the excitation beam was cut with a filter, and just the probe beam was introduced into the pinhole and focused onto the photodiode. The output of the photodiode was fed into a lock-in amplifier (NF Corporation, LI5640), and the phase and amplitude in the lock-in amplifier were recorded as signals with a personal computer. For comparison with intensity-modulation mode (normal TLM), we carried out intensity-modulation of the excitation beam by inserting an optical chopper and turning the Pockels cell off.

Chemicals. Optically active $[Co(en)_3]I_3$ was synthesized as described previously.[35] The optical purities were determined by a polarimeter (JASCO Corp., DIP-360) and CD spectrometer (JASCO Corp., J720). The optical purities of synthesized (+)-$Co(en)_3^{3+}$ and (−)-$Co(en)_3^{3+}$ were 86% and 92%, respectively. For the calibration curve on ee with the CD-TLM, we prepared mixtures of (+)-$Co(en)_3^{3+}$ and (−)-$Co(en)_3^{3+}$ and predetermined the ee with the CD spectrometer. The molar absorptivity (ϵ) and the differential molar absorptivity ($\Delta\epsilon = \epsilon_{LCPL} - \epsilon_{RCPL}$) of pure (±)-$Co(en)_3$ are 10 M^{-1} cm^{-1} and ±0.32 M^{-1} cm^{-1}, respectively.[35]

RESULTS AND DISCUSSION

First, we compared the thermal lens signals between intensity-modulation and phase-modulation modes to verify the basic principle of CD-TLM. The racemic $Co(en)_3$, pure (+)-$Co(en)_3^{3+}$, and pure (−)-$Co(en)_3^{3+}$ samples with the same concentration of 16 mM were measured with intensity-modulation mode and phase-modulation mode (CD-TLM). The results are shown in Figure 3. For intensity-modulation mode (Figure 3a), the three samples showed approximately the same signal intensity (407 μV) and phase (−180°). This result is reasonable because intensity-modulation mode has no chiral selectivity, and response to the absorbance (A) should be the same value for three samples with the same concentration. In contrast to intensity-modulation mode, however, the signal intensity of CD-TLM (Figure 3b) is almost zero (<1 μV) for the racemic-$Co(en)_3^{3+}$ due to the cancellation of the chirality between (+)-$Co(en)_3^{3+}$ and (−)-$Co(en)_3^{3+}$ enantiomers. For pure (+)-$Co(en)_3^{3+}$ and (−)-$Co(en)_3^{3+}$, the signals are clearly observed, and the intensities are 10.8 μV and 11.7 μV, respectively. The small difference of the signal intensities can be

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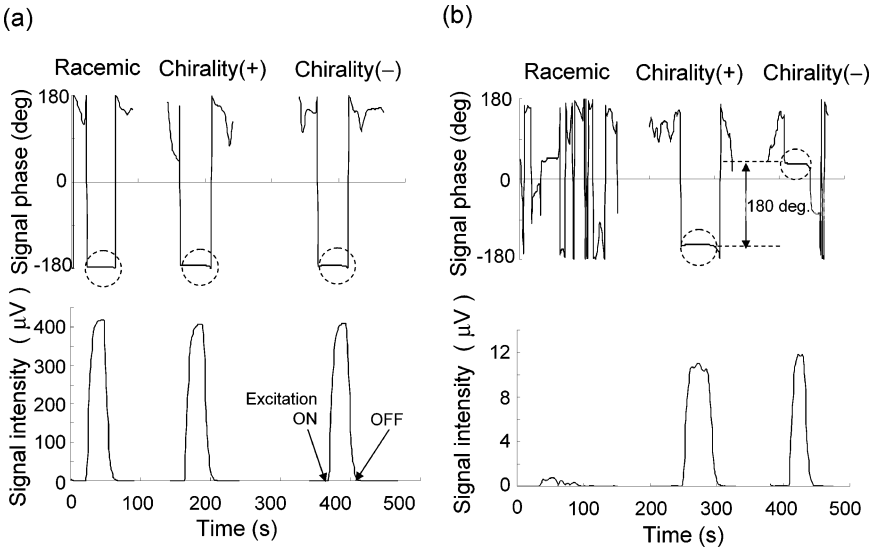

Figure 3. Comparison of (a) intensity-modulation mode (normal TLM) and (b) phase-modulation mode (CD-TLM) signals for racemic Co(en)$_3^{3+}$, (+)-Co(en)$_3^{3+}$, and (−)-Co(en)$_3^{3+}$ solutions. All the concentrations were 16 mM. The thermal lens signals were measured by turning the excitation beam on.

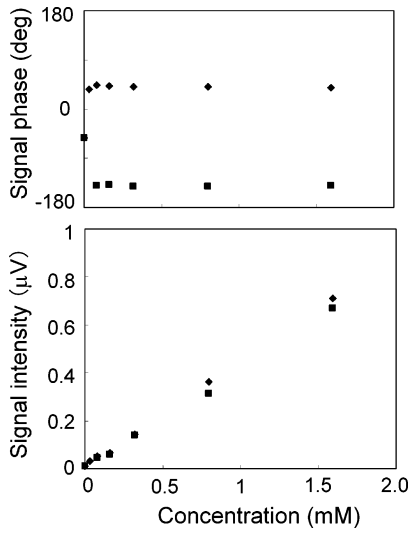

Figure 4. Dependence of CD-TLM signals on the concentration of pure (+)-Co(en)$_3^{3+}$ and (−)-Co(en)$_3^{3+}$ solutions: (■) for (+)-Co(en)$_3^{3+}$ and (♦) for (−)-Co(en)$_3^{3+}$.

explained by considering that the signal intensity is proportional to the optical purities (86% for (+)-Co(en)$_3^{3+}$ and 92% for (−)-Co(en)$_3^{3+}$). More important is that the phases change by 180°, which is also reasonable because of the difference of the sign in $\Delta\epsilon$. Furthermore, the g-factor ($\Delta A/A = \Delta\epsilon/\epsilon$) is significant for direct determination of ee. In TLM spectrometry, the $\Delta\epsilon$ and ϵ are proportional to thermal lens signal intensities in intensity-modulation mode and phase-modulation mode (CD-TLM) in Figure 3. From the values in Figure 3, the $\Delta\epsilon/\epsilon$ can be obtained as 0.026 for (+)-Co(en)$_3^{3+}$ and 0.029 for (−)-Co(en)$_3^{3+}$. They show good agreement with the values of 0.026 and 0.028 measured with a CD spectrometer at the same wavelength. On the basis of these results, we could verify the principles of CD-TLM for selective chiral analysis.

Next, we examined the sensitivity of the CD-TLM. Figure 4 shows the dependence of the CD-TLM signal intensity and phase on the concentration of pure (+)-Co(en)$_3^{3+}$ and (−)-Co(en)$_3^{3+}$. The signal intensities for both enantiomers have a linear relationship to the concentration. The phases differ by 180° between (+)-Co(en)$_3^{3+}$ and (−)-Co(en)$_3^{3+}$, as expected, without any significant dependence on the concentrations. The limits of detection (LODs) were calculated at $S/N = 2$ and are 9.4×10^{-5} M (2.6×10^{-7} abs) for (+)-Co(en)$_3^{3+}$ and 6.3×10^{-5} M (1.9×10^{-7} abs) for (−)-Co(en)$_3^{3+}$. For comparison, the same samples were measured by a CD spectrometer, and the LODs were calculated as 2.3×10^{-2} M (6.4×10^{-5} abs) and 2.5×10^{-2} M (7.3×10^{-5} abs), respectively. More than 250 times higher sensitivity for CD-TLM is obtained compared with that for the CD spectrometer. Therefore, our CD-TLM sensitivity meets the requirement to be >2 orders higher for chiral analysis on a microchip. Also, the maximum detection volume can be obtained by calculating the total sample volume that the excitation beam irradiated, and this value is calculated to be ~6 pL. Then, the LOD in mass units is less than ~88 fg, which is 8 times lower than the value (0.68 pg) reported by Tong's group.[34] On the basis of our previous results (7 fL detection volume with NA 0.46),[25] the detection volume can be calculated to be as small as ~44 fL, assuming that detection volume is proportional to the third power of NA. In this case, the LOD in mass units becomes 0.66 fg, which is 3 orders lower than the reported value. The exact detection volume is under investigation. By considering these results, we verified the advantage of CD-TLM for sensitive and selective chiral analysis in microsized spaces.

In addition to pure sample measurement, precise determination of ee is essential for on-line chiral detection on a microchip without separations. Then, we measured ee, which is defined as

$$\text{ee (\%)} = (C_+ - C_-)/(C_+ + C_-) \times 100\% \qquad (1)$$

where C_+ and C_- are the concentrations of (+)-Co(en)$_3^{3+}$ and (−)-Co(en)$_3^{3+}$, respectively. The positive value means that (+)-Co(en)$_3^{3+}$ is in excess. The total concentration ($C_+ + C_-$) was retained at 16 mM throughout the measurements. The ee values

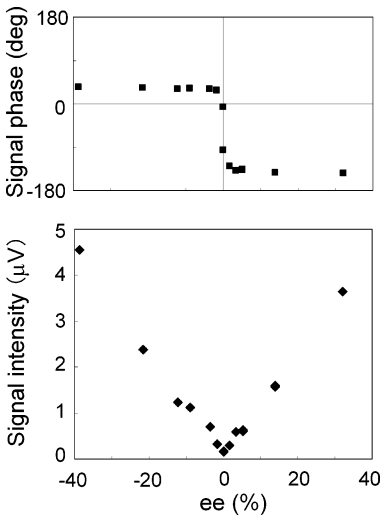

Figure 5. Determination of ee by CD-TLM. The concentration was retained at 16 mM for all ee values.

of the solutions were checked with a CD spectrometer to minimize the error of sample preparation. The calibration curve is shown in Figure 5. The signal intensity has a good linear relationship with the ee values. As expected, the phases turn by 180° at ee = 0, where the signal intensity becomes a minimum. The LODs are 1.8% (9.1×10^{-7} abs) for (+)-$Co(en)_3^{3+}$ and 1.7% (8.5×10^{-7} abs) for (−)-$Co(en)_3^{3+}$. In contrast, the sensitivity in the CD spectrometer was below the LOD, even for pure enantiomeric samples. Therefore, we used the LOD in Figure 4 for comparison of sensitivity, and the ee sensitivity in CD-TLM is still 1 order higher than a conventional CD spectrometer in absorbance. The lower sensitivity compared with that in Figure 4 may be caused by instability of LCPL and RCPL intensities. As one of the important functions of chiral analysis, direct determination of ee is also possible because we can measure $\Delta A/A$ by intensity-modulation and phase-modulation modes illustrated in Figure 3.

CONCLUSION

We have developed a circular-dichroism thermal lens microscope and evaluated its chiral detection performance on a microchip. More than 250 times higher sensitivity was realized versus that in a CD spectrophotometer for pure enantiomers detection. Also, we demonstrated ee determination on a microchip. The detection limit in absorbance units was >1 order higher than that in the CD spectrometer. The applicability of CD-TLM to microchip-based chiral synthesis and analysis system was verified. In this experiment, a visible laser source was used for the excitation beam. We have also realized UV-excitation TLM (UV-TLM) for nonlabeled and sensitive detection of nonfluorescent molecules.[36] The combination of UV-TLM and CD spectrometry is now being undertaken to realize various applications. Therefore, our system can be a powerful analytical tool for sensitive and selective chiral analysis on a microchip.

ACKNOWLEDGMENT

We would like to thank Dr. Haeng-Boo KIM and Mr. Shun Kubota of The University of Tokyo for valuable discussions.

Received for review November 9, 2005. Accepted February 10, 2006.

AC0519920

(36) Hiki, S.; Mawatari, K.; Hibara, A.; Tokeshi, M.; Kitamori, T. *Anal. Chem.*, in press.