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Determination of Subyoctomole Amounts of Nonfluorescent Molecules Using a Thermal Lens Microscope: Subsingle-Molecule Determination

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The photothermal effect of an ultratrace amount of nonfluorescent molecules in liquid was determined by optimizing the optical arrangement for a thermal lens microscope. The optimized experimental setup could be determined from the evaluation of probing volume and the concentration of the sample solutions even when the expectation of the molecule number in the probing region was less than a single molecule. The minimum expectation, which is explained as being the time average, was 0.4 molecule of Pb(II) octaethylporphyrin (OEP) in benzene. The concentrations in the $9.7 \times 10^{-11}-7.8 \times 10^{-10}$ M region used in this work corresponded to the expected number of 0.4−3.4 molecules, and the calibration curve in this region showed good linearity. Taking into account the enhancement factor of solvent, the molar absorption coefficient of solute, and the optimization of the optical arrangement, the present result, which was the determination limit of 0.34, was consistent with that previously reported. The relation between molecular behavior in the probing volume and the signal was discussed. The average temperature rise in the probing volume by the photothermal effect for the single OEP molecule was estimated as 3.1 μK, and this value was detectable, based on conventional thermal lens measurements for bulk scale sample.

Single-molecule detection in or on condensed-phase substances not only represents the ultimate goal in analytical chemistry but also may have great influence upon chemistry, biology, and physical and material sciences. The detection techniques of single or countable numbers of molecules, for example, may allow study of dynamics of molecules in a single quantum state, thermalization processes at a molecular level, and molecular behavior in intercellular space such as neurotransmitters in a synapse combination. Liquid media are especially important for chemistry and biology. However, technical difficulties always accompany detection in liquids because the numerous solvent molecules interfere with the detection by giving a large background. Reports concerning single-molecule detection in liquids, however, have been rapidly increasing in number during the past 10 years.[1,2]

Methods based on laser-induced fluorescence (LIF) have advanced single-molecule detection greatly; examples are near-field scanning optical microscopy[3,4] and confocal fluorescence microscopy.[5] However, these methods can measure only fluorescent molecules and are limited in applications. Other methods have also been used for single-molecule detection, including electrochemistry[6] and surface-enhanced Raman scattering.[7,8] However, these other methods also possess similar limitations in subjects, still leaving a more widely applicable method desired, especially for nonfluorescent species. Photothermal spectrometry is one candidate that satisfies the requirements, though there have been no reports on single-molecule detection using it. Photothermal spectrometry measures effects when light is converted to heat by matter including molecules.[9] Molecules in excited states relax via a radiation process such as fluorescence, but the quantum efficiency of fluorescence is small since only a few molecules are highly fluorescent, and thermal energy is evolved through nonradiation processes. Therefore, spectroscopic measurement methods using optical and mechanical effects caused by heat such as the photoacoustic effect, optical beam deflection, and thermal lens effect are applicable to a wide range of nonfluorescent molecules and they have been proved to be as sensitive as LIF.[10] These spectroscopic methods are known generically as photothermal spectroscopy.

Thermal lens spectroscopy and related techniques are within the family of photothermal spectroscopy methods, and some ideas have been proposed to measure very small quantities of molecules

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inside a capillary or on solid surfaces.[11−17] However, thermal lens measurements under an optical microscope have not been realized, because they are impossible using an aplanat lens which focuses both excitation and probe beams at the same point. We achieved true thermal lens measurements under an optical microscope using coaxial excitation and probe laser beams and utilizing chromatic aberration of an objective lens, and we proved that this thermal lens microscope is extremely sensitive.[18−20] As this thermal lens microscope is closely related to the established thermal lens optical configuration, we can estimate the detected quantity by the thermal lens theory.

This method allows ultrasensitive measurements on the surface and in the microspace in liquids including a single biological cell.[21−25] Generally, however, LIF signals possess selectivity to the fluorescence wavelength in addition to the excitation wavelength, while photothermal spectroscopy has only selectivity to the excitation wavelength because the thermalized energy, as heat, has already lost information related to the energy gap between the excited and ground states. It is often considered that this common characteristic of the photothermal methods is a great inconvenience for detection of an ultratrace amount of analytes in the presence of numerous solvent molecules. However, if the observation area is extremely limited and the number of solvent molecules included is reduced, only a very small amount of analyte molecules may be detected. Here we report that our idea has been proved and the method can detect the photothermal effect from molecules when the expectation number of these molecules in the probing region is countable, single, and even subsingle by optimizing the conditions of optical configurations, spectroscope, and thermal properties of the solute and solvent molecules.

EXPERIMENTAL SECTION

Apparatus. The thermal lens microscopy (TLM) system was described in detail elsewhere[21−23,25] and is only briefly noted here. The excitation and probe beams were made coaxial by the dichroic mirror and introduced into an optical microscope (Nikon, custom-made for our purposes) which was appropriately modified for TLM. This thermal lens microscope possessed focal point control units for both excitation and probe beams. The focal point of the laser beam can be controlled by adjusting the relative distance between two lenses in the units. The NA of the objective lens was 0.46. The excitation beam was the 488-nm emission line of an Ar^+ laser, and the beam power after passing through the objective lens was 2 mW at the sample. The excitation beam was mechanically chopped, and the chopping frequency was 1.6 kHz. The probe beam was a He−Ne laser of 632.8 nm, and its beam power was 0.1 mW after the objective lens. The probe beam, which passed through the sample, was separated from the excitation beam by a holographic notch filter (Kaiser Optical Systems, Inc., Ann Arbor, MI) and collected by another objective lens. The probe beam was detected by a photodiode and fed into a lock-in amplifier. The time constant of the lock-in amplifier was set to 4 s. The quartz microchip, which held a sample, was mounted on a 3-D stage which could be controlled in 1-μm steps in each direction; the step was precise enough for positioning the foci of the laser beams. In this optical configuration, we have confirmed that the signal generation mechanism corresponds to that of the thermal lens effect, based on the excitation power and frequency dependency.[20]

Reagents. The sample was selected to obtain high sensitivity, because the thermal lens effect depends on the optical and thermal properties of solutions.[9,25] Benzene was used as the solvent because of its high enhancement factor for the thermal lens effect. The solute, Pb(II) octaethylporphyrin (OEP) was synthesized from octaethylporphyrin. The absorption coefficient of the OEP at 488 nm is 3.2×10^4 M^{-1} cm^{-1}. Stock solution was prepared by dissolving OEP in benzene, and required concentrations were obtained by its stepwise dilutions.

Microchannel. The sample was introduced into a microchannel, which we fabricated in a quartz substrate. The details of the microchannel were reported elsewhere.[21−23,25] It was made by laser microfabrication and was 150 μm wide and 100 μm deep.

RESULTS AND DISCUSSION

The spatial arrangement of the focal points of the excitation and probe beams was adjusted to the optimal configuration, a confocal length apart from each other, as shown in Figure 1. It is well known that the optimal configuration of the thermal lens measurement, for which maximum sensitivity is obtained, is arranged at a distance $3^{1/2}Z_c$ that is the difference between the focal points of two beams, where Z_c is the confocal distance.[27] Figure 2 shows the dependence of the TLM signal on the distance between the focal points of two beams. Under our experimental conditions, the value of $3^{1/2}Z_c$ was calculated to be 4.7 μm. As can be seen in Figure 2, this calculated value was in good agreement with the experimental curve. All experiments in the present study were carried out with this optimal configuration, although our previous study did not strictly adjust the optical configuration of the focal points between two beams.[25] The probing volume is needed to calculate the number of molecules detected. Under the exact optical configuration of the thermal lens measurement, the volume can be estimated by the theory of thermal lens measurement. The probing volume is the same as the confocal volume, and the value for our experimental setup was calculated to be 7.2 fL (NA = 0.46). The concentrations, on the other hand, of the sample solutions measured were from 9.7×10^{-11} to 7.8×10^{-10} M. From these concentrations and probing volume, the expected number of molecules was calculated as 0.42−3.38 molecules.

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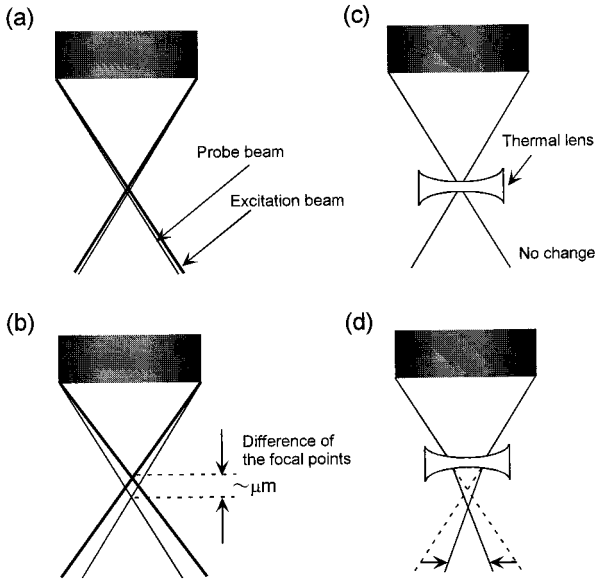

Figure 1. Optical configuration of the thermal lens measurement under the optical microscope. (a) The focal points of the excitation and probe beams are the same position; (b) the focal points of both beams are different positions. The influence of the probe beam by the thermal lens effect: (c) shows the (a) configuration and (d) shows the (b) configuration.

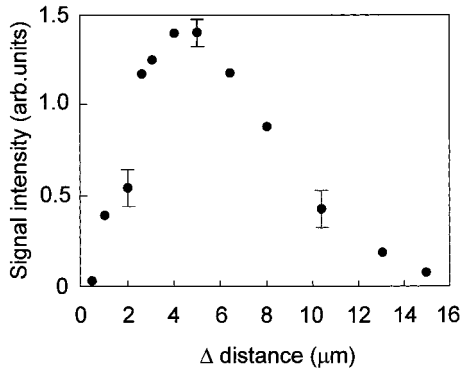

Figure 2. Dependence of the TLM signal intensity of 10 μM dye (sunset yellow) aqueous solution on the distance between the focal points of two beams.

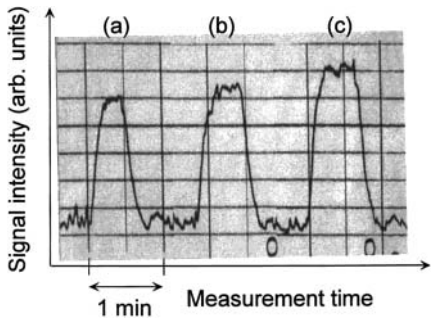

Figure 3. Photothermal signals from blank (a) and the samples with the expected numbers of 0.42 (b) and 1.69 molecules (c).

The detected photothermal signals from 9.7×10^{-11} M solution, 3.9×10^{-10} M solution, and pure benzene (blank) are shown in Figure 3. The expected molecule numbers of each solution were 0.42 and 1.69 molecules, respectively. Therefore, the signals in Figure 3b and c correspond to the photothermal signals from expectation numbers of 0.42 and 1.69 molecules, respectively. The

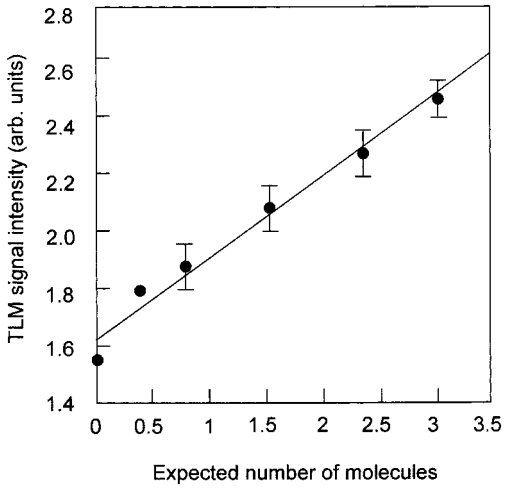

Figure 4. Calibration curve of the expected number of molecules in the probing volume. The solid line is the best fit weighted least squares. The error bars show twice the standard deviation, 2σ.

coefficient of variance (CV) was 3.0% for four repetitive measurements; hence, the reproducibility was satisfactory. Therefore, the difference from the background level was significant. A calibration curve in the range from 0.42 to 3.38 molecules is shown in Figure 4. The signal intensity was proportional to the expected number of the molecules. From these results, we judged the difference seen in Figure 2 as the photothermal signal from subsingle molecules. From the value of twice the standard deviation 2σ of this calibration curve, the lower limit of quantitative determination was estimated as 0.34 molecule. The lower limit of detection was estimated as 0.32 molecule from the conditions of signal-to-noise ratio S/N = 2 and signal-to-background ratio S/B = 0.1. Therefore, we verified photothermal signals could be obtained from a subsingle-molecule level as an expected molecule number.

The determination limit and photothermal and molecular properties of samples using the present and the previous studies are shown in Table 1. Thermal lens signal, S_{TL}, is expressed as

$$S_{TL} = EA = \frac{P_e(dn/dT)A}{\lambda_p \kappa} \quad (1)$$

where E is the enhancement factor, A is the absorbance ($= \epsilon Cl$: ϵ is the molar absorption coefficient, C is the concentration of the solution, and l is the path length of the cell), P_e is the power of the excitation laser, dn/dT is the refractive index temperature coefficient, λ_p is the wavelength of the probe laser, and κ is the thermal conductivity of the solvent.[9] In the previous study using aqueous solution of a dye, sunset yellow, we succeeded in obtaining the determination limit of 160 ymol (96 molecules) using TLM.[22] As can be estimated from Table 1, the thermal lens measurement in the present study is ~90-fold better than the previous one because of the enhancement factor and the absorbance. Therefore, the present determination limit of 0.34 seems reasonable enough to take into consideration the spectroscopic factor above and the optimization of optical arrangement. Since the success of this study derived from the above reasons, it is necessary to improve the apparatus including the optical components in order to apply aqueous solution.

Table 1. Photothermal and Molecular Properties of Samples in Two Experiments

solute	solvent	detn limit	P_e (mW)	dn/dT $(K^{-1} \times 10^4)^a$	λ_p (nm)	κ $(W \cdot m^{-1} \cdot K^{-1})^b$	ϵ $(M^{-1} \cdot cm^{-1})$
OEP	benzene	0.34	2	−6.52	632.8	0.137	32 000
sunset yellow	water	96	1	−0.91	632.8	0.598	22 400

[a] dn/dT. [b] κ data from ref 8.

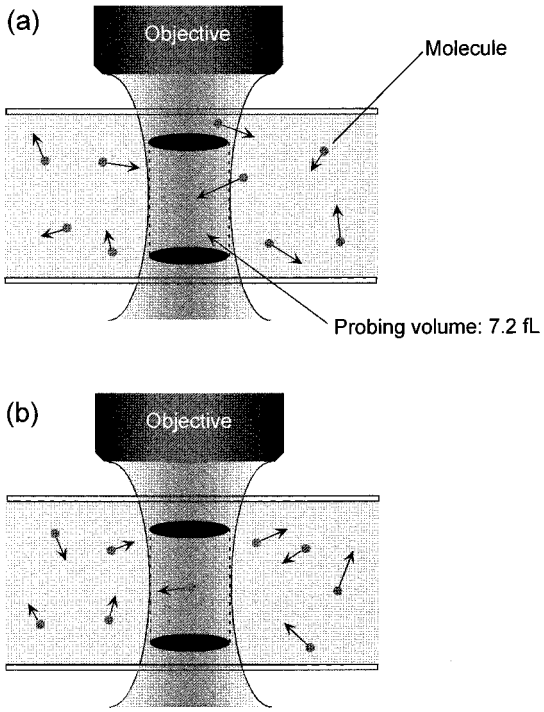

Figure 5. Illustrations showing molecule presence in the probing volume: when the probing volume contains no molecules (a) and one molecule (b). The number of molecules present in the probing volume changes with time.

We now discuss the physical meaning of the photothermal effect from the expected number of subsingle molecules. The behavior of the molecules in the probing volume is schematically illustrated in Figure 5. The number of molecules present in the probing volume rapidly changes with time because the molecules are violently moving in the liquid. There are two cases, i.e., the probing volume contains no molecules (Figure 5a) or one molecule (Figure 5b), for expectation of subsingle molecules. According to statistical theory, the probability distribution for the number of molecules present in the probing volume should be a Poisson distribution for such cases. From the normalized Poisson distribution, the probabilities for 0, 1, and 2 molecules in the probing region are 0.67, 0.27, and 0.054, respectively, when the expected molecule number is 0.42. Hence, in most cases, there are no molecules or only 1 molecule at any moment and there are hardly ever 2 or more molecules. The average time that a molecule goes across the probing volume is roughly estimated to be ~5 ms from Debye−Einstein−Stokes theory of the diffusion and the size of the probing volume. On the other hand, the time constant of the lock-in amplifier, which determines the representative value of the time scale of the measurement, was 4 s and it corresponds to the averaging time. The time scale of the probability event of the molecules coming into and going from the probing volume is, therefore, small enough compared to the time scale of the measurement, and this means that the event number is statistically sufficient. Therefore, the physical meaning of the expected molecule number of 0.42 is explained as being the time average of numerous events during a measuring time, i.e., the time constant of the lock-in amplifier (4 s).

We certainly measured the photothermal phenomenon from a single-molecule level as an expected molecule number, and this corresponded statistically to detection of a subsingle molecule. It was obvious from the above discussion that signal fluctuation of Figure 3 was not significant or resulting from a molecule's going in and out, but rather electric noises.

We estimated the average temperature rise in the probing volume by the photothermal effect for the single OEP molecule. The absorption cross section of the OEP molecule was obtained from the molar absorptivity, and the value was 5.3×10^{-17} cm^2. The photon flux was calculated to be 3.7×10^{23} photons$\cdot s^{-1} \cdot cm^{-2}$ from the optical power of the excitation beam and the spot size, 1.3 μm, in the confocal region. Thus, the number of photons absorbed per unit time was 19.6×10^6 photons$\cdot s^{-1}$. The time that it takes for heat to escape from the probing region was calculated by using the following equation,

$$t = l^2/D \qquad (2)$$

where l is the radius of the spot size, 0.65 μm, and D is the thermal diffusivity for benzene, 9.1×10^{-8} $m^2 \cdot s^{-1}$.[9] Then, the number of photons participating in the temperature rise was 90 photons. Therefore, the heat generated in the probing volume was 3.7×10^{-17} J, so the temperature rise was estimated to be 3.1 μK. Skogerboe and Yeung[28] applied conventional thermal lens spectroscopy to the determination of benzopurpurin 4B and achieved an absorbance limit of detection of 4×10^{-6} AU. Using the experimental parameters described in this paper, temperature rise could be estimated to be 0.5 μK. Considered from the standpoint of temperature rise, the estimated value of 3.1 μK is, therefore, sufficiently detectable. Bornhop et al.[29] reported temperature changes in a capillary tube could be measured at a level of 5.8×10^{-5} °C using refractive index measurements based on interferometric backscatter detection (IBD). The TLM is ~10 times superior in measuring temperature changes to the IBD.

The photothermal effect from a single molecule is different from that of the conventional thermal lens effect because the single molecule should work as a moving point heat source. The conventional thermal lens effect assumes that the molecules are

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homogeneously distributed in the solution and the intensity distribution of the excitation beam induces a temperature distribution, resulting in a refractive index distribution that acts as a lensing effect.[9] Thus, our experiment involves a momentary photothermal deflection by the moving point heat source rather than the ordinary thermal lens effect, as shown in one of our previous papers dealing with single-nanometer-sized particles.[21] Although further investigations are necessary, this is a very interesting photothermal effect of single molecules in liquid.

In summary, we could measure the photothermal signal from a subsingle molecule as an expected molecule number. The thermal lens microscope is a promising tool for ultrasensitive measurements in a microspace in liquids. For example, determination at a countable molecule level is very useful for cell biology, and it can be applied to ultrasensitive detection in a microchannel of an on-chip integrated chemical laboratory.[30−33] In the region of cubic micrometers (i.e., fL) at nanomolar concentration, the absolute amount becomes 10^{-24} mol, which means subsingle molecules.

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