Micro-scale temperature measurement method using fluorescence polarization

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Micro-scale temperature measurement method using fluorescence polarization

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Abstract. A novel method that can measure the fluid temperature in microscopic scale by measuring the fluorescence polarization is described in this paper. The measurement technique is not influenced by the quenching effects which appears in conventional LIF methods and is believed to show a higher reliability in temperature measurements. Experiment was performed using a microchannel flow and fluorescent molecule probes, and the effects of the fluid temperature, fluid viscosity, measurement time, and pH of the solution on the measured fluorescence polarization degree are discussed to understand the basic characteristics of the present method. The results showed that fluorescence polarization is considerably less sensible to these quenching factors. A good correlation with the fluid temperature, on the other hand, was obtained and agreed well with the theoretical values confirming the feasibility of the method.

1. Introduction

Microfluidic device are considered to be powerful tools in the fields of chemistry, biology and medicine in terms of enhancing the accuracy, sensitivity, and throughput and reducing the cost and time of the diagnoses and analysis. They are called lab-on-a-chip and micro total analysis system (μTAS). In order to develop and design these devices, measurement methods that can measure the fluid temperature in microchannels is an important issue to be solved. For example, precise temperature management with accuracy of 0.1°C is generally required to control the chemical and biological reaction occurring in the microchannel, represented by the process in the DNA hybridization [1].

The conventional methods employed to measure the fluid temperature in microchannels are the thermocouples, resistance temperature probe and laser induced fluorescence (LIF) method. Among these, thermocouples and resistance sensors are relatively reliable [2]. However, the spatial resolution is low (>10μm), and the location of the temperature measurement is fixed and the number of points are limited. The fabrication process of the wire patterning on the microchip, which is complicated and time consuming, is another problem. LIF, which is based on fluorescence intensity measurement of the fluorescent molecules solve in the fluid, is therefore often used since it can perform a noninvasive and two-dimensional measurement [3]. Nevertheless, there are issues which affects the accuracy of the measurement using LIF. One is the variation of the light intensity due to the light source and pathways. The other is the quenching effect.

Quenching effects [4][5][6] is a phenomena which decreases the fluorescence intensity and results from several reasons: temperature, oxygen, concentration and photobleaching. Temperature quenching is a type of excited-state reactions caused by encounters related to molecular rotational motion and the motions of quencher substance. Due to this quenching effect, the fluorescence intensity decreases with

temperature and the LIF measurement is based on this relationship. Oxygen plays an important role as quencher in the quenching process due to its high solubility in solutions. The collision between fluorescent molecules and oxygen forms an unilluminated substance and decreases the fluorescence. Therefore, fluorescence intensity decreases with oxygen concentration.

If the concentration of the fluorescent molecules are relatively high, concentration quenching or in other words, self-quenching takes place. This phenomenon is due to resonant transfer of energy from an excited molecule to an unexcited one, a transfer that makes spatial delocalization of the excitation. The increase of the absorption rate due to the high concentration will result in the elimination of excitation light and decreases of the fluorescence. Photobleaching is another significant phenomenon in terms of the fact that fluorescent molecules lose the ability of fluorescence emission when exposed to a strong excitation light. This results from the chemical damage of the molecular structure and is generally irreversible. The quenching effects mentioned here will affect the relation between the temperature and fluorescence intensity and deteriorate the performance of LIF method.

To tackle these problems, a novel fluid temperature measurement method based on the fluorescence polarization measurement is proposed in this study. As will be discussed in detail in Section 2, when the molecule is exposed to a polarized excitation light, a polarized fluorescence is emitted from the fluorescent molecule. Depolarization, a decrease of the polarization degree, takes place when the molecule experience the Brownian rotational motion in fluid. The degree of this depolarization depends mainly on the fluid viscosity, temperature and volume size of the molecule. Therefore, if the molecule size is fixed, the polarization degree of the fluorescence will depend on the fluid temperature and can be an index to measure the fluid temperature.

In the present study, the relation of the polarization degree of the fluorescent and fluid temperature is measured for microchannel flow. In addition to this, the relation of the fluid viscosity and polarization degree P is measured and compared with the theoretical values to validate the present method. Further, the effects of the fluid pH on the fluorescence polarization and intensity are measured under stationary fluid case to evaluate the measurement performance in the presence of quenching effect.

2. Measurement physics

The present method measures the polarization degree of the fluorescence emitted from the molecules. The fundamental principles of the measurement method will be described in this section.

Figure 1 shows the relationship between the polarization directions of the excitation light, fluorescence, and absorption moment of the fluorescent molecule. As shown in Fig. 1(a), when the fluorescent molecules are exposed to an excitation light linearly polarized (z axis in Fig. 1 (a)), the fluorescent molecules of which the absorption moment direction is parallel to the z axis are excited. The absorption degree of the molecule to the excitation light depends on the angle between the absorption moment and the polarization direction of the excitation light, θ. As θ increases, this absorption rate decreases in the rate of $\cos^2 \theta$. Therefore, if the direction of the absorption moment is parallel to the polarization direction of the excitation light (z direction), the absorption rate of the molecule becomes maximum. On the other hand, the molecule will not be excited if the direction of the absorption momentum is perpendicular to the excitation light (x and y directions).

The emitted fluorescence is also polarized in the same direction with the absorption moment. In other words, if an infinite number of fluorescent molecules randomly positioned are exposed to a linearly polarized excitation light, the molecules with the components of the absorption moment parallel to the excitation light are stochastically excited. The fluorescence observed from these molecules is, therefore, mainly polarized in the same direction with the excitation light.

When the molecules are suspended in the fluid, the rotation of the molecule due to the Brownian rotation significantly influences the polarization direction of the fluorescence in addition to the stochastic effects of the absorption moment. That is, since the molecule rotates during the period of the fluorescence excitation and emission, the polarization direction of the fluorescence shows larger

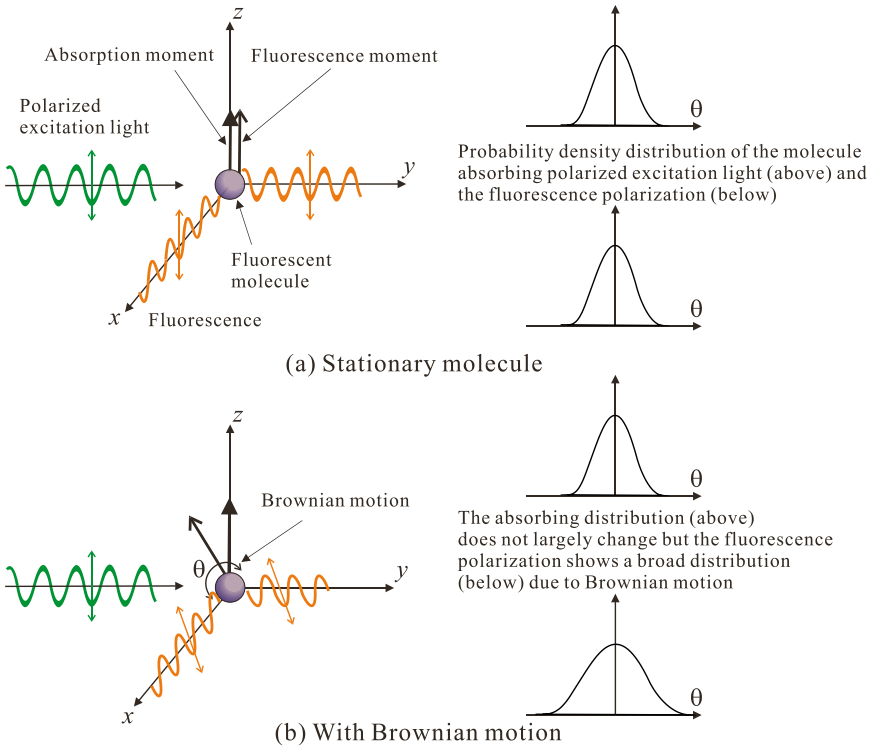


Figure 1. Schematic of the relationship between the polarized excitation light and the polarization degree of the fluorescence in the cases of the molecules in stationary state and the presence of Brownian motion. The probability density distributions show the probability of the directions of the absorption and fluorescence moments.

variance with the direction of the excitation light compared with those of fluorescent molecule in stationary condition (See Fig. 2(b)). This is the depolarization of the fluorescence.

To consider the relationship between the depolarization degree and other parameters, a variable called the polarization degree P is first defined as follows.

$$P = \frac{I_{//} - I_{\perp}}{I_{//} + I_{\perp}} \tag{1}$$

$I_{//}$ and $I_{\perp}$ present the fluorescence intensities of the components that are parallel and perpendicular to the polarization direction of the excitation light, respectively. Perrin [7] and Weber [8] have theoretically derived Eq. (2) to express the characteristic of P of a fluorescent molecule with Brownian motion.

$$\left(\frac{1}{P} - \frac{1}{3}\right) = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{k_B T}{\mu V}\tau\right) \tag{2}$$

P_0 is the polarization degree of the molecule in steady state (without rotation). k_B is the Boltzmann constant. μ and T are the viscosity and temperature of the fluid, respectively. τ and V are the fluorescence relaxation time and the volume of the molecule, respectively. As one can see in Eq. (2), the reciprocal number of P shows a linear relation with the T/μ. Therefore, if the molecular volume remains constant during the measurement, the fluid temperature can be obtained by measuring the polarization degree of the fluorescence. The present measurement is based on this physics.

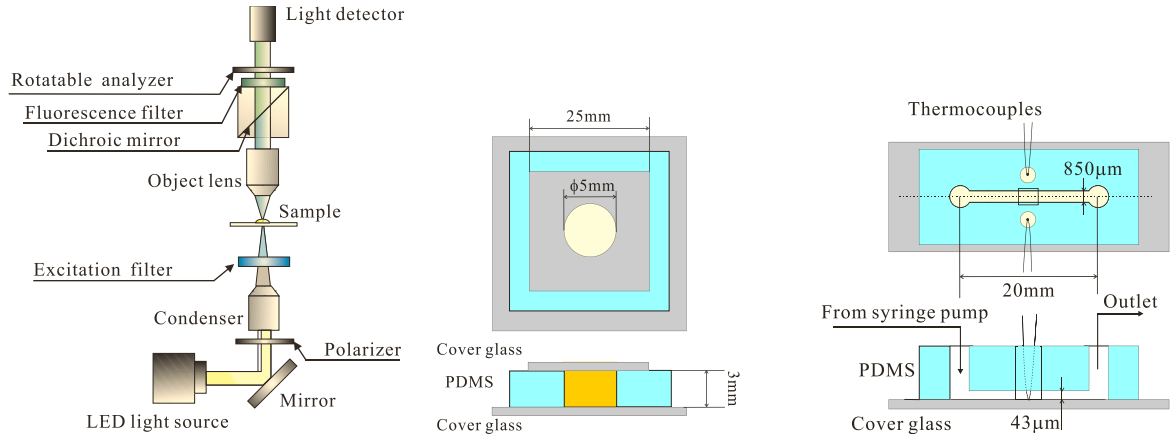
The total fluorescence intensity I can be influenced by various factors. For example, I can be presented by Eq. (3).

$$I = I_0 c \phi \varepsilon \tag{3}$$

I_0 is the intensity of the excitation light and c is the concentration of the florescent molecules. ϕ is the quantum efficiency, and ε is the absorption coefficient presenting the rate that the excitation light is absorbed while it passes through a unit length of solution.

As mentioned in Section 1, the quenching effect occurs attributed to various reasons. The change of the fluid temperature also produces a change in the vibration motion of the molecule, hence, the fluorescence intensity. Fluid temperature measurement using the laser induced fluorescence (LIF) method employs this relationship between the fluid temperature and fluorescence intensity. On the other hand, this means that the measurement performance can be influenced by other quenching effects and the disturbance of the light intensity at the light source and optical path.

The present method measures the polarization degree, P. As shown in Eq. (1), P is a value normalized by the total fluorescence intensity $I_{//}+I_{\perp}$. P is, therefore, not influenced by the variation of the fluorescence intensity. This indicates that the previously mentioned quenching effects can be neglected in this method.



(a) Optical path of measurement (b) reservoir (stationary fluid) (c) microchannel

Figure 2. Schematics of the (a) optical path in the microscope, (b) cylindrical reservoir with stationary fluid used to measure the pH, and (c) straight microchannel used to measure the fluid viscosity and temperature effects.

3. Measurement method

Measurement was conducted using an uplight optical microscope (Olympus, BX51), LED (Thorlabs, M490L3) as the light source, and photomultiplier (Hamamatsu Photonics, H5783-03) as the detector. Figure 2(a) shows the schematic of the optical pathway. The light from the LED passes the condenser with polarizer (Olympus, U-POC-2), fluorescent excitation filter (Semrock, FF01-482/35-25) and objective lens (Olympus, LMPlanFLN) and illuminates the fluid in the microchannel. The fluorescence is measured through the fluorescence filter (Semrock, FF01-536/40-25) and analyzer (Olympus, U-AN360P-2).

Measurement of the fluid pH effects on polarization was carried out under the stationary fluid condition using a PDMS reservoir. Measurements of the fluid viscosity and temperature effects on the polarization were performed using the microchannel flow. Figure 2(b) shows the schematic of the

reservoir. The reservoir was made of PDMS(poly-dimethylsiloxane) with cover glasses sealing the top and bottom sides. The diameter and height of the reservoir are 5mm and 3mm, respectively

Figure 2(c) shows the schematic of the microchannel. The channel is a straight one with the height of 43μm and width of 850μm. The microchannel was fabricated by PDMS using the standard photolithography and soft lithography. The channel pattern was first printed on the photomask using the laser lithography system. The pattern was then transferred to SU-8 (MicroChem Inc.) spin coated on silicon wafers using photoresists and lithography systems, and was applied as the mold in the soft lithograph. PDMS was first degassing by decompression and then casted using the SU-8 mold. Holes were cored at the inlet and outlet of the channel with biopsy puncher. The PDMS was then attached to the cover glass. To control the fluid temperature, the microchannel was placed on the temperature controlled stage. Thermocouples were attached to the cover glass at the locations 1mm aside of the channel and are referred as channel temperature.

Two fluorescent molecules are employed in the measurement. One is the fluorescein isothiocyanate (FITC: Nacalai Tesque, #V9E9889) and the other is casein-FITC conjugated (C-FITC: AAT Bioquest, #13440). C-FITC is a casein which is labeled by several FITCs. The molecular weight MW of FITC and C-FITC are different and are MW=389 and 26000, respectively. Therefore, the size of the molecules are approximately 1nm and 10nm, respectively. As shown in Eq. (2), the polarization degree P of the fluorescence emitted from the molecule relies on the temperature, the fluid viscosity and the volume of the molecule. Larger volume implies larger P leading to increase of the S/N ratio of the measurement. Further, the gradient of P against temperature T increases under larger P conditions showing that a higher sensitivity and accuracy of temperature measurement can be obtained with larger P. Therefore, C-FITC with larger MW (volume) compared with FITC was examined in this study.

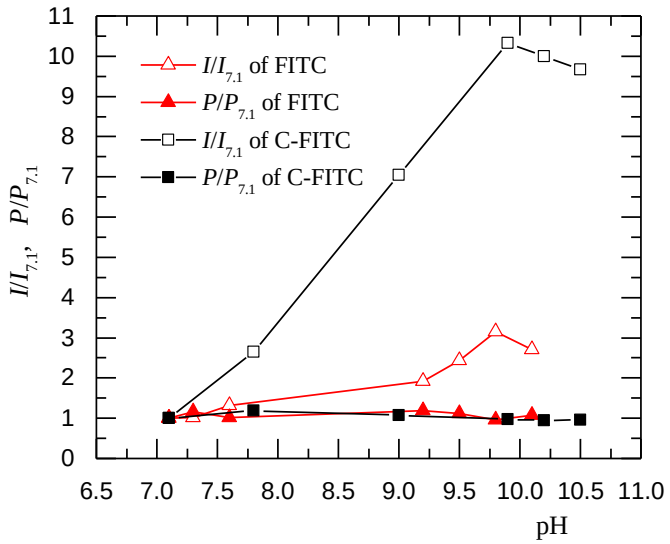


Figure 3. pH effects on fluorescence intensity I and polarization degree P in the cases of FITC and C-FITC solutions. I and P are normalized by the values of pH=7.1.

4. Results and discussion

4.1. Fluid pH effects

Fluorescent molecules are strongly pH dependent [9] and the effect of the fluid pH on the fluorescent intensity and polarization are evaluated in this study. The pH of the solution was controlled my adding NaOH to the solution following the next procedure. A solution of 0.75 mol/L NaOH was prepared initially and added to Millipore ultrapure water to obtain the target pH value. The sample solutions of casein-FITC with different pH were prepared at a concentration of 0.01wt% using the pH controlled

ultrapure water as solvent. For the FITC solution, 0.001wt% FITC of the same aqueous solution was prepared.

Figure 3 shows the effects of the pH on the fluorescence intensity $I=I_{\parallel}+2I_{\perp}$ and the fluorescence polarization P. The results are normalized by the values of pH=7.1, respectively. $I/I_{7.1}$ takes the maximum peak approximately at pH=10, which agrees well with the results of Obare [9]. The maximum peak shows 3 and 10 times larger values than those of pH=7.1 in the cases of FITC and C-FITC, respectively. This indicates that quenching by the pH will significantly affect the measurement accuracy.

On the other hand, the variation of $P/P_{7.1}$ in the cases of FITC and C-FITC are both less than 20%. These values are much smaller than those of $I/I_{7.1}$. This shows that the fluorescence polarization is less affected by the pH conditions and high reliability and accuracy of temperature measurement can be performed applying the measurement.

4.2. Fluid viscosity effects

Fluid viscosity effects on the fluorescence polarization is discussed in this section to compare with the theoretical value and evaluate the effective fluorescent molecule for the measurement. To control the viscosity of the fluid glycerol (Nacalai Tesque, #17018-25) was mixed with the solution.

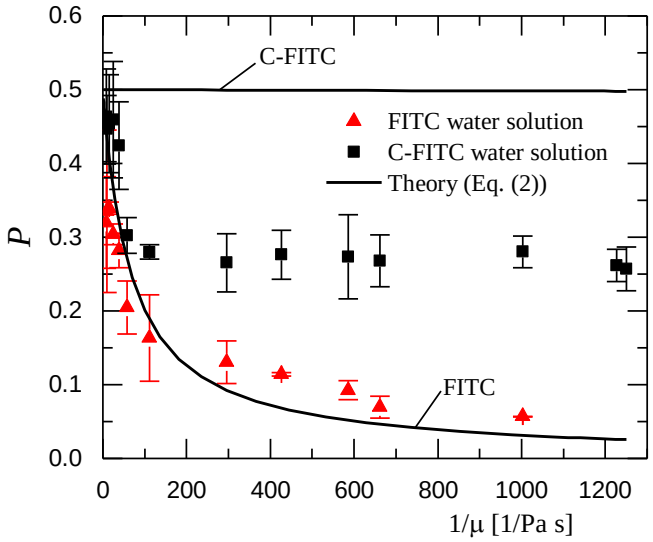


Figure 4. Fluid viscosity μ effects on P in the cases of FITC and C-FITC solutions. The solid lines presents the theoretical values obtained by Eq. (2) applying the values shown in Table 2.

Table 1. Properties applied to Eq. (2) to calculate P of FITC and C-FITC.

properties	**FITC**	**C-FITC (casein-FITC conjugated)**
P_0	0.5	0.5
κ_B	1.38×10^{-23} J/K	1.38×10^{-23} J/K
T	303K	303K
τ	4ns	4ns
d	1.08 nm	10 nm

Figure 4 shows the relation of P and the fluid viscosity μ in the case of FITC dissolved in water. The theoretical values derived from Eq. (2) are shown by the solid line in the figure for comparison. This

line is calculated applying the condition shown in Table 1, in which the fluorescence lifetime and diameter, τ and d_{FITC}, in the case of FITC referred to Magde [10] and Fowlkes [11] are shown.

In Fig. 4, the polarization degree P of the FITC decreases as the solution viscosity decreases. As the fluid viscosity decreases, the Brownian motion of fluorescent molecules become more active and the direction of the absorption moment vector become random. This leads to larger depolarization. In this case, $1/P$ and $1/\mu$ show a linear relation as presented in Eq. (2). Comparing the experimental results with the theoretical ones, the two values agree reasonably well showing the validity of the present measurement apparatus.

Together with FITC case, the results of fluorescence polarization in the case of casein-FITC are shown in Fig. 4. Same as the FITC case, the solid lines present the theoretical values obtained from Eq. (2) and Table 1, in which the diameter refers to Dalgleish [12]. It should be noted that several assumptions were made to apply this equation to the casein-FITC-conjugated molecules. One is that the FITC molecules are attached to the casein with random position and orientation. The other is that the FITC molecules are fixed to the casein and will not move and change its relative direction to the casein. In this case, the fluorescence polarization is related to the rotation of casein and is solved by Eq. (2).

Compared with the FITC case, the theoretical value shows that the depolarization of the casein-FITC due to Brownian motion is negligible and P remains nearly constant against the fluid viscosity $1/\mu$. This is attributed to the large diameter of the casein. The measurement, however, shows a noticeable depolarization in the region approximately at $1/\mu$=50. Although not shown here, the particle diameter was measured based on dynamic light scattering method (Otsuka electronics, ELSZ-2plus). The results showed that the diameter largely decreases at approximately $1/\mu$=50. Further, the fluorescence intensity increased markedly in the region of $1/\mu$>50. These results imply the possibility that the FITC (or a part of the casein cluster) has been detached from the casein at this condition. In this case, by having the FITC, which has been concentrated on the casein, detached from the casein and apparently diluted in the solution, the concentration quenching effect can be reduced and larger fluorescence intensity was obtained. In addition to this, the separated molecule or cluster possess a smaller volume which decreases the polarization degree.

Under the fluid viscosity condition of water, $1/\mu \cong 1000\sim1200$, P of C-FITC case shows a larger value compared with those of the FITC case. This indicates that measurement of higher reliability and sensitivity can be performed by using the C-FITC as the fluorescence probe. The temperature measurement discussed in the next section was conducted using the water solvent, and therefore, C-FITC was used as the fluorescent molecule.

4.3. Fluid temperature effects

Results of temperature effects on fluorescence polarization are shown in Fig. 5. Measurement was performed in the temperature range of 28 ~ 38°C, which is a reasonable range considering the temperature applied to the biochemical assay. The symbols plotted in the figure are the average values of data measured for several times. The line drawn in the figure was derived on the basis of the least square mean approximation using all measured data.

As shown in Fig. 5, the reciprocal value of fluorescence polarization $1/P$ increases linearly against fluid temperature T. This agrees well the Perrin's equation (Eq. (2)).

The empirical relationship between $1/P$ and T in the temperature range shown in Fig. 5 is presented as Eq. (4).

$$T=\frac{\frac{1}{P}-6.41}{0.139} \tag{4}$$

The sensitivity and accuracy of the present method was therefore 1.26%/°C and ±1.23°C, respectively. These results confirms the reliability and feasibility of applying the fluorescence polarization measurement to measurement of the fluid temperature.

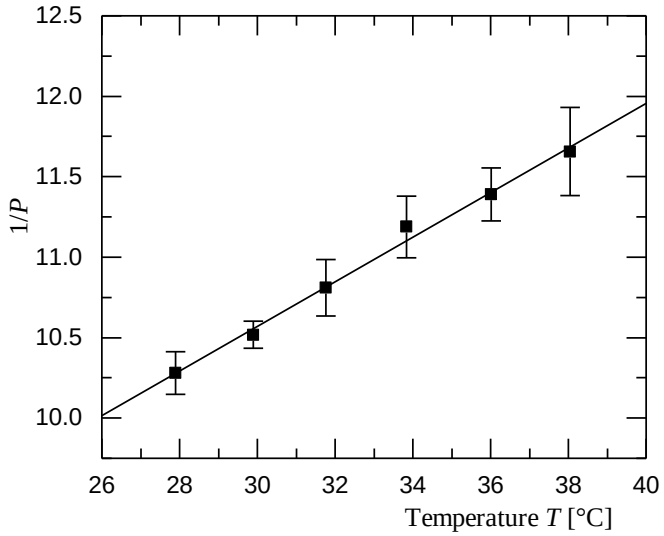


Figure 5. Relation of fluid temperature *T* and reciprocal value of *P* in the cases of C-FITC solutions. The solid line is based on the least mean square approximation.

5. Conclusion

Measurement was performed to study the feasibility and accuracy of the fluid temperature method using the fluorescence polarization *P* measurement. pH quenching effects varied *P* by approximately 20%, which was largely reduced compared with the measurement of the fluorescent intensities the variation of which was 300~1000%. Measurement of the fluid viscosity effects showed that the casein-FITC-conjugated probe provides high performance in the polarization measurement. Using the casein-FITC fluorescent molecules, the reciprocal value of *P* showed a good linear relationship with the fluid temperature with the sensitivity and accuracy of 1.26%/°C and ±1.23°C. The results agreed well with the theory and confirmed the reliability and feasibility of the present method.

Acknowledgments

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