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Application News

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Spectrophotometric Analysis

Evaluation of Fluorescence Anisotropy in Fluorescein and Rhodamine B Solutions

The evaluation of fluorescence anisotropy can be used for research into physical and chemical molecular properties, for example, as a method of investigating the electronic structure and solvent-solute interactions of small molecules as well as the conformation and dynamics of natural and synthetic macromolecules [1].

This article introduces the results of investigating the changes in fluorescence anisotropy of fluorescein solution and rhodamine B solution while varying the temperature of these solutions.

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Fluorescence Anisotropy

Measurement of fluorescence anisotropy in solution is published in the referenced IUPAC Technical Report [1]. The basic relations defined in this report are given below.

Fluorescence anisotropy (r) is generally defined by the following equation.

$$r = (I_{VV} - I_{VH}) / (I_{VV} + 2I_{VH}) \quad (1)$$

where I_{VV} is the intensity measured with the excitation polarizer and emission polarizer in the vertical position (V), and I_{VH} is the intensity measured with the excitation polarizer in the vertical position (V) and the emission polarizer in the horizontal position (H). The denominator is the total fluorescence intensity and the numerator is the degree of polarization.

The fluorescence polarization ratio (p) is generally defined by the following equation.

$$p = (I_{VV} - I_{VH}) / (I_{VV} + I_{VH}) \quad (2)$$

The relationship between fluorescence anisotropy and fluorescence polarization ratio is given by the following expression.

$$r = 2p / (3 - p) \quad (3)$$

Generally, the fluorescence anisotropy is expressed using the angle α between the absorption transition moment upon molecular excitation and the emission transition moment upon fluorescence emission, in the following way.

$$r = 0.2(3\cos^2\alpha - 1) \quad (4)$$

Equation (4) shows that the fluorescence anisotropy will be in the range of $-0.2 \le r \le 0.4$ (and decreases due to molecular rotation in the time between absorption and emission).

Note that in actual measurement, sensitivity differences between the polarized components (vertical and horizontal directions) of the light emitted from the instrument must be corrected. The G-factor, which is defined by the following equation, is used for correction.

$$G = I_{HV} / I_{HH} \quad (5)$$

In actual measurement, the fluorescence anisotropy (r_{exp}) is calculated using the following equation which incorporates the G-factor into equation (1).

$$r_{exp} = (I_{VV} - GI_{VH}) / (I_{VV} + 2GI_{VH}) \quad (6)$$

Temperature Changes and Fluorescence Anisotropy

Fluorescein was dissolved into glycerol solution (glycerin and pure water prepared to a 95:5 ratio) to obtain a concentration of 0.2 mg/L. We determined the optimal excitation wavelength using 3D spectrum measurement, and performed measurement while varying the solution temperature under the conditions listed in Table 1. Figs. 1 and 2 and Table 2 show the measurement results.

As the temperature rises, energy dissipation, internal conversion, and intersystem crossing due to collisions between molecules occur more readily leading to a decrease in fluorescence intensity (temperature quenching) [2]. Fig. 1 illustrates the decrease in fluorescence intensity as the temperature rises.

Table 1 Measurement Conditions

Instrument used	: RF-6000, polarized light measurement attachment, constant-temperature single cell holder
Excitation wavelength	: 497 nm (fluorescein solution) 555 nm (rhodamine B solution)
Fluorescence wavelength range	: 505 nm to 650 nm (fluorescein solution) 565 nm to 700 nm (rhodamine B solution)
Scan speed	: 600 nm/min
Sampling interval	: 1.0 nm
Bandwidth	: Ex 3.0 nm/Em 5.0 nm
Sensitivity	: Low

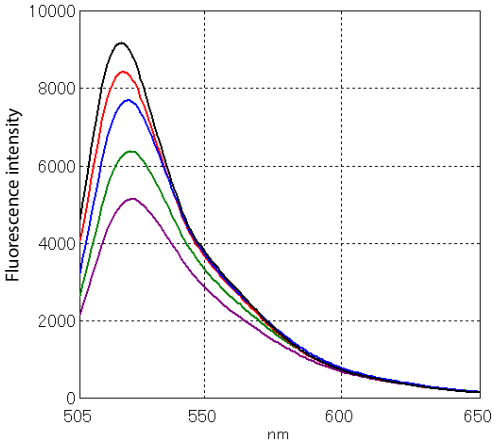

Fig. 1 Fluorescence Spectra of Fluorescein Solution (Without Polarizer)
Black: 10 °C, Red: Room Temperature, Blue: 40 °C, Green: 60 °C, Purple: 78 °C

As shown by Fig. 2, the intensities required to calculate the fluorescence anisotropy were measured through the combination of excitation and emission polarizers. We can see that the sensitivity of the horizontal component is higher on the instrument used.

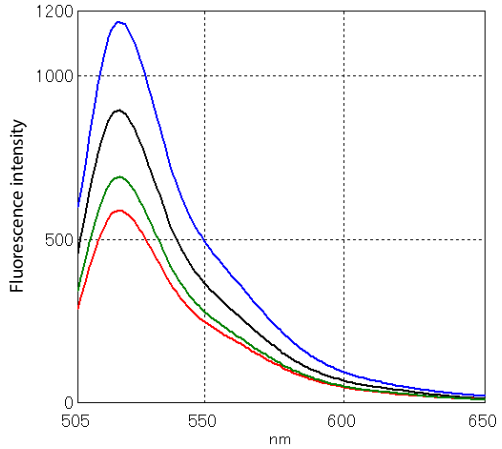

Fig. 2 Polarization Spectra of Fluorescein Solution (10 °C)
Black: I_{VV}, Red: I_{VH}, Blue: I_{HH}, Green: I_{HV}

Table 2 Fluorescence Anisotropy Values and Fluorescence Polarization Ratios of Fluorescein Solution at Various Temperatures

Temperature	Fluorescence wavelength	I_{VV}	I_{VH}	G	r	p
10 °C	520 nm	931.4	588.2	0.59	0.36	0.457
Room temperature	521 nm	804.6	525.2	0.59	0.35	0.444
40 °C	522 nm	644.8	501.4	0.59	0.28	0.371
60 °C	523 nm	450.7	437.9	0.59	0.20	0.271
78 °C	524 nm	304.1	359.3	0.59	0.13	0.178

Table 2 shows the decrease in fluorescence anisotropy as the temperature rises. The three causes of depolarization are generally considered to be intramolecular relaxation, rotational Brownian motion, and intermolecular excitation energy transfer [3]. Intermolecular excitation energy transfer does not need to be taken into account for this sample because fluorescein is the only fluorescent molecule in the solution. Furthermore, since intramolecular relaxation is not really affected by temperature changes, we can infer that the decrease in fluorescence anisotropy is occurring due to the increased rotational Brownian motion as the temperature rises.

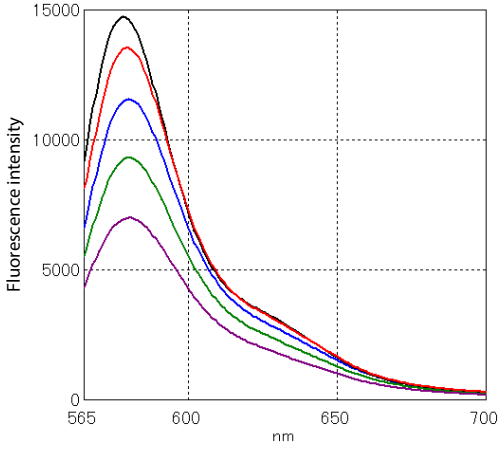

Fig. 3 Fluorescence Spectra of Rhodamine B Solution (Without Polarizer)
Black: 10 °C, Red: Room Temperature, Blue: 40 °C, Green: 60 °C, Purple: 78 °C

Next, rhodamine B was dissolved into glycerol solution (glycerin and pure water containing 0.5 mM NaOH prepared to a 95:5 ratio) to obtain a concentration of 0.2 mg/L. We determined the optimal excitation wavelength using 3D spectrum measurement, and performed measurement while varying the solution temperature under the conditions listed in Table 1. Figs. 3 and 4 and Table 3 show the measurement results.

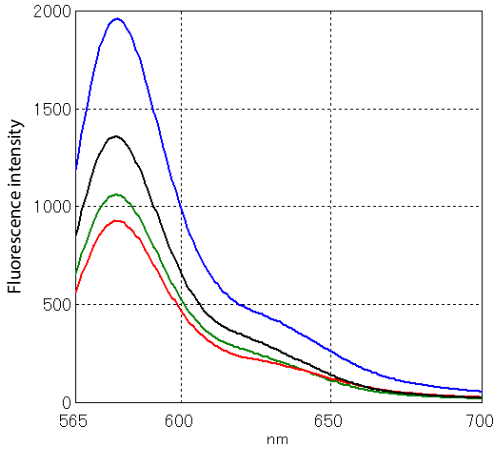

Fig. 4 Polarization Spectra of Rhodamine B Solution (10 °C)
Black: I_{VV}, Red: I_{VH}, Blue: I_{HH}, Green: I_{HV}

Table 3 Fluorescence Anisotropy Values and Fluorescence Polarization Ratios of Rhodamine B Solution at Various Temperatures

Temperature	Fluorescence wavelength	I_{VV}	I_{VH}	G	r	p
10 °C	578 nm	1359.8	928.2	0.54	0.36	0.461
Room temperature	579 nm	1244.3	885.4	0.54	0.35	0.445
40 °C	580 nm	987.1	764.5	0.54	0.32	0.410
60 °C	580 nm	710.5	627.6	0.54	0.27	0.354
78 °C	580 nm	489.3	481.2	0.54	0.23	0.306

As with the fluorescein solution, the rhodamine B solution also showed a decrease in fluorescence intensity and fluorescence anisotropy as the temperature rises. We can again infer that the decrease in fluorescence anisotropy is caused by the increased rotational Brownian motion as the temperature rises, which is consistent with the fluorescein solution.

Conclusion

We measured the fluorescence anisotropy of fluorescein solution and rhodamine B solution while varying the temperature of these solutions. We confirmed that decreases in fluorescence intensity and fluorescence anisotropy coincide with temperature increases. The decrease in fluorescence anisotropy is most likely caused by the increased rotational Brownian motion as the temperature rises. This demonstrates that measuring fluorescence anisotropy helps to investigate information related to physical and chemical molecular properties.

References

1) Marcel Ameloot, Martin vandeVen, A. Ulises Acuna, and Bernard Valeur. Fluorescence anisotropy measurements in solution: Methods and reference materials (IUPAC Technical Report). *Pure Appl. Chem.*, 2013, vol. 85, No. 3, p. 589-608
2) Yasuharu Nishikawa, Keizo Hiraki, "Fluorescence and Phosphorescence Spectrometry", Kyoritsu Shuppan (in Japanese)
3) Kazuhiko Kinoshita, Koushin Mihashi (Editor), "Fluorometry", Gakkai Publishing Center (in Japanese)

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