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Site-Dependent Fluorescence Decay of Malachite Green Doped in Onion Cell

Hiroki NAKATSUKA*, Masaya HIRAI, Shunji SEKINE, Yuji SUZUKI and Toshiaki HATTORI
Institute of Applied Physics, University of Tsukuba, Tsukuba, Ibaraki 305-8573, Japan

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Time-resolved fluorescence measurements of malachite green dye molecules doped in onion cells were carried out. The fluorescence decay time was dependent on the individual cell and on the position of the dye in a cell, which reflect the microscopic dynamics of each bound site. Upon cooling, the decay time increased and this increase was accelerated at around the freezing point of the onion cell.

KEYWORDS: fluorescence decay, fluorescent microprobe, malachite green, microscopic viscosity, onion cell

Malachite green dye molecules (MG), a kind of triphenyl methane dye, are known for their very rapid nonradiative relaxation from the optically excited state S_1 to the ground state S_0. In MG the central carbon is joined by three phenyl rings (Fig. 1), and the excited-state dynamics is strongly influenced by the microscopic viscosity of the local environment. The fluorescence decay time varies with the solvent viscosity from picoseconds to nanoseconds at room temperature. The viscosity-dependent nonradiative process is thought to be due to diffusive rotational motion of the phenyl rings of MG around their axes, which causes the internal conversion from the excited state to the ground state.[1–3] The internal potential of the excited state of the rotational motion of the phenyl rings of MG has been found to be barrierless.[3,4] It renders the dye a very sensitive fluorescent microprobe since the friction provided by the surrounding molecules is the only impediment to the rotational motion of the phenyl rings. The fluorescence decay time of MG is essentially determined by the microscopic dynamics of the local environment. Kemnitz and Yoshihara explained the site-dependent fluorescence decay of MG adsorbed on a quartz plate, by using the free-volume effect.[5] In our previous work MG microprobes were used to investigate the microscopic dynamics of the glass transition of various organic glasses.[6,7]

The application of the fluorescent microprobe to biological objects is of interest, because fluorescence measurement is very sensitive and noninvasive. Single-photon counting is now a widely used technology. Moreover, by using an optical microscope, we can observe the microscopic dynamics of each cell or even the position-dependent microscopic dynamics within a cell.

For staining onion cells with MG, we prepared a special solvent. If onion cells are dipped in an aqueous solution of MG for staining, they take up water and swell because of the difference in osmotic pressure between the cell and the aqueous solution. The friction applied to the phenyl ring rotation of MG in the cell is changed by the swelling. In order to prevent this effect, we obtained a solvent from an onion by squeezing. The osmotic pressure of the solvent can be considered to be equal to that of onion cells. We first dissolved MG (obtained from Excition) in the solvent (1.2×10^{-5} M), and a sheet of onion cells was immersed in the solution for 2 hours. Then the extra solution on the surface of the onion sheet was wiped off with paper tissue. In order to prevent drying, it was sandwiched between two glass plates. A photograph of stained onion cells is shown in Fig. 2(a). The nucleus and the cell wall are preferentially stained. This implies that MG is not dissolved in the water in a cell but is preferentially bound to large organic molecules in the cell such as DNA and/or proteins.

The experimental setup for fluorescence measurement is shown in Fig. 3. The excitation light source is a cw mode-locked dye laser (Coherent, Satori) whose pulse repetition rate is 76 MHz. The output from the dye laser with 300 fs pulse width at 640 nm wavelength was attenuated to 0.1 mW and focussed on the onion cell by an optical microscope. The red spot in Fig. 2(b) shows the focussed laser beam on the onion cell. By monitoring this spot using the microscope, we can move the position of focus over the sheet of onion cells. The emitted fluorescence was wavelength-resolved using a spectrometer and time-resolved using a synchroscan streak camera (Hamamatsu Photonics, model M1955). The time resolution of the whole apparatus was about 25 ps. No wavelength dependence of the fluorescence decay was observed; the observation wavelength window was set to be from 670 nm to 690 nm.

Figure 4 shows typical fluorescence decay curves of MG doped in the onion cell at room temperature, where the excitation laser beam was focussed on the nucleus (closed circles) and on the wall (open circles) of the onion cell. The decay curves cannot be fitted with single-exponential functions, but can be well fitted with biexponential functions $I(t) = A_\mathrm{f} \exp(-t/\tau_\mathrm{f}) + A_\mathrm{s} \exp(-t/\tau_\mathrm{s})$ with two time constants τ_f and τ_s, and amplitudes A_f and A_s.[3–7] The fitted curves are shown in Fig. 4. The obtained fast and slow decay times, τ_f and τ_s, and the amplitude ratio, $A_\mathrm{f}/A_\mathrm{s}$, vary slightly from one cell to another, but there are clear differences between the nucleus and the wall of the onion cell, as shown in Table I. The averages were taken over 10 different cells in an onion sheet for both the nucleus and the wall. The noteworthy point here is that even the fast decay time, $\tau_\mathrm{f} = 43$ ps, for the wall is much longer than the fluorescence decay time of MG in pure water, less than a few picoseconds.[3,8] This strongly suggests that MG is not dissolved in water in the onion cell, but binds to some biological substrate.[9,10] This binding results in a substantial decrease in the rate of phenyl-ring rotation. Although we cannot identify the bound site at present, the biexponential nature of the fluorescence decay indicates that there are two kinds of bound sites for MG: a weakly bound site and a strongly bound site. Recently the site-sensitive decay of crystal violet molecules, another triphenyl methane dye, was clearly observed by Ye et al. using single-molecule spectroscopy.[11] Although the slow decay time τ_s is not significantly different between the nu-

*E-mail address: nakatsuk@bk.tsukuba.ac.jp

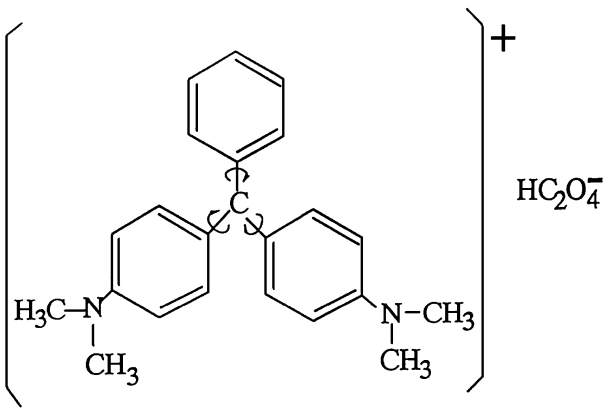

Fig. 1. Molecular structure of MG.

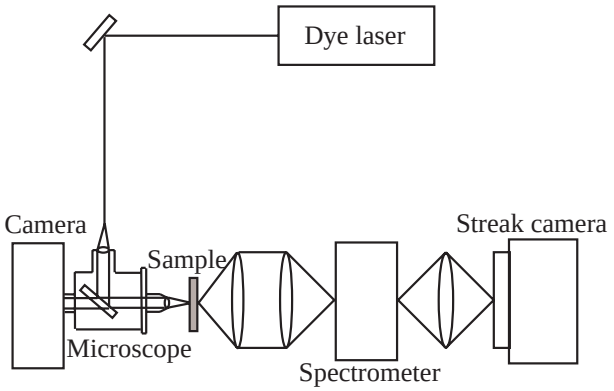

Fig. 3. Experimental setup for fluorescence measurement.

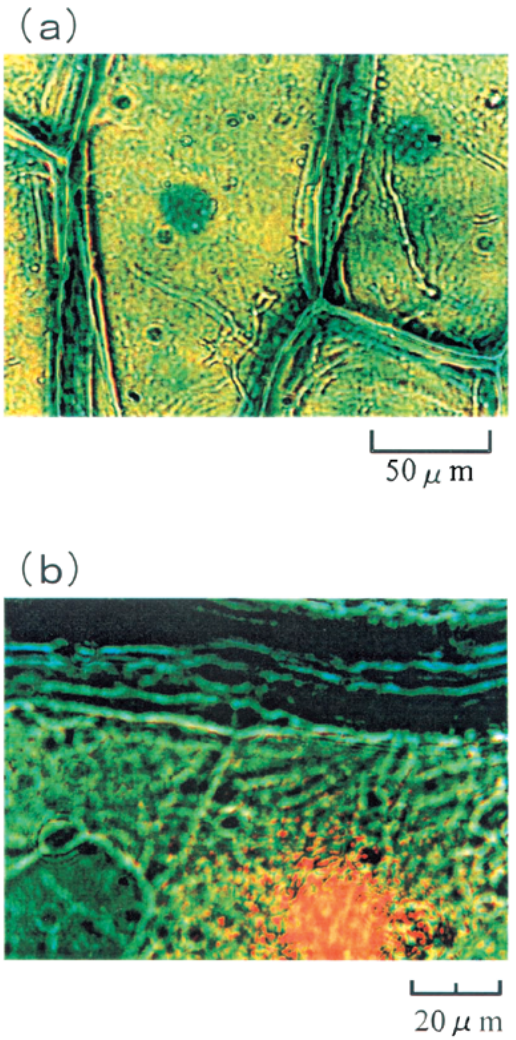

Fig. 2. (a) A photograph of onion cells stained with MG. (b) A photograph of a stained onion cell where the red spot shows the focussed laser beam.

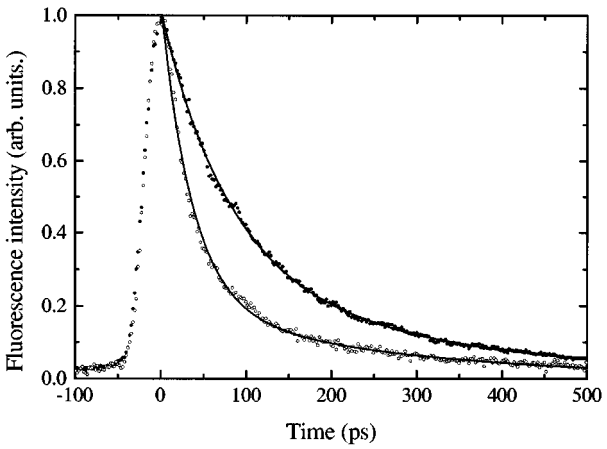

Fig. 4. Typical fluorescence decay curves of MG doped in the onion cell at room temperature. Closed and open circles represent the values for the nucleus and wall, respectively, and the solid curves are the biexponentially fitted curves.

Table I. Measured fluorescence decay times and the amplitude ratio of MG doped in the onion cell.

	Nucleus		Wall	
	Average value	Standard deviation	Average value	Standard deviation
τ_f (ps)	70	10	40	10
τ_s (ps)	200	20	300	40
A_f/A_s	1.5	0.3	4.0	1.0

cleus and the wall, the fast decay time τ_f and the amplitude ratio A_f/A_s are considerably different. This means that the wall has more weakly binding sites for MG than the nucleus.

In order to see whether MG is dissolved in water or binds to some organic substrate of the onion cell, we measured the temperature dependence of the fluorescence decay time of the doped MG. The sample onion cells sandwiched between two glass plates were mounted in a cryostat (Edwards: C91140). They were protected from drying by sealing off the plate edges with silicon rubber. In this case the excitation laser beam could not be focussed to the size smaller than a cell because of the long distance between the sample and the outside window of the cryostat, therefore more than ten onion cells were irradiated simultaneously, and the measured fluorescence was composed of that from the nucleus and from the wall of the onion cell. Figure 5(a) shows the temperature dependence of the fast and slow fluorescence decay times, τ_f and τ_s, and the amplitude ratio, A_f/A_s, of MG in the onion cell. For comparison, the fluorescence decay of MG dissolved in the solvent (1.2×10^{-5} M) which was squeezed from an onion is also shown in Fig. 5(b). In both cases, τ_f is almost

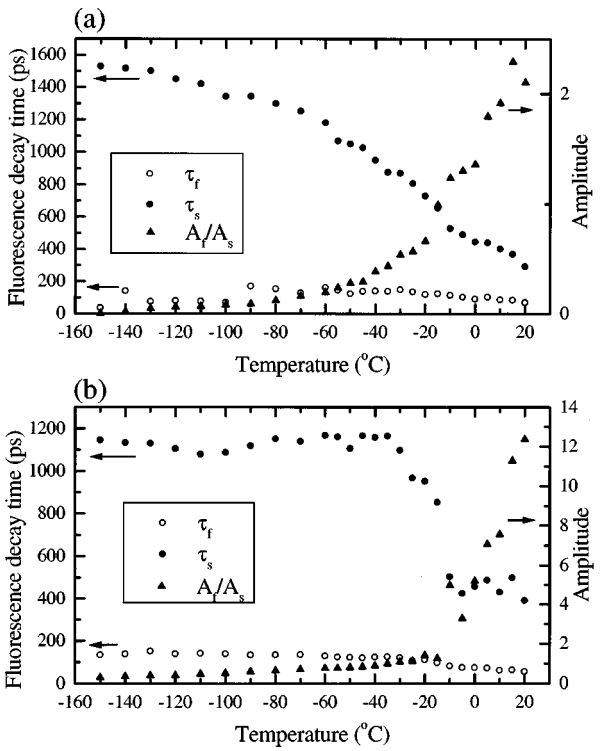

Fig. 5. Temperature dependence of the fast and slow fluorescence decay times, τ_f and τ_s, and the amplitude ratio, A_f/A_s, of MG in the onion cell (a), and in the squeezed solvent (b).

independent of temperature all through the temperature, but τ_s and A_f/A_s change greatly at around -15°C which corresponds to the freezing point of the onion cell. The temperature dependence of the heat capacity measured with a differential scanning calorimeter (Seiko Instruments lnc. SSC5200H) was similar for both the onion cell and the squeezed solvent (Fig. 6), and it shows a sharp peak at around the freezing point -15°C.

Upon cooling, τ_s increases because the strongly bound site of MG becomes more strongly bound and the friction at the site increases. Although the origin of the temperature-independent nature of τ_f is not clear, the temperature dependence of A_f/A_s may be explained if the free energy of the strongly bound site is smaller than that of the weakly bound site. The effect of freezing is much more marked for the squeezed solvent where DNA or protein molecules are directly attached to water molecules. On the other hand, in the case of the onion cell, MG binds to an organic substrate and is more or less shielded from the effect of freezing of the outside water molecules.

In the case of MG dissolved in pure water, above the freezing temperature, the fluorescence decay time was much shorter than the time resolution, 25 ps, of the present experimental system, and when frozen, MG molecules were pushed

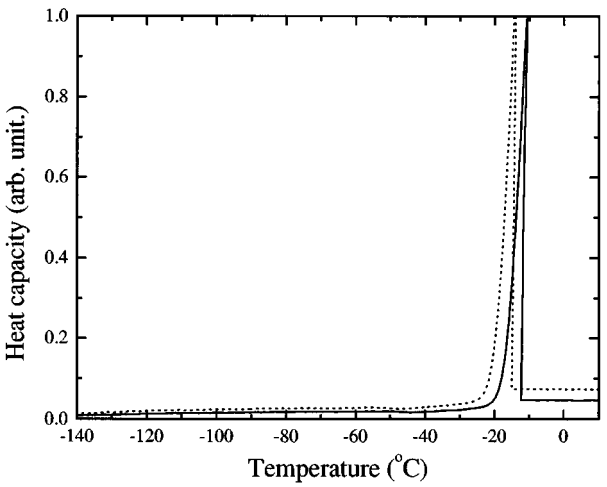

Fig. 6. Temperature dependence of heat capacities of the onion cell (solid curve) and the squeezed solvent (dotted curve).

away from the crystal structure of ice and aggregated at the crystal domain boundaries and did not fluoresce upon laser excitation.

All these results suggest that MG binds to large organic molecules within the cell, such as DNA and/or proteins. Therefore the fluorescence decay reflects the microscopic dynamics at the bound site. We can observe the microscopic dynamics at the site by distinguishing one cell from another or even by identifying its position in a cell.

We measured the fluorescence decay time of MG doped in an onion cell by distinguishing one cell from another or even by identifying position in a cell. The decay time reflects the microscopic dynamics at the bound site. The application of a fluorescent microprobe to biological objects is of interest, because fluorescence measurement is very sensitive and non-invasive. If this method is combined with the use of optical fibers, it may serve as a future medical diagnostic tool.

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