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Ultrafast transient absorption spectra of photoexcited YOYO-1 molecules call for additional investigations of their fluorescence quenching mechanism

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ABSTRACT

In this report, we observed that YOYO-1 immobilized on a glass surface is much brighter when dried (quantum yield 16 ± 4% in the ambient air) or in hexane than in water (quantum yield ~0%). YOYO-1 is a typical cyanine dye that has a photo-isomerization reaction upon light illumination. In order to understand this quenching mechanism, we use femtosecond transient absorption spectroscopy to measure YOYO-1's electron dynamics after excitation directly. By deconvoluting the hot-ground-state absorption and the stimulated emission, the dynamics of electronic relaxation and balance are revealed. The results support the intermolecular charge transfer mechanism better than the intramolecular relaxation mechanism that has been widely believed before. We believe that the first step of the relaxation involves a Dexter charge transfer between the photo-excited YOYO-1 molecule and another guest molecule that is directly bound to the YOYO-1 giving two radicals with opposite signs of charges. The charges are recombined either directly between these two molecules, or both molecules start to rotate and separate from each other. Eventually, the two charges recombined non-radiatively via various pathways. These pathways are reflected on the complicated multi-exponential decay curves of YOYO-1 fluorescence lifetime measurements. This charge transfer mechanism suggests that (1) electrical insulation may help improve the quantum yield of YOYO-1 in polar solutions significantly and (2) a steric hindrance for the intramolecular rotation may have a less significant effect.

1. Introduction

The fluorescence quenching mechanisms of a typical organic dye have been summarized in the literature to be a vibrational or/and electrical relaxation of the excited electrons through major pathways shown in Fig. 1, namely, intramolecular relaxation, and intermolecular/intermoiety relaxation including energy transfer (e.g. Förster resonance energy transfer, FRET) and charge separation pathways [1]. Among these, photo-induced charge separation (via electron or hydrogen transfer) is one of the most fundamental processes in chemistry and biology that has been extensively investigated by diverse experimental and theoretical methods [2–12]. For example, ultrafast charge transfer (100 fs) from solvents to Nile Blue A perchlorate (NB) has been reported by Yashihara et al. to be responsible for NB's fluorescent quenching [10], and the same mechanism has been used to explain the fluorescence quenching of oxazines and coumarines [8,13–15].

Here we report a study on the fluorescent quenching mechanism of a typical cyanine dye, YOYO-1 (Fig. 2A), for which intramolecular charge transfer has been considered the major mechanism [16–20]. YOYO-1, an oxazole yellow (YO) dimer and a member of the YOYO-TOTO family of cyanine dyes belonging to the polymethine group, is a widely used DNA fluorescent staining dye [21–23]. YOYO-1 has a high photon molar absorptivity, with peak extinction coefficient at a visible wavelength approaching $10^5\,cm^{-1}M^{-1}$ [22]. The fluorescence quantum yield of YOYO-1 in water is usually very small (< 0.1%) and thus is nonfluorescent. Upon binding to DNA, its quantum yield enhances over 1000 fold and reaches up to 50% [22,24]. This huge fluorescence contrast has caused a revolution in molecular biology in the 1990s [25], which enabled visualization and detection of DNA molecules using fluorescent molecules instead of radioactive molecules. The quenching mechanism of the YOYO-1 fluorescence in water has been attributed to the nonradiative decay of excited electrons via the rotation/torsion of the benzoxazole and quinolinium moieties at the methine bridge (photo-isomerization) [17], a mechanism proposed for typical polymethine dyes [19,26,27,28,29,30]. Theoretical calculations have suggested that the initial rotation is induced by an intramolecular twisted internal charge transfer (TICT) [19]. In this report, we use

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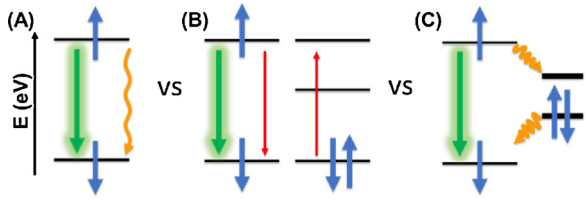

Fig. 1. Scheme of simplified fluorescence quenching mechanisms, self-quenched (intramolecular) or quenched by other molecules (intermolecular) [1]. (A) An electron energy diagram of fluorescent and vibrational relaxation of an excited molecule. Blue arrows are electrons with spin, each black line is an electronic orbital, green arrows are fluorescent emission, and orange arrows are thermal relaxation. (B) Energy transfer where the red arrows indicate the energy exchange between two molecules. (C) Charge transfer to another molecule that is directly connected to the molecule.

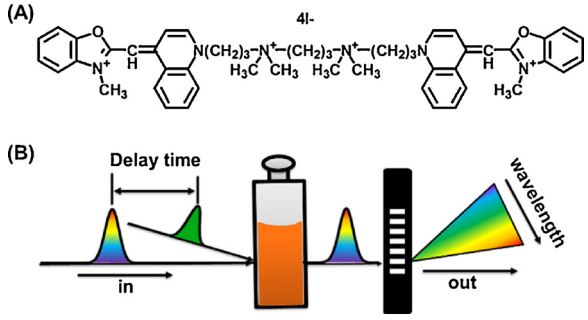

Fig. 2. (A) Chemical structure of YOYO-1. (B) Scheme of time-resolved transient absorption spectroscopy. Pump (green) and probe (rainbow) lasers pass through a sample with a delay time between them, and then the probe pulse is analyzed with a spectrometer. The TA signal is the difference in absorbance of the probe before and after excitation.

femtosecond time-resolved transient absorption (**TA**) spectroscopy (Fig. 2B) that has been widely used for photo-induced electron dynamics [18,31–36], to revisit YOYO-1's quenching mechanism. We are particularly comparing the intramolecular TICT relaxation and the intermolecular charge-transfer mechanism.

2. Experimental section

2.1. Fluorescent imaging of YOYO-1

It was carried out with a 473 nm solid-state excitation laser (Dragon Lasers, China) either under total internal reflection fluorescence (TIRF) or epifluorescence (EPI) mode; Nikon Ti-U inverted microscope with a Nikon 100× oil-immersed TIRF objective (CFI Apo 100×, NA 1.49, WD 0.12 mm); a fluorescent filter cube with ZET488/10 laser bandpass filter, ZT488rdc dichroic mirror, and ET500lp long pass filter (Chroma, USA); and an EMCCD camera (Andor iXon Ultra 897, USA) [21].

Glass coverslips and tweezers were cleaned by first sonicating in soap (Liquinox) water and secondly using "base piranha" solution (*caution* for corrosive and splashing). The glass coverslips were washed with ultrapure water after each step and were stored in ultrapure water before use. All cleaned glass coverslips used in these experiments were confirmed to have no fluorescent contamination at the single-molecule level by measuring them before experiments (*i.e.* no fluorescent spot was observed when searching around the nitrogen blown dry glass coverslips). A volume of 10 µL of the solvents, ultrapure water, ethanol (200 proof, Sigma-Aldrich), and hexane (GC grade > 99.9%, B&J Brand), were dropped on the clean glasses, dried, and measured. No detectable single-molecule fluorescent signals were observed.

YOYO-1 parent solution (0.1 mM in water) was diluted in ultrapure water to make 0.1 µM, 0.1 nM, and 1 pM solutions. Rhodamine 6 G (99%, Sigma-Aldrich) was dissolved in ethanol and its absorbance was measured to be 0.763 at 530 nm (extinction coefficient 116,000 cm^{-1}/M) that corresponds to the concentration of 6.6 µM. This solution was used to make a 1 pM solution.

The high-coverage YOYO-1/glass sample was made by dropping YOYO-1 aqueous solution (0.1 µM, 10 µL) on a glass coverslip and incubated for about 30 s before it was rinsed away by water for about 30 s and blown dry with nitrogen. This sample was imaged during which water and hexane were dropped on and dried alternately (room humidity 17%) both at TIRF and EPI mode. Excitation laser power density was tuned to 18 W/cm^2, camera electron-multiplying (EM) gain was set to 50, and the exposure time was 0.02 s per picture. The low-coverage YOYO-1/glass sample was prepared by dropping YOYO-1 solution (1 pM, 3 µL) on a glass coverslip and dried. Low-coverage rhodamine 6 G/glass sample was prepared by dropping rhodamine 6 G solution (1 pM, 3 µL) on a glass coverslip and dried. Laser power density was tuned to 12 W/cm^2, camera EM gain was set to 300, and the exposure time was 0.05 s for single-molecule fluorescence measurements. The histograms of single-molecule intensity were obtained by selecting molecules whose intensities were > 5 times the noise level of the fluorescent image.

2.2. UV–vis spectra

They were collected with an Ocean Optics USB2000 spectrometer and an Agilent 8453 spectrometer. UV–vis and steady-state fluorescent spectra were fitted using software Fityk (free version) with Voigt functions.

2.3. Femtosecond transient absorption spectroscopy

Three solutions for TA measurements were prepared using reagents without further purifications. The water used in sample preparation was an ultrapure deionized (DI) water (18.2 MΩ cm from Barnstead E-pure system). YOYO-1 in dimethyl sulfoxide (DMSO) (Invitrogen, Thermo Fisher Scientific) was diluted to 10 µM in ultrapure water, or DMSO (Sigma-Aldrich) for the two solutions of YOYO-1/water and YOYO-1/DMSO, respectively. This relative low concentration is chosen to avoid the formation of H-aggregation or J-aggregation of YOYO-1 in the solutions. YOYO-1/DNA solution (300 µg/mL) was made by adding phage Lambda DNA (48,502 basepairs, Thermo Fisher Scientific) to the buffer solution of 25 mM HEPES (pH 7.4, Sigma-Aldrich), 20 mM NaCl (Sigma-Aldrich), and 2 mM $MgCl_2$ (Ambion, Thermo Fisher Scientific). The YOYO-1 to DNA basepair ratio was calculated to be 1:46 assuming an average molecular weight per basepair of 650 daltons for double-stranded DNA with sodium salt. The relatively small dye-DNA ratio minimizes the concentration of free and non-intercalated YOYO-1. The solutions were heated to 40 °C and vacuumed two minutes to remove air bubbles, and the DNA samples were incubated overnight before the TA measurements.

Femtosecond TA spectra were collected on a HELIOS femtosecond transient absorption spectrometer (Ultrafast Systems, USA). The instrumental setup and the experimental procedures were reported previously [31,37,38]. The pump laser was set to 486 ± 1 nm whose power was adjusted to 185 ± 5 µW, and the probe white-light laser was measured at 30 ± 5 µW. Under these conditions, nonlinear laser effects or two-photon absorption, and photobleaching were negligible or minimal. The repetition rate was 1 kHz, and the pump-probe pulse convoluted width was ~150 fs. During the experiments, the sample solutions were irradiated with an absorbance of 0.2 measured at the excitation wavelength in a 2.0 mm path length cuvette under mild stirring. All TA data were corrected by subtracting spectral background features that persisted from the previous pulse and appeared pre-pulse, as well as applying chirp and t_0 corrections.

A home-written MATLAB code was used to analyze the TA spectral data which was represented from the Surface Xplorer Pro 1.1.5 software that came with the instrument (see its online manual for details) [39].