The Journal of Physical Chemistry A

 VOLUME 102, NUMBER 21, MAY 21, 1998

LETTERS

Millisecond Intensity Fluctuations of Single Molecules at Room Temperature

Kenneth D. Weston and Steven K. Buratto*

Department of Chemistry, University of California, Santa Barbara, California 93106

Received: September 26, 1997; In Final Form: February 25, 1998

We show that single $DiIC_{12}$ molecules adsorbed on a silica surface display a new type of rapid intensity fluctuations between an "on" level and a "dim" level distinct from "on"−"off" blinking. These fluctuations are correlated in time and exhibit two correlation times, one of the order of 10 ms and the other of the order of 100 ms, which are likely due to small motions of the nuclear coordinates of the adsorbed molecule. In addition, molecules that show these fast fluctuations also have emission spectra blue-shifted from the emission spectrum of the ensemble, indicating that this behavior is sensitive to the binding site of the molecule.

In the last seven years, ultrasensitive optical detection techniques have made possible detection and spectroscopy of single molecules at cryogenic[1,2] and room temperatures.[3−11] The observation of single molecules allows one to observe phenomena otherwise obscured in ensemble measurements such as the distribution of spectral positions and shapes,[4−10] discrete fluctuations in intensity [5,6,9,11,12] and spectral wandering.[4,10] All of these phenomena are extremely sensitive to the local environment, which makes single molecules perfect probes for nanoenvironments of a variety of systems including surfaces, polymers, membranes, and large biomolecules such as proteins and nucleic acids. The nature of this dependence, however, remains unclear. In this Letter, we show that the emission from single molecules dispersed on a glass surface exhibits fluctuations between an "on" level and a "dim" level and that these fluctuations are correlated in time indicative of molecular dynamics. The time scales for these dynamics range from milliseconds to tens of seconds and are due to changes in the nuclear coordinates resulting from transitions between minima of the potential energy landscape of the molecule that alter both the emission yield of the molecule as well as the emission spectrum. This model is analogous to that used to describe the slow (>100 ms) dynamics of single molecules in polymer hosts observed by monitoring spectral diffusion.[10,13] In addition, the rapid intensity fluctuations associated with the fast dynamics (<100 ms) are large (a factor of 10) and are most often observed for molecules with fluorescence spectra blue-shifted from that of the ensemble. This suggests that fast dynamics are dependent on the binding site of the molecule.

Figure 1a shows an image of single $DiIC_{12}$ molecules (Molecular Probes) dispersed on a glass coverslip. The sample was prepared by spin-casting (30 μL aliquot) from 0.1 nM dye solution in toluene. Each sample was imaged in the far-field with a 1.3 NA oil immersion objective, used to both illuminate and collect light from the dye-covered glass surface. The molecules were excited using the 514 nm line of an argon ion laser attenuated to 5 μW and directed into the back of a conventional optical microscope (Leitz Orthoplan). The laser light was then split with a 25/75 beam splitter, which directed 25% of the power of the beam into the objective to illuminate the sample with a spot size of approximately 400 nm (∼200 W/cm^2). The emission was collected with the same objective and passed through the same 25/75 beam splitter, sending 75% of the emission toward the detectors. The scattered laser light was isolated from the fluorescence with a holographic notch filter (Kaiser Optics). The fluorescence was split 50/50 with a beam splitter (Newport), sending 50% into a multimode fiber coupled to a liquid nitrogen cooled CCD spectrometer for

S1089-5639(97)03116-2 CCC: $15.00
Published on Web 04/30/1998

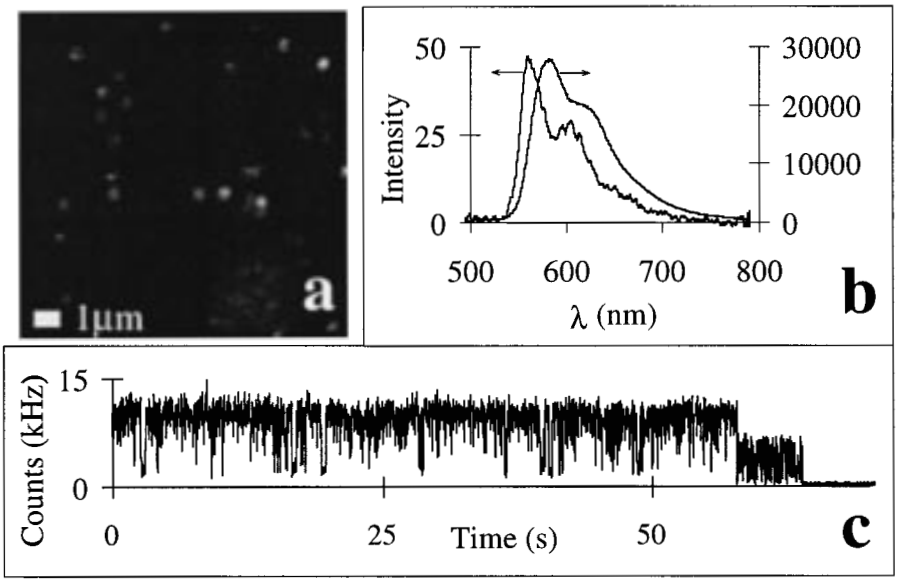

Figure 1. Representative data from single-molecule fluorescence experiments. Single $DiIC_{12}$ molecules (structure shown at top) on a glass surface imaged with a scanning confocal fluorescence microscope is shown in (a). Each bright spot in the image represents emission from a single molecule. The simultaneously acquired fluorescence spectra and time course from one of the molecules are shown in (b) and (c), respectively. The emission spectrum of this molecule (plotted with intensity units on the left axis) is blue-shifted from the ensemble average spectrum (plotted with intensity units on the right axis). Note the rapid intensity fluctuations in (c), which are correlated in time (see text).

spectroscopy and the remaining 50% to an avalanche photodiode (APD) photon-counting module (EG&G) with a 100 μm active area for imaging. A fluorescence image was acquired by rastering the sample using a four-quadrant piezotube (Stavely sensors) driven by commercially available scanning electronics (Digital Instruments).

A single molecule was selected for study by positioning the excitation beam over an emission spot in the image. The emission intensity vs time was then recorded (160 μs/bin) simultaneously with an emission spectrum (60 s integration/spectrum). Figure 1b shows the emission spectrum, and Figure 1c shows the intensity time course of a molecule that exhibits fast intensity fluctuations. An emission spectrum of an ensemble of molecules is also shown for comparison (Figure 1b). We note that the ensemble absorption and emission spectra of $DiIC_{12}$ molecules on a glass surface have maxima of 549 and 565 nm (a Stokes shift of 16 nm), respectively.

The intensity time course and spectrum for 103 different molecules were recorded until each underwent irreversible photobleaching. Of the 103 molecules, 49 (45%) photobleached before emitting enough photons to observe intensity fluctuations or a measurable emission spectrum. Of the remaining 54 molecules, 29 (54%) emitted photons with nearly constant intensity until an irreversible photobleach. An example of this type of single molecule emission is shown in Figure 2a. The emission spectra for these molecules did not change over the observation time. Seven of the remaining 54 molecules (13%), however, emitted at several discrete intensities with sudden jumps between them. One such time course is plotted in Figure 2b. For these molecules, constant intensity periods lasted for tens of seconds. The spectra of these molecules were observed to shift as a function of time, and the shifts corresponded directly to the changes in intensity. The spectra corresponding to the time course of Figure 2b are shown in Figure 3. During the first 60 s integration period, when the intensity was high, spectrum a was acquired. During the next 2 min, when the signal level dropped by a factor of 4, spectrum b was red-shifted by roughly 7 nm from the first spectrum. The last 18 of the 54

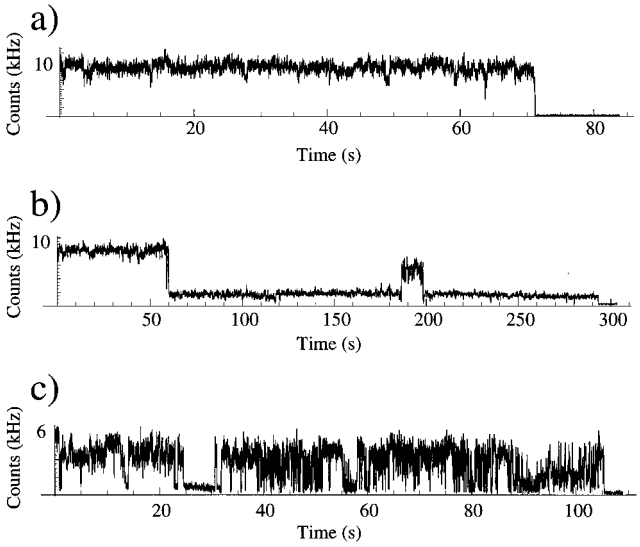

Figure 2. Fluorescence intensity of single $DiIC_{12}$ molecules plotted as a function of time. Data were acquired at 160 μs time resolution but have been binned (to 20ms/pt) in order to show the entire time course. The signal at the end of each trace shows the background after an irreversible photobleach. Part a shows an example of a molecule with nearly constant intensity. Part b shows an example of a molecule with several discrete intensity levels, each being maintained for several seconds. Part c shows an example of a molecule with rapid intensity fluctuations.

molecules (33%) showed switching between intensity levels on much faster time scales. This behavior is demonstrated in Figure 2c and also is similar to the time course plotted in Figure 1b. The change in emission levels for molecules that showed rapidly fluctuating intensity was always large, approaching an order of magnitude.

To quantitatively analyze the intensity fluctuations, we employed the intensity autocorrelation method previously used to analyze intensity fluctuations of single molecules at cryogenic temperatures,[14] and more recently at room temperatures.[12] The

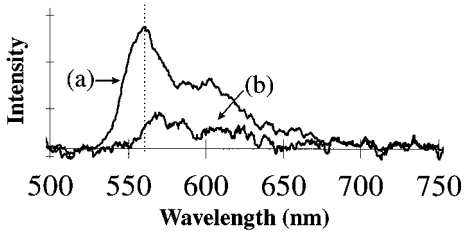

Figure 3. Fluorescence spectra taken simultaneously with the time course of Figure 2b. The higher intensity spectrum (a) corresponds to the first 60 s of emission, and the peak wavelength of the lower intensity spectrum (b) shifts peak wavelength shifts to the red by 7 nm after the transition to lower intensity.

intensity autocorrelation function is defined as the probability of detecting pairs of photons separated in time by an interval τ and is given by

$$g^{(2)}(\tau) = \frac{\langle I(t)I(t+\tau)\rangle}{\langle I(t)\rangle^2} \equiv \frac{\langle N(t)\,N(t+\tau)\rangle}{\bar{N}^2} \qquad (1)$$

where I is the emission intensity, N is the number of photon counts observed during the 160 μs bin time, and $\bar{N}$ is the average number of photon counts observed during a 160 μs bin time. For photons correlated in time, $g^2(\tau) > 1$ and dynamics are determined from $g^2(\tau)$ by monitoring the approach of $g^2(\tau)$ to unity. An example of $g^2(\tau)$ corresponding to the time course data of Figure 2c is shown in Figure 4. The data of Figure 4a fit very well to a double-exponential function of the form

$$g^{(2)}(\tau) = 1 + c_1 e^{(-k_1\tau)} + c_2 e^{(-k_2\tau)} \qquad (2)$$

with the rate parameters $k_1 = 1/\tau_1$ and $k_2 = 1/\tau_2$. For all of the molecules that showed rapid intensity fluctuations, we observed two independent time constants, a fast time constant of the order of 10 ms and a slow time constant of the order of 100 ms.

Previous observations of millisecond intensity fluctuations for single Texas Red molecules linked to individual DNA strands adsorbed on a glass surface have been attributed to long-lived triplet states.[11] In these experiments the intensity fluctuated between an "on" level and an "off" level (intensity equal to the background signal) as expected for molecules in the triplet state. In our, experiments, however, the "dim" level is easily distinguished above the background level. In addition, we do not observe a strong intensity dependence for our fluctuations as would be expected if they were due to triplet states. We have also performed experiments under conditions of flowing N_2 and wet air with negligible effect on the fluctuations. Thus, it is unlikely that our rapid intensity fluctuations are due to long-lived triplet states. It is important to note that at higher powers and lower bin times (16 μs), we have observed "on"−"off" blinking with correlation times of the order of 100 μs, which we do attribute to triplet states. This is consistent with the results of Macklin et al.[8] where the authors report an average triplet lifetime of 400 μs for single $DiIC_{12}$ molecules. We stress that this "on−off" blinking is distinct from the millisecond fluctuations that are the focus of this paper.

Another explanation to consider for the rapid intensity fluctuations is random spectral diffusion.[10,13−15] In this model, each emission spectrum arises from the molecule accessing different potential minima of the molecule's energy landscape. Transitions between the potential minima have been attributed to either intramolecular motions (such as conformational changes of the side chains of the molecule) or to intermolecular motions (such as small motions of the host matrix). The intensity fluctuations

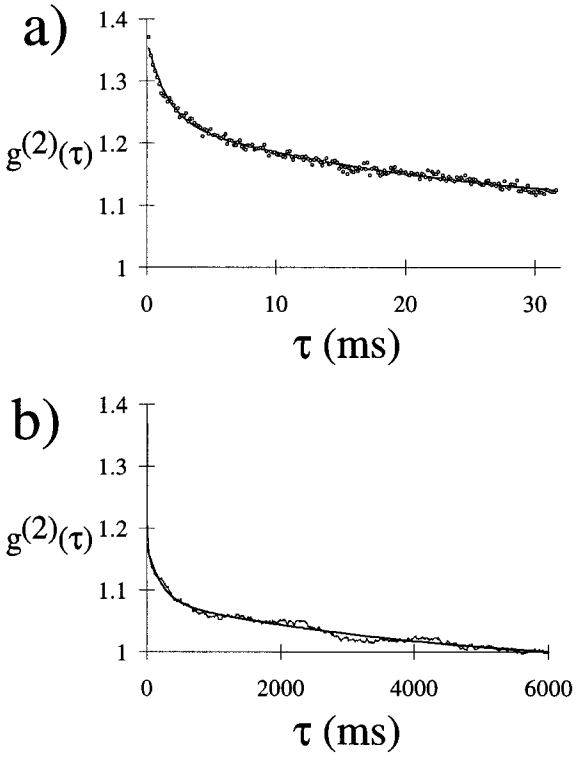

Figure 4. Intensity autocorrelation plots obtained from data shown in Figure 3c. In (a), a biexponential fit to $g^2(\tau)$ from 0.16 to 100 ms reveals dynamics associated with two independent processes, one occurring with a time constant of 1.2 ms and another of 23 ms. In (b), $g^2(\tau)$ is shown for longer times and produces additional time constants of 250 ms and 1.7 s.

that accompany the spectral shifts result from a change in the absorption cross section at the new resonance frequency. The most dramatic observation of this phenomena occurs for single molecules at cryogenic temperatures where the absorption and emission spectra are narrow and the spectral shifts are sufficiently large that the exciting laser is no longer resonant with the new absorption frequency. The off level in these experiments is the same as the background level.[14,15] At room temperature, however, where the absorption bandwidth is much larger (~100 nm fwhm), the spectral shift is not large enough to cause the excitation to go completely off-resonance. Thus, the change in absorption cross section and the corresponding change in emission intensity are much smaller at room temperature than at low temperature. In our experiments the exciting laser is positioned near the center of the absorption band, even for the molecule with the shortest emission wavelength ($\lambda_{max} \approx 540$ nm) assuming a Stokes shift of 16 nm (equal to that observed in the ensemble). In this situation, a change in absorption cross section due to a spectral shift of $\leq$10 nm (the range observed in our experiments) is not far enough to account for the large intensity changes observed in our experiments. It is important to note that we also observe much slower dynamics of the order of seconds that are due to small intensity fluctuations as seen in Figure 4b. These dynamics we attribute to spectral diffusion of the sort observed in ref 10, indicating that it is possible to observe 100 ms to 10 s dynamics by measuring correlations in either intensity or spectral position and that the two techniques are complementary.

To be consistent with our data, we propose a refinement to the model described in the previous paragraph in which each potential minimum is characterized by both a new emission spectrum *and* a new emission yield. We rule out the possibility that the intensity fluctuations were due to changes in the

orientation because we observe the same dynamics using either linearly polarized excitation or circularly polarized excitation. This lack of changes in the molecular orientation is consistent with previous single molecule experiments at room temperature.[10,11] Since we can eliminate trapping in triplet states, changes in the absorption cross section due to spectral shifts, and changes in molecular orientation as causes for the observed intensity fluctuations, we attribute the intensity fluctuations to changes in the emission yield of the molecule. One specific type of molecular motion consistent that would result in a change in both the emission frequency and emission yield is a twist about the conjugated bridge that links the two indole groups of the $DiIC_{12}$ molecule (see Figure 1). Such twisting is known to significantly alter the emission yield owing to a delocalization of electron density and greater access to nonradiative decay channels. Firm confirmation of this could be established by measuring the decay rates of both the light and dim states, but since the molecule is resident in each state for a time of the order of 10 ms, it is not possible to acquire a decay transient with sufficient signal-to-noise over such a short time. It should be noted that even if the lifetime of the "on" and "dim" states cannot be measured separately, a time-averaged lifetime of a molecule undergoing intensity fluctuations would exhibit a multiexponential fluorescence decay. Lifetimes of single $DiIC_{12}$ molecules in a PMMA matrix have been measured for 60 s of signal averaging.[8] While the lifetimes in this study were fitted to single exponentials, the reduced χ^2 values for the fits ranged between 0.94 and 1.4, allowing for the possibility of a second exponential. In addition, for molecules with long "on" and "dim" times such as the one described in Figure 3, it will be possible to directly measure the excited state lifetimes of each state. We are currently in the process of adding instrumentation for lifetime measurements.

By comparing the intensity time course and peak emission wavelength for all of the molecules detected, we have found that of the molecules that exhibit rapid intensity fluctuations 78% have emission peaks blue-shifted from that of the ensemble (see Figure 1b), a much higher percentage than the 49% of the remaining molecules that have emission peaks blue-shifted from that of the ensemble. This implies that there is a correlation between the peak position and rapid intensity fluctuations. Since each peak position is characteristic of the nanoenvironment of the molecule, the observation of intensity fluctuations provides more detailed information about the nanoenvironment since these particular sites influence the twisting about the bridge of the chromophore.

Our results show a new type of intensity fluctuations for single molecules on glass surfaces, which are strongly correlated to the binding site of the molecule. The dynamics indicated by our fast intensity fluctuations are due to transitions between potential minima that have different degrees of twisting about the bridge of the molecule. The presence of multiple exponentials in the fits to our data indicate that more than one pair of potential minima are responsible for the dynamics. In addition we observe a correlation between the peak emission wavelength and the existence of fast fluctuations and that this phenomenon provides additional insight into the nanoenvironment of the molecule. The possibility of utilizing this behavior as a method of analysis for such substrates as self-assembled monolayers, Langmuir−Blodgett films, and bioploymers is currently under investigation.

Acknowledgment. The authors acknowledge Jay Trautman for helpful discussions, Paul Carson for critical reading of this manuscript, and the National Science Foundation (CHE -9501773) for funding.

References and Notes

(1) Moerner, W. E.; Kador, L. *Phys. Rev. Lett.* **1989**, *62*, 2535.
(2) Orrit, M.; Bernard, J. *Phys. Rev. Lett*. **1990**, *65*, 2716.
(3) Betzig, E.; Chichester, R. *Science* **1993**, *262*, 1422.
(4) Trautman, J. K.; Macklin, J. J.; Brus, L. E.; Betzig, E. *Nature* **1994**, *369*, 40.
(5) Ambrose, W. P.; Goodwin, P. M.; Martin, J. C.; Keller, R. A. *Science* **1994**, *265*, 364.
(6) Xie, X. S.; Dunn, R. C. *Science* **1994**, *265*, 361.
(7) Nie S.; Chiu, D. T.; Zare, R. N. *Science* **1994**, *266*, 1018.
(8) Macklin, J. J.; Trautman, J. K.; Harris, T. D.; Brus, L. E. *Science* **1996**, *272*, 255.
(9) Ha, T.; Enderle, Th.; Chemla, D. S.; Selvin, P. R.; Weiss, S. *Phys. Rev. Lett.* **1996**, *77*, 3979.
(10) Lu, H. P.; Xie, X. S. *Nature* **1997**, *385*, 143.
(11) Ha, T.; Enderle, Th.; Chemla, D. S.; Selvin, P. R.; Weiss, S. *Chem. Phys. Lett.* **1997**, *271*, 1.
(12) Dickson, R. M.; Cubitt, A. B.; Tsien, R. Y.; Moerner, W. E. *Nature* **1997**, 388.
(13) Reilly, P. D.; Skinner, J. L.*Phys. Rev. Lett.* **1993**, *71*, 4257.
(14) Zumbusch, A.; Fleury, L.; Brown, R.; Bernard, J.; Orrit, M. *Phys. Rev. Lett.* **1993**, *70*, 3584.
(15) Ambrose, W. P.; Moerner, W. E. *Nature* **1991**, *349*, 225.
(16) Weston, K. D., Carson, P. J.; Metiu, H.; Buratto, S. K. Submitted.
(17) Turro, N. J. *Modern Molecular Photochemistry*; University Science Books: Sausalito, 1991; p 113.