Time-Varying Triplet State Lifetimes of Single Molecules

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(Received 1 December 1998; revised manuscript received 8 June 1999)

It is found that triplet state lifetimes and intersystem crossing yields of individual molecules embedded in a polymer host at room temperature are not constant in time. The range over which the triplet lifetime of a single molecule varies during long observation times shows a strong similarity with the distribution of lifetime values obtained during short observation times of many individual molecules dispersed in space. The similarity is an elegant manifestation of the ergodic principle of statistical physics.

PACS numbers: 33.50.–j, 07.79.Fc, 33.80.–b

Single molecule microscopy and spectroscopy give new and unique insight in the complex behavior of individual emitters on the nanometer scale [1–16]. Measurements on individual molecules have two distinct advantages. First, phenomena that are usually hidden due to ensemble averaging can be directly observed, such as spectral and rotational jumps [1–8], photon (anti-)bunching [7,9–13], and discrete photobleaching. Second, the monitoring of single molecule fluorescence forms a powerful way to probe the dynamics of the local environment on a nanometer scale. Therefore, single molecule detection allows the inhomogeneity of the ensemble to be directly related to the real time dynamics of the heterogeneity of the environment. In contrast, alternative techniques to study the composition of an inhomogeneously broadened ensemble like spectral hole-burning or pump-probe spectroscopies still average over a subset of the ensemble.

For the purpose of this Letter, fluorescent molecules are considered as a three-level system. Besides the repetitive transitions between the singlet ground state (S_0) and the lowest singlet excited state (S_1) giving rise to fluorescence, the molecule has a small chance to undergo intersystem crossing (ISC) from S_1 to the lowest excited triplet state (T_1). As long as the T_1 state remains occupied, the $S_0 - S_1$ transition does not occur and the fluorescence is interrupted temporarily. After decaying to S_0 the molecule starts fluorescing again. As a result, the fluorescence photons are emitted in bunches separated by dark periods when the molecule is in T_1. This so-called photon bunching can be investigated in two ways. First, autocorrelation of the time intervals between detected photon counts yields the typical duration of the dark periods and thus the T_1 lifetime [7]. The disadvantage of this indirect method is that it yields only a mean value for the T_1 lifetime. Second, integration of the detected fluorescence photons over time intervals shorter than the duration of the dark periods can identify the time length of each excursion to T_1 in a direct way [11–13]. We have used the second method to obtain time-resolved T_1 state dynamics.

The first measurements on the T_1 state of individual molecules were performed at cryogenic temperatures [1,7–9,11]. It was found that T_1 lifetimes and intersystem crossing rates could vary among different molecules, which was attributed to local static disorder in the crystal host. At room temperature and under ambient conditions, due to increased dynamics, molecules exhibit increased dissociation probability, reduced absorption cross section, and shortening of the T_1 lifetime when compared to cryogenic conditions. Not surprisingly, due to the severe experimental demands on the determination of single molecule T_1 lifetimes, only a few experiments at room temperature have been reported [12,13].

For the first time, the conditions to observe T_1 state excursions of single molecules at room temperature have been fulfilled with near-field scanning optical microscopy (NSOM). We describe measurements on the T_1 state of single molecules which show that T_1 lifetimes vary in time. We find a suitable length of time of about 10 s over which the distribution of T_1 lifetimes of one molecule becomes similar to the distribution of constant T_1 lifetimes obtained from many individual molecules during short observation times.

In our experiments the individual molecules are excited by a local probe with a subwavelength aperture light source in a NSOM configuration [4,5,14,15], enabling subnanometer accuracy for locating molecules. Moreover, the three-dimensional orientation of individual molecules can be determined [14,15]. Through the use of high brightness probes [15] (typical excitation intensity 10 kW/cm^2) we obtained a single molecule fluorescence signal up to 10^6 photon counts/s (close to saturation of the $S_0 - S_1$ transition), pushing the time resolution of the apparatus down to 30 μs. Furthermore, with a lateral position feedback system, nanometer accuracy for positioning the probe above a molecule of choice was achieved. We have used the same 135-nm-diameter probe emitting circularly polarized light for all measurements described in this Letter. The measurements were performed on DiIC$_{18}$ molecules embedded in either of two different polymer hosts (10 nm

thin films of PMMA and polystyrene, respectively) and for two different oxygen concentrations. DiI has been studied before at the single molecule level, yielding information about the fluorescence spectrum [5,6,16], fluorescence lifetime [16], orientation [4,14,16], quantum jumps to T_1 [13], and intensity fluctuations [6].

Figure 1 shows two typical fluorescence gray-scale images of $DiIC_{18}$ molecules in PMMA obtained by raster-scanning the probe at a height of 10 nm above the sample surface. Each image shows the collected fluorescence of one single $DiIC_{18}$ molecule with an integration time of 200 μs per pixel. The presence of several isolated dark pixels within the bright spots indicates that the fluorescence is not continuous in these images. The dark periods (different length for both molecules) are attributed to a temporal residence of the molecule in the T_1 state.

In order to monitor the fluorescence of a single molecule continuously as a function of time, we positioned the NSOM probe directly above a molecule. All molecules in this study (a total number of 247) were illuminated and monitored until irreversible photobleaching occurred (typically after 10^6 detected photon counts). Figure 2(a) shows a 15 ms interval of the fluorescence of a single $DiIC_{18}$ molecule, which emitted fluorescence for 2.8 s in total. The emission undergoes abrupt transitions from a high to a low intensity level and back due to quantum jumps of the molecule from S_1 to T_1 and from T_1 to S_0, respectively. From such a fluorescence time trace both a T_1 state lifetime (τ_T) and intersystem crossing yield (Y_{ISC}) can be extracted. In good approximation, Y_{ISC} is given by the ratio between the ISC rate from S_1 to T_1 and the fluorescence rate from S_1 to S_0. The duration of the dark periods was determined directly by selecting a signal level (defined as the 1% lower limit confidence level for the Poissonian photon noise distribution of a time-integrated fit of the intensity) to discriminate between emission (light periods) and dark periods. A histogram of the duration of all dark periods within a certain observation time interval yields an exponential decay with a typical decay time (τ_T) for that interval. Figure 2(b) shows such a histogram that was obtained by evaluating the fluorescence during an observation time interval of 2.8 s. Similarly, a typical Y_{ISC} for a certain observation time interval is obtained from the exponential decay of the number of photon counts in the light periods [Fig. 2(c)], as $(Y_{\mathrm{ISC}})^{-1}$ is the typical number of counts of the light periods divided by the detection efficiency.

We have determined τ_T and Y_{ISC} of 80 different molecules embedded in PMMA and 72 molecules in polystyrene. For a fraction of these molecules, the histograms of the dark period duration and the number of counts of the light periods could be described by a single exponential relation. This indicates that both τ_T and Y_{ISC} were constant during the entire observation time for each of these molecules at their specific location. In Figs. 3(a) and 3(b) the distributions of τ_T are given for these subsets in PMMA (51 molecules) and polystyrene (55 molecules), respectively. The uncertainty of the τ_T values ($<10\%$) is smaller than the bin widths in these distributions. Note that both peak position and width of the distributions are different. In polystyrene, the distribution peaks at 40 μs, while in PMMA this value is 170 μs. The distributions of Y_{ISC} for these sets of molecules in PMMA and polystyrene yield peak values of 3.3×10^{-4} and 2.2×10^{-4}, respectively. The different peak values of both τ_T and Y_{ISC} clearly demonstrate the

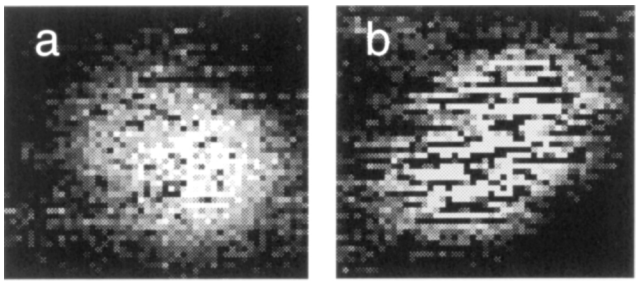

FIG. 1. (a) and (b): Gray-scale NSOM fluorescence images (370×370 nm) of two different single $DiIC_{18}$ molecules embedded in a 10 nm PMMA layer (200 μs integration time per pixel). Dark pixels are caused by a temporal cease of fluorescence due to quantum jumps to the T_1 state. Molecule (b) has a longer T_1 lifetime than molecule (a).

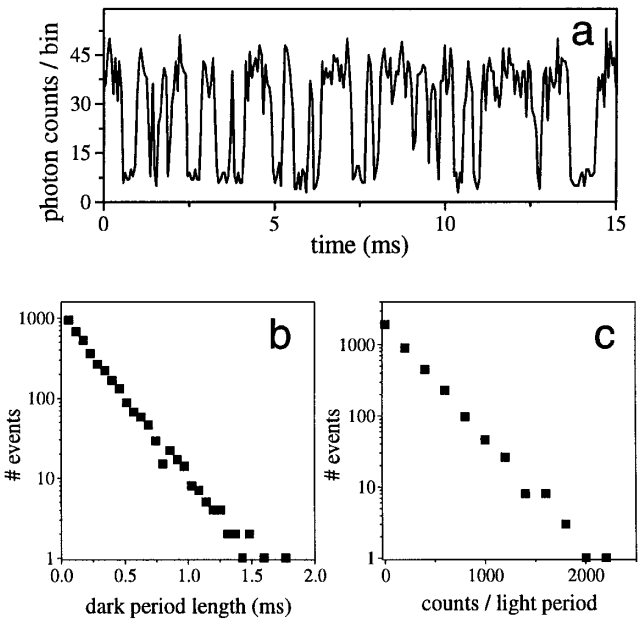

FIG. 2. (a) Single molecule fluorescence as a function of time (integration time per point = 57 μs). The fluorescence drops repeatedly to a low level due to transitions to the T_1 state. (b) Histogram of the length of the "dark" periods for the same molecule over an observation time of 2.8 s. The decay can be described with a single exponential giving a mean T_1 lifetime of 216 ± 5 μs. (c) Histogram of the number of photon counts of the "light" periods for the same molecule, resulting from an ISC yield of $(3.6 \pm 0.5) \times 10^{-4}$.

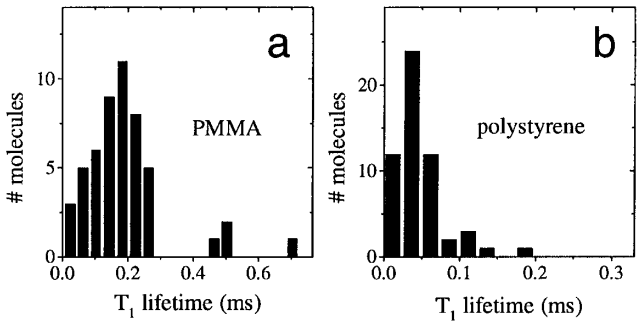

FIG. 3. Distributions of T_1 lifetimes of 51 spatially dispersed $DiIC_{18}$ molecules in PMMA (a) and 55 molecules in polystyrene (b). Heterogeneity of the polymer environment of the molecules accounts for the range of different T_1 lifetimes. Note the difference in peak position and overall shape of both distributions, due to differences in polymer properties that influence the T_1 lifetime.

influence of the different environments on the molecular photophysical properties. The width of the distributions is caused by the spatial heterogeneity of each sample.

Surprisingly, we found that the histograms of τ_T and Y_{ISC} for a considerable fraction of the molecules (35% in PMMA and 25% in polystyrene) could not be described by a simple single exponential decay. This could imply the presence of either multiple decay channels or values for τ_T and Y_{ISC} that are not constant in time. To verify this, we have performed a time-resolved analysis of τ_T and Y_{ISC}. Figures 4(a) and 4(b) show, respectively, τ_T and Y_{ISC} of a molecule embedded in PMMA as a function of the observation time. To evaluate a typical τ_T or Y_{ISC}, a minimum number of T_1 excursion events and thus a minimum observation period is required. A value for τ_T and Y_{ISC} was evaluated with a single exponential in time intervals of 466 ms, containing approximately 600 T_1 state excursions each, resulting in an accuracy for τ_T of 5%–10%. The molecule indeed displays τ_T variations, ranging from 0.12 to 0.28 ms, and Y_{ISC} variations between 2.3×10^{-4} and 4.9×10^{-4}, until irreversible photobleaching occurs.

Histograms of the relative occurrence of the different values of τ_T and Y_{ISC} for the long-lived molecule of Figs. 4(a) and 4(b) are shown in Figs. 4(c) and 4(d), respectively, over the entire observation period (13 s). Superimposed to the histograms are also the distributions of τ_T [as plotted in Fig. 3(a)] and Y_{ISC}, respectively, for spatially dispersed short-lived molecules. It is found that the relative occurrence in time of τ_T and Y_{ISC} for the individual molecule matches a large part of the distributions of the constant τ_T and Y_{ISC} for the set of spatially dispersed molecules. We find that this similarity is exhibited for all molecules with a time-varying τ_T and Y_{ISC}, both in PMMA and polystyrene. Furthermore, the longer the observation time, the larger the overlap becomes. We find that variations in τ_T and Y_{ISC} for a single molecule occur on a time scale of 0.2 to 20 s. The combined histogram of the relative occurrence of τ_T and Y_{ISC} for all molecules with a time-varying τ_T and Y_{ISC} overlaps the entire distribution of τ_T [Fig. 3(a)] and Y_{ISC}, respectively, of spatially dispersed molecules.

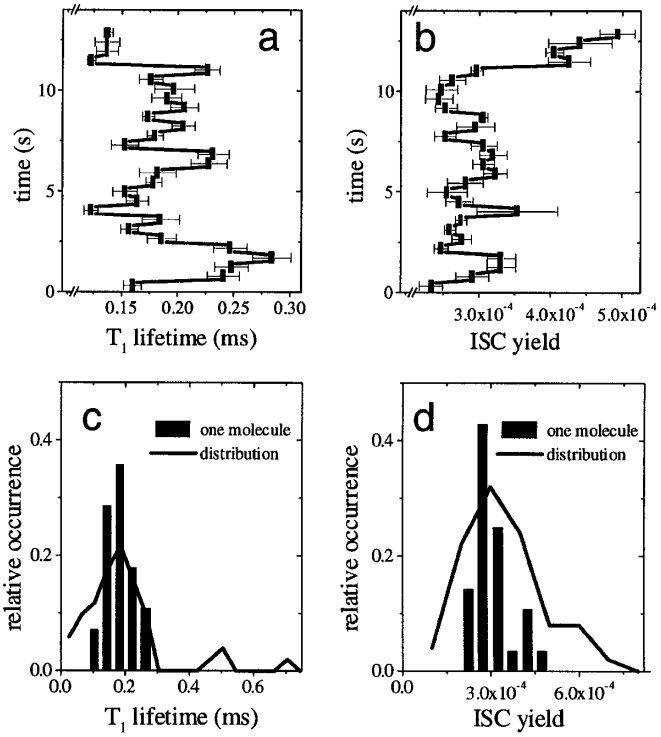

FIG. 4. Trajectories of the T_1 lifetime (a) and ISC yield (b) of a single $DiIC_{18}$ molecule in PMMA as a function of time. Histograms of the relative occurrence of the T_1 lifetimes (c) and ISC yields (d) for the single molecule in (a) and (b) (bars), and distribution of T_1 lifetimes (c) and ISC yields (d) for spatially dispersed molecules in PMMA (line). The similarity of both distributions in (c) as well as those in (d) indicates that environmental sites become indistinguishable from each other in time.

The time-dependent behavior of τ_T and Y_{ISC} can be understood if one considers that the polymer host constitutes a semirigid environment, which is dynamic in nature at room temperature [4,5,16,17]. As a result, photodynamical parameters such as τ_T and Y_{ISC} can indeed vary with time. For instance, variations of the local oxygen concentration related to local conformational dynamics of the polymer host [17] could be involved in the observed variation in τ_T. Oxygen is a well-known quencher of the T_1 state of many aromatic dyes [18]. The diffusion constant of oxygen in PMMA is 1 order of magnitude smaller than in polystyrene, while the solubility of oxygen is approximately the same [19]. For a single molecule in polystyrene at ambient conditions, the number of oxygen collisions in a 40 μs time interval is of the order of one to ten [19]. To verify if oxygen quenching plays an important role for the τ_T distribution in polystyrene, we have reduced the oxygen concentration by continuous flushing of the sample with argon. The

flushing indeed resulted in lengthening of τ_T to a mean value of 150 μs for a set of 95 different individual molecules. Thus, we propose that variations of the local oxygen concentration are involved in the observed variation in τ_T, both in space and time.

However, we also find other mechanisms that change τ_T and Y_{ISC}. We sometimes observe that an abrupt change in τ_T or Y_{ISC} of a molecule is directly correlated with a sudden change in emission intensity [12]. We can exclude orientational movements as a cause for these intensity jumps due to our orientation sensitive measurement. Therefore, we attribute the intensity jumps to spectral jumps [2,5,6,12] (probably related to changes of the local polarity of the environment) or abrupt changes of the fluorescence quantum yield [6] (probably related to twisting of the conjugated bridge of DiI molecules). Molecules exhibiting intensity or orientational jumps were excluded from the T_1 state analysis and will not be further discussed here.

Finally, a major conclusion can be drawn from the similarity of the two distributions of Fig. 4(c) for τ_T and Fig. 4(d) for Y_{ISC}, one of them representing the behavior of one molecule in time, and the other representing the behavior of different molecules separated in space. The similarity indicates that all environmental sites become indistinguishable from each other in time. A molecule that would be observable for a sufficiently long period would indeed exhibit the same distribution of τ_T and Y_{ISC} occurrences as a set of molecules dispersed in space at a single moment in time. Therefore, we observe that our measurements satisfy the ergodic principle of statistical physics, stating that for a physical stationary system a time average is equivalent to an ensemble average. Moreover, we obtain a minimum time of approximately 10 s that is needed to observe this manifestation of ergodicity.

In summary, this Letter reports the first observation of time-varying T_1 lifetimes and ISC yields of single molecules at room temperature with NSOM. The observations illustrate the potential of single molecules to locally probe physical and chemical dynamics in time on a nanometer scale at ambient conditions. We have shown that τ_T and Y_{ISC} reveal the dynamical nature of the polymer host. The similarity between the distributions of τ_T and Y_{ISC} for different molecules dispersed in space, and for individual molecules in time is an elegant demonstration of the ergodic principle of statistical physics.

This work is financially supported by the "Stichting voor Fundamenteel Onderzoek der Materie" (FOM).

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