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ARTICLES

Parameters Influencing the On- and Off-Times in the Fluorescence Intensity Traces of Single Cyanine Dye Molecules

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On−off kinetics observed in fluorescence intensity traces of single organic dye molecules have been assigned to molecules being either continuously emitting photons (on state) or occupying the triplet state (off state). To date this was mainly substantiated by the oxygen dependence of this behavior. By means of various experimental approaches, we show that indeed the occupation of the triplet state principally causes the off periods in some cyanine dyes structurally related to frequently used DiI or Cy3. Comparing recorded traces for various cyanine dyes bearing different atoms at the 1- and 1′-positions, we demonstrate for the first time the consequences of the internal heavy atom effect on the on−off dynamics of single organic dye molecules. An additional pathway to populate the triplet state is proposed in order to explain the enhanced intersystem crossing quantum yield for the transition S_1 to T_1 for these molecules, which demand less free volume for rotation.

Introduction

In recent years, single molecule detection and spectroscopy have developed rapidly. Generally, by means of confocal fluorescence spectroscopy or near field scanning optical microscopy (NSOM) insight could be gained into the excited-state dynamics of single molecules as well as into the influence of the local environments on these properties.[1−3] It has been demonstrated that the influence of the environment leads to differences in the photophysical properties. This is reflected by multimodal distributions of different parameters, such as the fluorescence lifetime and the spectral position.[1,4−8] One of the most intriguing characteristics in the photodynamics of a fluorescent single molecule is the occurrence of off states in its fluorescence transient. These are time periods during which the molecule does not emit photons on continuous irradiation with light. The occurrence of on−off behavior (so-called blinking) has attracted a lot of interest and multiple causes have been suggested for the origin of off states.[1,9−12] For a single chromophoric organic dye molecule, the occupancy of the excited triplet state T_1 was established to be the main reason for these off states. This interpretation allows the inverse approach, i.e., the triplet lifetime of an individual molecule can be extracted from its off-time statistics. Several examples of this approach can be found in the literature. Information could be obtained about the single molecule itself or its environment.[1,13] For the determination of triplet lifetimes from fluorescence transients, one has to assume that the photophysics occurring in the chromophore can be described by a three-level system. Excitation and subsequent fluorescence correspond to the on state and occur between the singlet ground state (S_0) and the lowest excited singlet state (S_1). When intersystem crossing (ISC) from S_1 to the lowest triplet state (T_1) takes place (ST_{ISC}), the molecule goes to an off state until it returns to the ground state in a second intersystem crossing step (TS_{ISC}). This process is schematically illustrated in Figure 1.

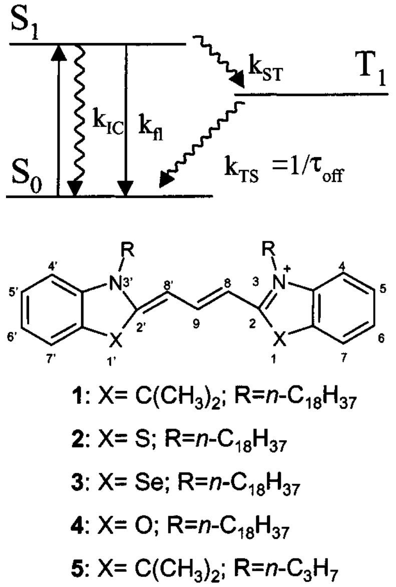

Figure 1. Three-level schematic diagram showing the singlet ground and excited state, S_0 and S_1, respectively, and the triplet state T_1. The two intersystem crossing rates are denoted as k_{ST} ($S_1 \rightarrow T_1$) and k_{TS} ($T_1 \rightarrow S_0$). The lower part demonstrates the structure of all dyes used for this study.

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Despite their low quantum yields of fluorescence in solution, cyanine dyes that are embedded in a rigid matrix are excellent study objects for single molecule spectroscopy. In such kind of environment, efficient pathways of deactivation, involving torsional motion around the conjugated double bonds, are largely frozen out.[14,15] Cyanine dyes fulfill all requirements for single molecule spectroscopy. They have high extinction coefficients, high quantum yields of fluorescence in a rigid matrix and slow photobleaching rates, i.e., a substantial amount of emitted photons (10^6-10^7) can be obtained from a single cyanine molecule before the final bleaching step occurs. Among the cyanine dyes, the indocarbocyanine DiI (**1**), with varying R substituents, is the most popular in single molecule spectroscopy. Single molecules of dye **1** revealed triplet lifetimes longer than 100 ms in a rigid and carefully deoxygenated environment.[1,9] Long triplet lifetimes were also obtained in a matrix which possessed a low diffusion constant for oxygen, such as poly(vinyl alcohol).[13] In the presence of oxygen the triplet state is quenched and far shorter triplet lifetimes in the μs range were obtained.[16]

In competition with intersystem crossing, torsional motion leading to a twisted geometry or even trans-cis isomerization may still be a relaxation pathway, though inefficient, in rigid matrixes. Whether the isomerization of cyanine dyes occurs from the S_1 or the T_1 state is still a point of discussion in the literature.[17−23] Isomerization will yield a compound with different absorption and emission properties and may be another reason for off times. As a consequence, an additional process needs to be considered during analysis of the fluorescence intensity traces. Therefore, we decided to investigate whether the on−off blinking characteristics are exclusively caused by the population of the triplet state or whether a pathway linked to torsional freedom is involved as well.

Our approach to study single cyanine dye molecules was 3-fold. First, as it is in general very likely to record in the course of the experiment fluorescence intensity traces with highly different signal-to-noise ratio, we introduced an analysis program for single ion channel traces in order to determine the on−off kinetics and to ensure that the extracted values for the rate constants were reliable and not an artifact of the analysis. This program was proven to be capable of recovering accurate values even for traces with low signal-to-noise ratio and off times just longer than the bin time per data point.[24−27] To illustrate that this program retrieves reliable values we recorded fluorescence intensity traces of single molecules for which an electrooptical modulator (EOM) was used in order to change the direction of the linear polarization of the excitation light during the acquisition of the traces. On one hand, this is supposed to have an impact on the number of absorbed photons per time unit, and accordingly, also on the number of emitted photons, but on the other hand should not influence the extracted photophysical rate constants. Furthermore, this experiment intrinsically provides a power dependence study. To date the power dependence of the on−off kinetics in fluorescence intensity traces is still a point of discussion in the literature,[1,11,28,29] and this could be clarified by means of this excitation polarization modulation experiment.

Second, we studied the internal heavy atom effect on single molecule fluorescence on−off kinetics. We looked at the cyanine dyes **1**, **2**, and **3**, with a similar structure, but where the atoms on the 1- and 1′-position were either carbon, sulfur or selenium (see Figure 1). Focusing only on these three compounds, the heavier sulfur and selenium atoms should have an impact on the on−off kinetics, since heavy atoms increase ISC rates via spin−orbit coupling. As for most cyanine dyes the quantum yield of intersystem crossing from S_1 to T_1 (Φ_{ST}) is very low in solution ($10^{-2}-10^{-4}$), the heavy atom effect does not influence the fluorescence behavior substantially in solution.[21,30] Φ_{ST} was determined to be 0.004 for THIA2 (R = ethyl) assuming that trans−cis isomerization occurs from the singlet excited state.[18,31] However, Serpone and Sahyun reported a quantum yield of intersystem crossing of 0.55.[17] They proposed that intersystem crossing and isomerization are coupled such that the intermediate in the trans−cis isomerization process is located on the T_1-potential surface. Accordingly, isomerization is accompanied by a transition from S_1 to T_1. This additional pathway to populate T_1 accounts for the higher intersystem crossing quantum yield. The ensemble triplet lifetime τ_T, the inverse of k_{TS}, was measured to be 6.5 ms in poly(vinyl alcohol) films at room temperature.[15]

Third, we studied two cyanine dyes, which only differed in the length of their R substituent (octadecyl chain (**1**) and propyl (**5**), respectively). As sufficiently free volume is a prerequisite for torsional motion, the kinetics for these two dyes should be affected, if torsional motion or even isomerization influences the kinetics significantly.

Experimental Section

The dyes were commercially available (DiI−C3 (**5**), FEW Chemie, Wolfen, Germany, used as received) or synthesized (DiI−C18 (**1**), THIA18 (**2**), SE18 (**3**), and OXA18 (**4**)) following standard procedures and purified by recrystallization.[32]

The fluorescence traces of the single molecules, excited at 543 nm by a cw-HeNe laser (Spectra Physics) or with the 488 nm line of an argon-ion laser (Stabilite, Spectra Physics) (compound **4**), were detected using a confocal microscope (Diaphot 200, Nikon) with an oil immersion, high-numerical aperture lens and an avalanche photodiode (EG&G, Canada) serving as the detector. A notch filter (Kaiser Optics) was used to suppress remaining excitation light. The fluorescence intensity traces were acquired with integration times of 500 μs or 1 ms. The overall detection efficiency was about 10%.

An electrooptical modulator (Lasermetrics, 3079 FW 450-650) was used for part of the experiment. Changing the applied voltage from −180 to 108 V resulted in a change in polarization of 90° for the transmitted light. A frequency generator (Wavetek 10 MHz DDS model 29) was used in order to control the polarization changes.

The samples were prepared by spin-coating a 0.5 nM solution of the respective dye in a 10 wt % solution of Zeonex, an aliphatic polynorbonene, in $CHCl_3$ on top of a glass cover slip. This procedure resulted in a film thickness of about 300 nm. The samples were placed in high vacuum (5×10^{-5} mbar) for more than 2 h to remove oxygen and successively covered with aluminum in order to keep oxygen from penetrating the film.

Results and Discussion

1. Appearance and Analysis of the Fluorescence Intensity Traces. In general, the appearance of fluorescence intensity traces, which are obtained from a single chromophoric molecule and show blinking behavior between an on and an off state, can be classified into three categories. Even though this is only a consequence of the way of data acquisition and presentation, there is a justification for the classification. Under the first limiting condition, the ideal single molecule fluorescence trace has a box-like shape, where each data point refers unambiguously to one of the two states, either to the on state or to the off state. Almost ideal box-like traces can be recorded from

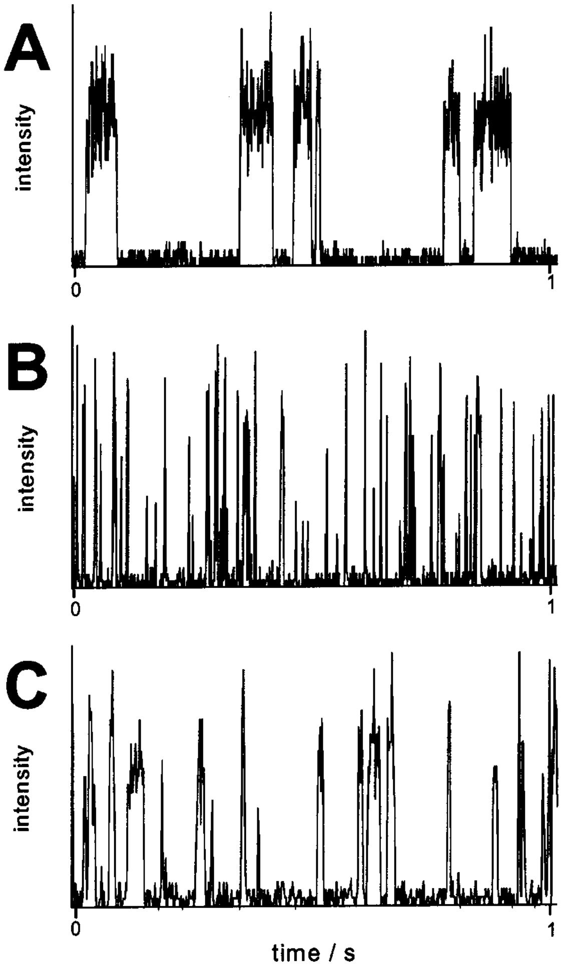

Figure 2. Presentation of the various appearances of fluorescence intensity traces recorded of single molecules. (a) Box-like trace, recorded from compound **1**; (b) spiky trace, recorded from compound **3**; (c) trapezoidal-like trace, recorded from compound **2**.

compound **1**, as depicted in Figure 2a. However, single molecule data are always recorded with a finite time interval referred to as the bin time. This is a consequence of the type of data acquisition. A fixed time interval is set during which all detected photons are accumulated. The sum of all counted photons is the output data point for the specific bin. However, this means that the transition from an off to an on state or vice versa is very likely to happen within one bin and not at the very beginning or at the very end. Under the second limiting condition, the off-on transition as well as the on−off occur within one bin. Traces, which principally show this behavior, have a spiky appearance as the trace recorded from compound **3** depicted in Figure 2b. These two ideal cases can be easily analyzed in terms of on−off kinetics, if the signal-to-noise ratio is sufficiently high.[1,9,13,16]

However, the majority of the fluorescence intensity traces has an appearance, which is lying between the two limiting conditions and which we denote as trapezoidal. This is shown in Figure 2c, a trace recorded from compound **2**. The first and the last bin of each on period have a statistically lower number of photocounts than the on level. We have to emphasize again that the different appearance of fluorescence intensity traces is a consequence of the time bin used. If the bin size was set to very small duration times, all traces would have a box-like appearance, as there would be in the extreme case only 0 or 1 count per bin. However, fluorescence intensity traces have very different signal-to-noise ratios. To always have analyzable traces with a satisfactory signal-to-noise ratio, the bin size has to be of a certain length, which results in the three different appearances.

Moreover, the fact, that the analysis is applied to traces with different signal-to-noise ratios is very relevant. For traces with a very low signal-to-noise ratio, there is no clear distinction whether a data point refers to the on or the off level, as the intensity of the two levels will interfere. As a consequence, the discrimination for each data point whether it belongs to an off or an on level, can no longer be done like for the two limiting cases. The rate constants $k_{\mathrm{ON/OFF}}$ (a value related to Φ_{ST}) and $k_{\mathrm{OFF/ON}}$ (equals k_{TS}) can only be calculated by means of an appropriate algorithm. Therefore, we analyzed the fluorescence intensity traces with a program, which was originally designed to retrieve rates from single ion channel data.[26,27] The suitability of this program to analyze fluorescence intensity traces has been shown.[33] An in-depth evaluation of the analysis and a comparison of possible mathematical models to analyze on−off behavior will be published elsewhere.

Prior to the discussion about the on−off kinetics in fluorescence intensity traces, we present some general results, which are based on the statement that the values calculated for Φ_{ST} and k_{TS} should not depend on the kind of trace that was recorded. In advance, it cannot be predicted which of the three patterns, shown in Figure 2, a fluorescence trace will display, as this depends on the absorption rate (expressed in number of absorbed photons per second) and thus on the relative position of the transition dipole of the dye molecule with respect to the incident excitation polarization. If the transition dipole of the molecule is oriented parallel to the excitation polarization, the probability is higher to obtain a spiky trace, whereas for the dipole being oriented more perpendicular to the excitation polarization, the acquisition of a box-like trace or a trapezoidal-like trace is more likely, if the set time bin is equal. As a trace can exhibit any kind of the three discussed appearances, the extracted values of Φ_{ST} and k_{TS} have to be independent of the shape of the trace, and as a consequence, they should be independent of the excitation polarization. If this is fulfilled, one can deduce that the on−off behavior originates indeed from a process that is linked to the amount of absorbed photons. And as stated before, this method provides furthermore an alternative to a power dependence study. The absorption efficiency is changed, without changing the excitation light intensity. The advantage of this procedure versus the common approach to directly increase the excitation power is that such a dependence study can be done under the same conditions for one individual molecule. To check the independence of the extracted values on the excitation conditions we modulated the excitation polarization with a frequency of 0.025 Hz (square pulse), changing every time the incident polarization by $\pi/2$ and recorded traces for compound **1**.

Figure 3 displays a zoom in a part of a recorded trace. Clearly, the modification of the polarization gives rise to an altered appearance of the fluorescence trace. For this specific molecule, the characteristics of the fluorescence trace change significantly from a box-like structure and a relatively low maximum intensity to a fairly spiky trace with much higher signal-to-noise ratio. These patterns reflect the relative orientation of the transition dipole of the dye molecule with respect to the polarization of the excitation light. For the part of the trace which shows a box-like shape, the molecule is oriented more perpendicular with respect to the excitation polarization, as for the time showing a spiky trace, illustrated in Figure 3. The analysis of the trace displayed in Figure 3 proves also the suitability of the analysis

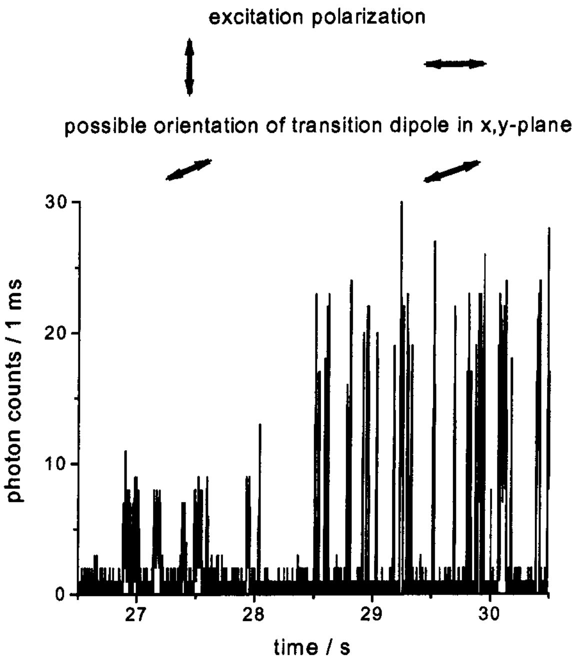

Figure 3. Zoom in a fluorescence intensity trace recorded for compound **1** for which light was guided through an electrooptical modulator working with 0.025 Hz (square pulse). In the graph the pronounced change of appearance is illustrated. While for the first part the transition dipole of the molecule is fairly perpendicular with respect to the incident light polarization, in the second part the transition dipole and the excitation polarization are almost parallel.

program for the determination of the photophysical rate constants. The extracted values for these two appearances are the same within the experimental error (for the left part, $\tau_T = 69.6$ ms, $\Phi_{ST} = 4.9 \times 10^{-4}$; for the right part, $\tau_T = 66.1$ ms, $\Phi_{ST} = 4.6 \times 10^{-4}$). Moreover, such an abrupt change in pattern was also observed for less than 0.5% of all analyzed traces which were recorded without modulation of the polarization of the excitation light. Under these conditions we attribute this change to a direct observation of a movement of the respective molecule. This means that from time to time even in a fairly rigid polymer matrix sudden orientational leaps take place.

Figure 4 demonstrates the analysis of an entire trace for one specific molecule in terms of Φ_{ST} and k_{TS}. It is a representative example, where the signal intensity does not change as pronounced as shown in Figure 3. Each 20 s time segment, corresponding to a different orientation of the polarizer, was analyzed independently. As expected, for most of the consecutive periods switching the excitation polarization does not change Φ_{ST} or k_{TS}, although the appearance of the various segments is very different. However, dynamic disorder is observed for a time scale larger than a few segments. This disorder is correlated, as shown in the inset of Figure 4c. Whenever one rate is altered, the second rate also undergoes a change toward the same direction.

As stated before, the different excitation polarizations involve a change in excitation power. In literature, there is no agreement about the power dependence of the rate constants.[1,11,28,29] However, from Figure 4, it is clear that there is no power dependence, consistent with the results reported by English et al.[1] To further substantiate this finding, experiments were conducted using excitation with peak irradiances ranging from 0.3 to 12 kW/cm^2, ensuring that saturation was not reached even at the highest irradiation intensity. The values extracted from these traces are power independent.

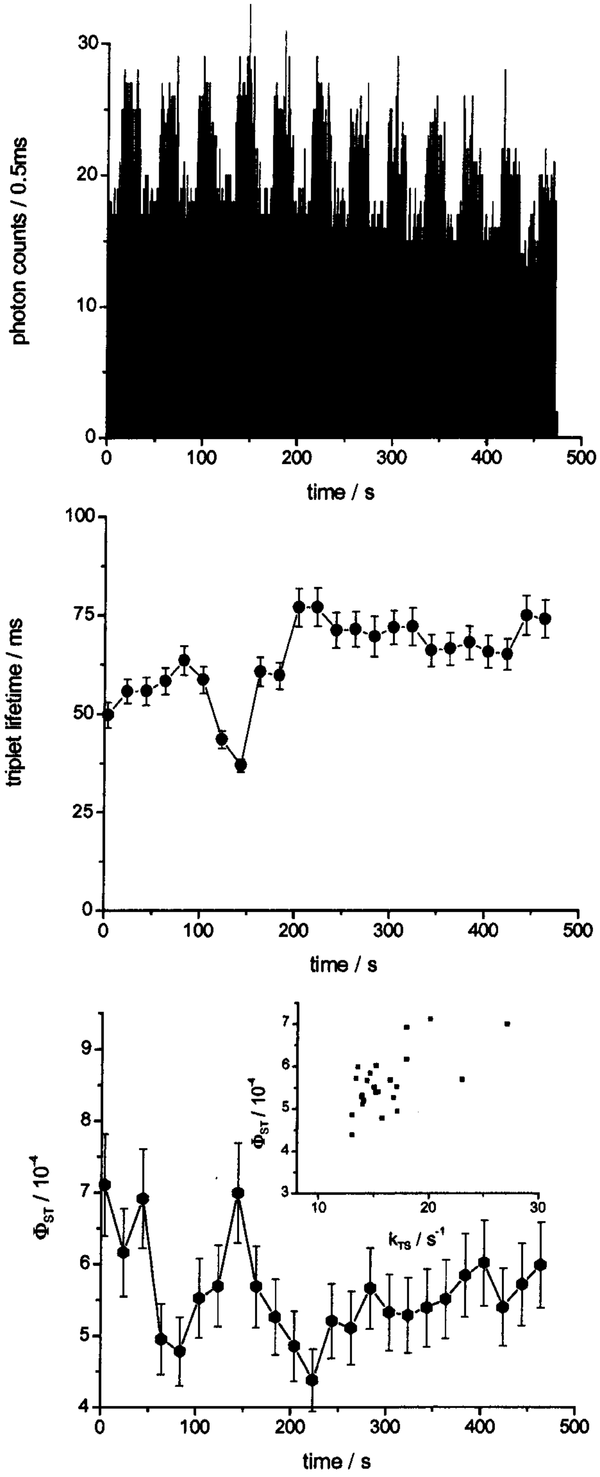

Figure 4. Upper part: Fluorescence intensity trace for a single molecule of compound **1**. The excitation polarization was changed by $\pi/2$ every 20 s. Middle part: Extracted triplet lifetime in milliseconds for every 20 s segment. Lower part: Values for the quantum yield of intersystem crossing from S_1 to T_1 for every 20 s segment. The small inset displays the correlation of the changes of the two intersystem crossing parameters.

2. Photophysical Parameters of the Cyanine Dyes Studied. The histograms of the Φ_{ST} for the transition $S_1 \rightarrow T_1$ and of the rate constant, k_{TS}, for $T_1 \rightarrow S_0$ are depicted in Figure 5 for the compounds **1**, **2**, **3**, and **5**. Traces were only recorded for molecules that displayed blinking behavior in the acquired image. Apparently, a few spots exhibited a very uniform intensity distribution. These spots were assigned to derive from

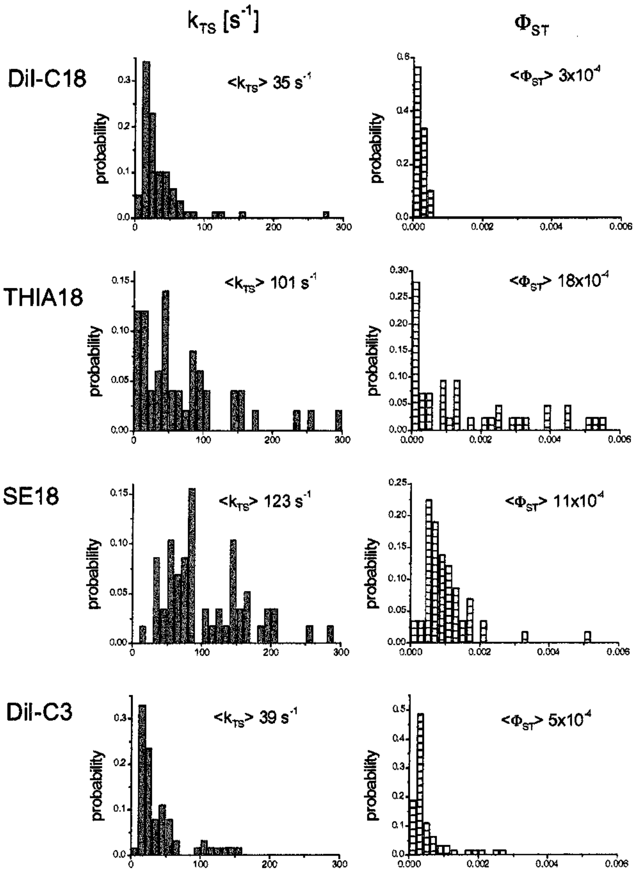

Figure 5. Probability distributions of the rate constant for intersystem crossing from T_1 to S_0, k_{TS}, in the left row and the quantum yield of intersystem crossing from S_1 to T_1, Φ_{ST}, right parts. From top to bottom: compound **1**, DiI−C18, (79 molecules); compound **2**, THIA18, (52); compound **3**, SE18, (56); compound **5**, DiI−C3, (69)

molecules interacting with the metal coating as such uniform spots were not observed for uncoated samples. The interaction of dye molecules with metal surfaces can result in altered behavior.[34] Unfortunately, neither the degree nor the depth of the penetration of aluminum into the polymer film is known.

To determine the on and off periods, always the first 20 s of each recorded trace were analyzed, for it was shown that in rigorously degassed samples the dynamic disorder is smaller than 2% for this time range.[1] A clear increase of the k_{TS}-values, the inverse of the triplet lifetime, is observed by increasing the atom number at the 1- and 1′-position, which demonstrates that the internal heavy atom effect has a genuine impact on the on−off kinetics of single molecules. As to compounds **1** and **2** the mean value of the rate constant k_{TS} triples from 35.2 s^{-1} for **1** to 100.6 s^{-1} for **2**, resulting in a mean triplet lifetime of 28.4 ms for **1** and 9.9 ms for **2** (see Figure 5). The second value is in the same order of magnitude as the 6.5 ms that was found for bulk measurements in poly(vinyl alcohol).[15] The smaller triplet lifetime obtained for compound **1** compared to the ones published by English et al.,[1] can be explained by the different host matrix. The polymer used in this study was purely aliphatic, whereas in Ref. (1) the host was poly (methyl methacrylate). The differences in triplet lifetime can be either due to environmental properties or to an overall higher remaining oxygen content in the sample, as oxygen dissolves better in less polar matrixes.[35]

Also the Φ_{ST} is affected by the heavy atom substitution. Values of 2.8×10^{-4} for compound **1** and 18.1×10^{-4} for **2** are found, which is a 6-fold enhancement for the heavy atom compound.

One of the strongest points of single molecule spectroscopy is the elucidation of inhomogeneous distributions. Not only the mean value shifts for the two distributions for Φ_{ST} and k_{TS}, but upon visual inspection an increase of the variance seems to be present as a consequence of the substitution of the atom at the 1- and 1′-position. While for compound **2** many molecules exhibit a decreased triplet lifetime compared to compound **1**, there are still molecules that exhibit a very long off time. It is quite unlikely that the samples used to measure the sulfur compound were more inhomogeneous than the ones for **1**, as they were prepared in exactly the same manner. Thus, we conclude that the variance in the distributions for compound **2** are produced by an additional parameter. This could be the presence of conformations of the molecules for which the spin−orbit coupling is weaker than for other ones.

Compound **3**, bearing selenium atoms at positions 1 and 1′, was also studied. It was reported about cyanine dyes that the intersystem crossing rate increases by approximately one order of magnitude on going from sulfur to selenium at the 1- and 1′-position.[19] The values of Φ_{ST} and k_{TS} retrieved from single molecule fluorescence intensity traces do not reveal such an enhancement, but as displayed in Figure 5 the distributions are shifted to faster rate constants and higher quantum yields. However, the mean values are 122.9 s^{-1} and 10.2×10^{-4} for k_{TS} and Φ_{ST}, respectively. The reason for this is the small number of photons that are emitted by molecules that possess an enhanced Φ_{ST}. Assuming an enhancement of the intersystem crossing rate constant by an order of magnitude on going from sulfur to selenium and having in mind the 6-fold enhancement from carbon to sulfur, this sixty times higher rate constant eventually means that if compound **1** emits on average sixty photons in each on period, compound **3** only exhibits an average of one photon in each on period. Consequently, these molecules can hardly be addressed and the distributions of the retrieved values are biased to lower rate constants. The variance of these distributions also seems to be larger than for the distributions of compound **1**, following the same trend as the data acquired for **2**.

To complete the series of frequently used cyanine dyes, the oxacarbocyanine OXA18 **4** was studied. However, despite the fact that this compound is widely used in research,[36−38] it photobleaches very fast, even when the samples are freshly deoxygenated. Accordingly, only a few molecules had a sufficient survival time to collect enough data for analysis. This fast photobleaching could be explained by the high electronegativity of the oxygen atoms inducing an increased partial positive charge on the vicinal carbon atoms at the 2- and 2′-position. Addition of any nucleophile will result in the loss of conjugation or a retro aldol reaction,[39] and hence, in the loss of absorption and fluorescence. Very likely this step also accounts for photobleaching of the other cyanine dyes.

To date the reason for the fluctuations in the triplet lifetime during the course of the acquisition of the fluorescence intensity traces (dynamic disorder) is not well understood for systems similar to the studied cyanines. These fluctuations have a 2-fold appearance. Sudden changes of the triplet lifetime can be observed or very rarely off times in the order of several seconds are found. A common explanation is trans-cis isomerization, which was previously proposed to explain temporal fluctuations for DiI molecules on glass.[5] However, free volume is needed in order to undergo conformational changes. If a triplet is populated from a highly twisted state, there could be occasionally sufficient free volume to allow a further relaxation to a cis-triplet and the corresponding ground state. Also internal conversion from a highly twisted singlet can yield with high probability a cis-ground state, if sufficiently free volume is present. While in solution the latter is supposed to live micro- or nanoseconds,[22,23] it will persist much longer in the solid state. This can explain the very long off times (lasting seconds) occurring very infrequently.

We examined the impact of the torsional freedom by studying two different DiIs having either long aliphatic chains at the nitrogen atoms (DiI−C18, **1**) or short aliphatic chains (DiI−C3, **5**). The smaller demand for free volume for compound **5** to twist its skeleton could favor the formation of the twisted state. Figure 5a and d display the histograms for Φ_{ST} and k_{TS}, with the mean values given in each panel. For convenience the integrals are normalized to unity. As one can see, the rate constant for the depletion of the off state is hardly affected, whereas Φ_{ST} doubles. The relevance of this increase is further corroborated by the fact that the mean survival time until the molecules photobleach is longer for DiI−C18 **1** (236 s) than for DiI−C3 **5** (146 s), as photobleaching is supposed to occur more likely in the triplet state. We attribute the doubling of Φ_{ST} to an additional pathway for the transition $S_1 \rightarrow T_1$. The off times do not exclusively stem from a triplet state that is directly fed by the transition $S_1 \rightarrow T_1$ in a planar conformation, but also via the formation of a twisted conformation which is most probable a zwitterionic or biradical-ion state.[22,23,40] This twisted state has a higher probability to undergo ISC to the triplet state, as the energy difference between S_1 and T_1 in the twisted conformation is smaller.[17,21] Hence, the process is more efficient. Actually, in the framework developed by Momicchioli and Ponterini[23] the singlet triplet splitting should go to zero in this perpendicular conformation. Although Ponterini argued that the symmetry of the perpendicular singlet and triplet states is unfavorable for efficient spin−orbit coupling,[41] the same argument holds for the planar states.[42] Hence there is no direct argument why in this perpendicular state the smaller singlet triplet splitting should not lead to faster intersystem crossing compared to the spectroscopic accessible trans and cis conformations. While the planar triplet state will relax in this framework to the all trans conformation the perpendicular triplet state can relax to a trans or cis triplet state followed by intersystem crossing to the trans or cis ground state. As the equilibrated triplet state is generally a planar species its decay will not be enhanced by a larger conformational mobility in agreement with the experimental results.

From these considerations we deduce an energy diagram, which is portrayed in Figure 6.[20−23] The proposed parallel pathway to populate the triplet state very likely accounts for the increased Φ_{ST} for the short chain compound **5** and could also account for the changes in triplet lifetime for different time periods in one single molecule fluorescence intensity trace as a consequence of changes in the free volume experienced by the single molecule. It has to be pointed out that the two ways to populate the triplet state cannot be resolved in the analysis, because the transition takes place via competitive pathways from the same state (S_1) to the same state (T_1). However, assuming that the quantum yield for the population of the triplet state via the twisted conformation is virtually zero for DiI−C18, an upper limit for the quantum yield $\Phi_{ST,\ Twist}$ can be given. This is the difference between the two calculated Φ_{ST}-values, hence, 2×10^{-4}. Which modes induce internal conversion or intersystem crossing in the molecules with longer chains or experience a smaller free volume is highly speculative.[40] Although the conditions present in the single molecule experiments do not suppress the asymmetric stretching[40,44] as much as the disrotatory mode leading to the perpendicular conformation[40] and the corresponding conical intersection, we have no evidence to confirm the role of this asymmetric stretching.

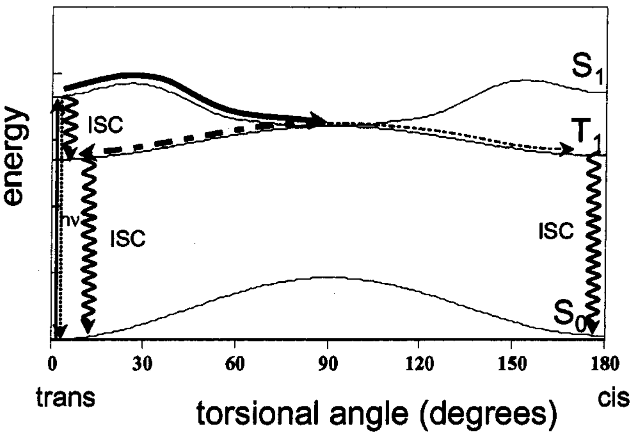

Figure 6. Schematic diagram of potential energy surfaces involved in radiative and nonradiative deactivation of a single cyanine dye molecule. The activation barriers from ground and excited state were taken from refs 22 and 23, while the singlet excitation energy and singlet triplet splitting were obtained from experimental data and a PPP calculation.[43]

The occurrence of persistent off states cannot be explained directly by this specific scheme. However, as mentioned before, assuming that the energy barrier to the cis-isomer will be different for each molecule, one can still imagine that isomerization can take place very rarely. The cis-configuration can in the framework of Figure 6 become populated from the twisted singlet excited state either directly by internal conversion or indirectly by intersystem crossing followed by relaxation to a cis-triplet. However, in a rigid matrix relaxation to a trans ground state or triplet is predominant after internal conversion or intersystem crossing from an excited twisted singlet state. This should have no influence on the triplet lifetimes, but could account for the long off times. As stated above, we observed also for 0.5% of all traces one sudden orientational leap, which requires substantial free volume. Whether there is a link between these orientational leaps and the probability for trans-cis isomerization still needs a more direct experimental verification.

Conclusions

The off-time characteristics of single molecule fluorescence intensity traces were investigated for different cyanine dyes. The hypothesis that off times observed in fluorescence traces of cyanine dyes are principally due to the formation of the triplet state, which to date was only based on the oxygen dependence of these kinetics, could be substantiated by means of several experiments. The heavy atom substitution on the 1- and 1′-position led to an altered appearance of the off-time kinetics, which proved that the triplet state accounted for the off times. In agreement with recent studies we found Φ_{ST}-values in the order of $10^{-3}-10^{-4}$.[1,5] These values do coincide nicely with the values found in solution. However, for part of the recorded data an additional pathway was proposed involving torsional motion of the chromophore. This affected the on−off behavior in such a way that cyanine dyes with a smaller demand on free volume showed enhanced triplet formation.

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