Cent. Eur. J. Chem. • 12(9) • 2014 • 937-952
DOI: 10.2478/s11532-014-0544-0

VERSITA

Central European Journal of **Chemistry**

A multi-property fluorescent probe for the investigation of polymer dynamics near the glass transition+

RICCCE 18

Joanna R. Siekierzycka[1], Catharina Hippius[2], Frank Würthner[2], René M. Williams[1], Albert M. Brouwer[1*]

[1]*Van't Hoff Institute for Molecular Sciences, University of Amsterdam, P.O.Box 94157, 1090 GD Amsterdam, The Netherlands*

[2]*Institut of Organich Chemistry, Würzburg University, 97074 Würzburg, Germany*

Received 5 September 2013; Accepted 20 December 2013

Abstract: In addition to the commonly observed single molecule fluorescence intensity fluctuations due to molecular reorientation dynamics, a perylene bisimide-calixarene compound (**1**) shows additional on-off fluctuations due to its ability to undergo intramolecular excited state electron transfer (PET). This quenching process is turned on rather sharply when a film of poly(vinylacetate) containing **1** is heated above its glass transition temperature (T_g), which indicates that the electron transfer process depends on the availability of sufficient free volume. Spatial heterogeneities cause different individual molecules to reach the electron transfer regime at different temperatures, but these heterogeneities also fluctuate in time: in the matrix above T_g molecules that are mostly nonfluorescent due to PET can become fluorescent again on timescales of seconds to minutes.

The two different mechanisms for intensity fluctuation, rotation and PET, thus far only observed in compound **1**, make it a unique probe for the dynamics of supercooled liquids.

Keywords: *Fluorescence • Electron transfer • Rotation • Single molecule*

1. Introduction

Numerous amorphous materials, such as glassy solids, viscous liquids or polymers have been studied during the past decades [1,2] yielding a variety of reports on heterogeneities of the structural relaxation of these systems [3,4]. Relaxation times in polymers spread over a very broad time range. The reorientation of the side groups of polymer chains occurs on the nanosecond timescale [5] whereas the so called "physical aging" can be observed within hundreds of hours [2,6]. There are also several other viscoelastic mechanisms, for example sub-Rose modes and Rose modes [7-9], which contribute to the softening of the polymer matrix upon increasing the temperature. When the temperature is lowered toward the glass transition temperature (T_g), the relaxation times increase drastically until the system becomes frozen in the glassy state.

Reported heterogeneities in polymers can be classified in two groups; the first group refers to spatial diversity [10-17] of the probed property that is not the same for different parts of the sample: static spatial heterogeneity, which can be found below the T_g of the material. Another group describes temporal inhomogeneity referring to fluctuating local properties [18]. These "dynamic heterogeneities" rely on an exchange between "fast" and "slow" relaxations at the same spatial point. In order to obtain reliable information on the material dynamics the experimental method chosen has to be sensitive to the changes occurring on the timescale of the material relaxation. Many experimental techniques have been used to study these relaxation inhomogeneities, such

* *E-mail: a.m.brouwer@uva.nl*

+ *The article has been presented at the 18th Romanian International Conference on Chemistry and Chemical Engineering - RICCCE18 - held in New Montana, Sinaia, Romania on 4-7 September, 2013.*

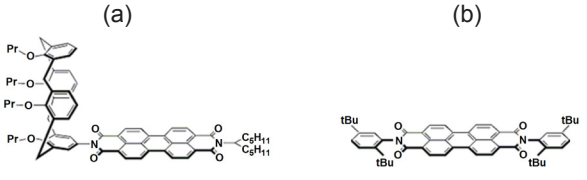
as differential scanning calorimetry (DSC), [6] dielectric spectroscopy (DS) [19-22], time-resolved emission spectroscopy (TRES) [5], nuclear magnetic resonance (NMR) [23], atomic force microscopy (AFM) [14], mechanical spectroscopy [24], and single molecule spectroscopy (SMS) [13,18,25,26]. These studies have yielded a variety of models describing the observed relaxation phenomena. Application of ensemble techniques has shown that most relaxation processes in amorphous polymer matrices are very complex and cannot be described using a simple monoexponential model as a response function to an external perturbation. Two phenomenological explanations of this phenomenon have been proposed. According to one explanation, all relaxation decays in different parts of the sample are nonexponential, yielding a nonexponential relaxation of the ensemble. Another model assumes that the nonexponential decay is a result of a sum over many monoexponential relaxations with different time constants.

In order to investigate all the heterogeneities in polymer films, it is important to be able to observe distributions of the properties and not only to probe their averages. One such method that avoids ensemble averaging is single molecule fluorescence detection [27-30]. The orientation changes of fluorescing molecules [13,31], fluorescence decay time variations [32-34], blinking behavior [35] or/and spectral shape changes [36,37] give information about their direct environment. The dynamic heterogeneity can be revealed in the measurement on individual molecules over time while the static heterogeneity will show up by comparing populations of molecules embedded at different locations in the matrix.

Here we describe measurements with ensemble and single molecule methods, which allow to access and investigate polymer dynamics in the glassy and viscoelastic states appearing at different timescales. Performing confocal microscopy and wide field imaging of individual molecules allows analyzing the rotational dynamics, correlation decays of this dynamics, average intensities of molecules, decay times of fluorescence as well as fluorescence on/off behavior of the molecular probe embedded in the polymer matrix at different temperatures.

In the experiments we use a calixarene-functionalized perylene bisimide (PBI) probe molecule (**1**, Scheme 1). This molecule is composed of the electron-poor fluorescent reporter dye PBI and a bulky electron-rich calix [4]arene subunit. Investigations by steady-state and time resolved fluorescence spectroscopy showed that an efficient photoinduced electron transfer from the calixarene to the PBI leads to efficient quenching of the PBI fluorophore even in solvents of low polarity such as toluene [38]. The molecule has already been shown to be a useful probe for sensing viscoelastic properties of polymers, *i.e.*, for poly(methyl acrylate) [39]. In previous work [39], we showed using wide field fluorescence imaging (WFI) that the fluorescence emission of the probe molecules **1** embedded in a polymer matrix can be switched on and off reversibly by changing the environment from the supercooled liquid to the glassy state and back. The switching-off of the fluorescence of our molecular probe was attributed to the availability of sufficient free volume for the molecule to reach the geometry needed for efficient excited-state electron transfer, which quenches the fluorescence.

In this study, the measurements were performed on **1** embedded in poly(vinyl acetate)) (**pvac**, T_g = 28°C, M_W = 26 kg mol^{-1}) as the matrix. All measurements were performed in a temperature window from 21 to 41°C. In this paper, we zoom in on the fluorescence dynamics of individual molecules using confocal detection. Intensity fluctuations due to overall molecular rotation occur, as seen in other studies [26,40], but there is an additional blinking process related to the apparent on/off switching which was detected in the wide field experiments [39]. The fluorescence of **1** depends very strongly on the state of the matrix and can be switched on and off reversibly many times by changing the temperature around T_g. The frequency of the off/on switching and duration of the off periods at different temperatures may give information on the polymer rheology.



Scheme 1. Molecular structures of (a) 1 and (b) reference dye 2.

2. Experimental procedure

2.1. Materials

Spectroscopic grade toluene and *N,N'*-bis(2,5-di-*tert*-butylphenyl)-perylene-3,4:9,10-bis(dicarboximide) (**2**) were purchased from Sigma Aldrich Co.; poly(vinyl acetate) (**pvac**) was purchased from Scientific Polymer Products Inc. Both were used without further purification. Compound **1** was available from previous work [38,41].

2.2. Confocal microscopy measurements

The confocal measurements were performed using an inverted light microscope (IX71, *Olympus*) equipped with an oil immersion, 100×, 1.4 NA objective (UPlanSApo, *Olympus*), mounted on a piezo-scanning stage (*Physik Instruments GmbH*). The excitation light source was a Ti:Sapphire laser (Chameleon ULTRA-II, *Coherent*) combined with second harmonic generator (*APE*) resulting in an excitation wavelength of 488 nm and repetition rate of 80 MHz. Light emitted from the sample was passed through a dichroic mirror and a pinhole of 100 μm in diameter, and imaged onto or two avalanche photodiodes (SPAD). In the experiments on the ensemble level, the emitted light passed a polarizing beam splitter and the two orthogonally polarized beams were sent to SPAD 1 and SPAD 2. In the case of experiments on individual molecules the emission was detected by a single SPAD. The limiting anisotropy at the ensemble level was evaluated using *N,N'*-bis(2,5-di-*tert*-butylphenyl)-perylene-3,4:9,10-bis(dicarboximide) **2**, as a reference compound. Compound **2** was embedded in a rigid medium, poly(styrene), in order to avoid the effects of Brownian motion. The limiting anisotropy was calculated according to Eq. 1 [42]

$$r(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{|}(t) + 2I_{\perp}(t)} \quad (1)$$

where $I_{\parallel}$ and $I_{\perp}$ are fluorescence intensities detected in the parallel and the perpendicular channel, respectively.

The fluctuations in the fluorescence intensity of molecules due to rotational dynamics were measured using the same setup. Rotational dynamics were characterized by calculating the autocorrelation function of the fluorescence trajectory for each molecule according to Eq. 2:

$$C(\tau) = \frac{\left\langle \left(I(t) - \left\langle I(t)\right\rangle\right)\left(I(t-\tau) - \left\langle I(t)\right\rangle\right)\right\rangle}{\left\langle I^2(t)\right\rangle - \left\langle I(t)\right\rangle^2} \quad (2)$$

where the angled brackets < > indicate an average over time.

The excitation power for experiments performed at higher temperatures was about 14 μW allowing for long term measurements on single molecules. Due to the relatively fast degradation of molecules of **1** excited with such a power at low temperature (no rotational diffusion of the compound), these measurements were performed with 10-fold lower excitation power. In order to decrease photobleaching of dye molecules, experiments were performed in a nitrogen atmosphere.

2.3. Wide field imaging (WFI)

WFI was performed with excitation at 488 nm. The sample was contained in an atmosphere of nitrogen. A Berek polarization compensator (*New Focus*) was used to modify the polarization of the laser light from linear to circular.

2.4. Temperature control

Decreasing temperature of the specimen below T_g of **pvac** was achieved by flushing the sample with cold nitrogen. The flow was faced directly to the spin-coated polymer film. For heating and temperature control the sample was enclosed in a climate chamber (INU, *Tokai Hit Co. LTD*). The temperature was measured in one point of the sample using a thermocouple Fluke 51 II.

3. Results

3.1. Confocal measurements – ensemble

The emission anisotropy measurement of **1** in **pvac** allows to obtain information on molecular mobility in the polymer matrix on the timescale of the excited state lifetime of the probe molecule (~ 3.5 ns [39]). When the sample is excited with linearly polarized light, a population of molecules with the transition moments oriented in a direction close to that of the electric vector of the excitation light is excited most efficiently. The emission ought to be anisotropic since the distribution of orientation of the excited dye molecules is anisotropic. Any change in the orientations of molecules during the lifetime of the excited state, however, will induce depolarization of the fluorescence and decrease of the anisotropy. As was reported [43], a temperature dependence of the anisotropy can be observed; for anthracene derivatives in polyisobutylene ($T_g \approx -75^{o}$C), as an example, it was shown that at temperatures below 7°C the anisotropy may be considered as constant with its value of about 0.4 whereas at higher temperatures it decreases linearly with the temperature to 0.2 at 167°C.

In the case of molecules with parallel absorption and emission transition moment the theoretical value of the so-called fundamental anisotropy, that is, the theoretical anisotropy in the absence of any motion, is 0.4. Values obtained experimentally are usually lower and range from 0.32 to 0.39. Some of the factors influencing the accurate measurement of the anisotropy comprise instrumental artifacts such as large cone angles of the incident and observation beams or imperfect polarizers. Observed differences of anisotropy may also be related to the fluorophore itself. At high enough concentrations of dye molecules (when the distance between them is of

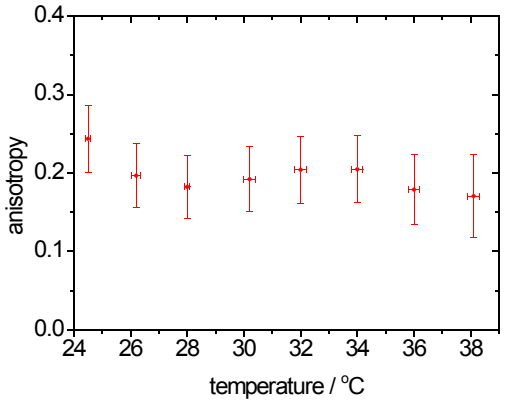


Figure 1. Anisotropy of **1** in **pvac** as function of temperature.

the order of a few nanometers) energy transfer can take place resulting in depolarization of the fluorescence from the sample. The fundamental anisotropy is an intrinsic property of the fluorophore and was shown to be 0.369 ± 0.002 for perylene and a number of its derivatives [44]. In the present case, the anisotropy was measured using confocal microscopy with linear polarization of the excitation light. Emission from the sample was collected by two independent SPADs, detecting two perpendicular polarizations of fluorescence light separated by a polarization beam splitter. The anisotropy obtained for perylene orange (**2**) in a rigid poly(styrene) (**ps**) matrix at 24°C was 0.32 ± 0.01. The lower than expected value we attribute mainly to the use of a large NA (1.4) objective used for the experiment [45,46]. In the case of **1** in **pvac** the anisotropy was lower (Fig. 1).

The samples were prepared using 5×10^{-8} M concentration of **1** in toluene containing 2% of **pvac**, which leads to average distances between molecules of a few hundred nanometers (100–200 nm) in a spincoated film. Such a concentration efficiently decreases the possibility of energy transfer between the dye molecules that could decrease the measured anisotropy. The value of the anisotropy of **1** in **pvac** is lower than the fundamental value. This suggests that depolarization of the fluorescence takes place. The dependence on the temperature in the small temperature window is insignificant. The decrease of the measured anisotropy in comparison to **2** in **ps** can be caused by photoinduced small librations of **1** in the polymer voids. Besides that, the movements during the changes of the geometry of **1** in excited and ground states may influence the position of the molecule in the polymer on a timescale of a few nanoseconds. A clear decrease of anisotropy in the rubbery state (above T_g = 28°C) is not observed. Thus, the freedom of the molecule to undergo large-amplitude rotational motion on the nanosecond timescale is strongly restricted even 10 degrees above T_g. As will be shown in the next section, such rotations in fact occur on a much longer timescale.

3.2. Confocal measurements – single molecules

Confocal microscopy measurements allowed us also to record fluorescence time traces and evaluate the fluorescence decay times of over 450 individual molecules at different temperatures (151 at room temperature, 250 at T_g = 28°C and 121 at 33°C). For each molecule **1** the fluorescence time trace was recorded (see Fig. 2a) upon excitation with linearly polarized light. The emission intensity of the individual molecule, fluctuating in time, reflects the changes in its in-plane and out-of-plane orientation resulting from Brownian diffusion; the excitation efficiency of the molecule is the biggest when the molecule's transition moment is oriented in a direction close to that of the electric vector of the excitation laser beam. When the molecule rotates, the probability of being excited changes and thus the fluorescence intensity fluctuates.

A single detection channel used in this experiment obtains a higher signal to noise ratio using lower excitation power than in the case of two detectors. Using lower excitation power leads in turn to longer photochemical lifetimes of the measured molecules. The disadvantage of using a single detector is the sensitivity of the measurement to laser power fluctuations; in our case the laser stability was satisfactory for performing experiments using one detector only. The fluorescence trajectories were built using a binning time of 20 ms.

According to Nyquist's theory [47], the length of the fluorescence time trace is one of the limiting factors for determining the maximum relaxation time of the environment of the probe molecule. For all molecules characterized by significant fluctuations in the fluorescence intensity only those with traces longer than 200 s were used for calculation of the autocorrelation function (for example: Fig. 2b). Each transient was characterized by a different correlation function with a different decay time. In order to extract the information on relaxation times from such functions, the curves are usually fitted with the Kohlrausch-Williams-Watt stretched exponential function [48] but also with a single exponential decay or just characterized by giving the time value at which the autocorrelation curve decayed to a certain value. None of the decays could be well fitted using a monoexponential model indicating possible temporal inhomogeneities of the relaxation time constants of individual molecules. The relaxation times were therefore obtained by simply reporting the time value for which the autocorrelation curve decayed to its half value (Fig. 2b).

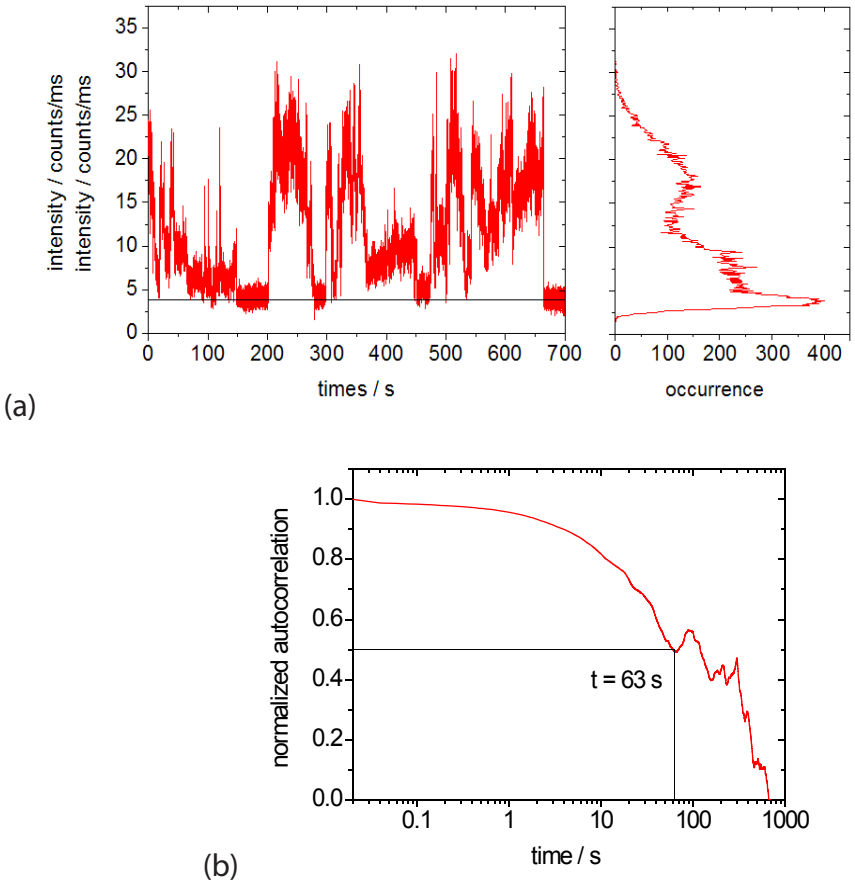


Figure 2. An example of a single molecule of **1** measured at 33°C (T_g + 5°C) (a) fluorescence trace with fluorescence intensity histogram (the black horizontal line shows the background level) (b) autocorrelation curve of the fluorescence trajectory with indicated time at which the autocorrelation function drops to its half value.

The half-decay times of the autocorrelation function of rotational motion of **1** at 2 different temperatures are shown in Fig. 3. The central values of these time distributions are 68 s and 22 s for measurements at T_g and T_g + 5°C respectively, showing that the correlation of the fluorescence signal drops faster at higher temperatures. In both cases the relaxation dynamics is found in the range of several tens of seconds.

A similar result was obtained by adding together the autocorrelation curves of the individual molecules and taking the half-decay time of the sum (see Fig. 4); the half-decay times obtained using this method were 52 s and 16 s for measurements at 28°C and 33°C respectively. Like the autocorrelation curves of individual molecules, the curve presented in Fig. 4 cannot be fitted well using a monoexponential decay.

These rotational correlation decay times give an indication of the timescale of the structural fluctuations of the surroundings of the molecular probes. Autocorrelation functions were not obtained for molecules investigated at 21°C since the dynamics at this temperature is too slow in comparison to the measurement time (see Fig. 5).

As mentioned before, the fluorescence trajectories give information about the rotational motion of molecules embedded in the polymer matrix.

From the fluorescence trace shown in Fig. 6, one can see that the rotational motion of a single molecule at high temperature shows different dynamics in different time intervals. For example, in the first 28 s a rather high frequency of rotation is observed when compared with the time range from 28 s to 36 s, when the molecule has nearly constant fluorescence intensity. Such a phenomenon was already observed and the explanation is that such distribution does not originate only from the presence of rotational diffusion but is caused by hopping between different “sites” in the polymer. In such “sites” a molecule can spend different periods of time and be characterized by different rotation rate [18,49].

During our measurements 85% of **1** at T_g and 33°C showed very dynamic changes of the fluorescence

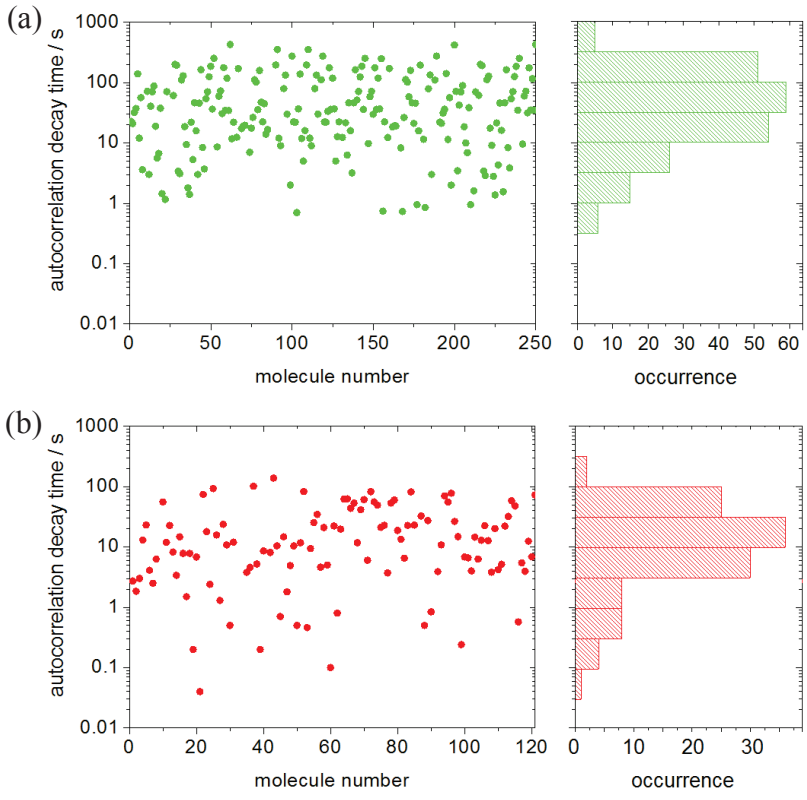


Figure 3. Distribution of the relaxation times obtained for 1 in pvac at (a) 28°C and (b) 33°C.

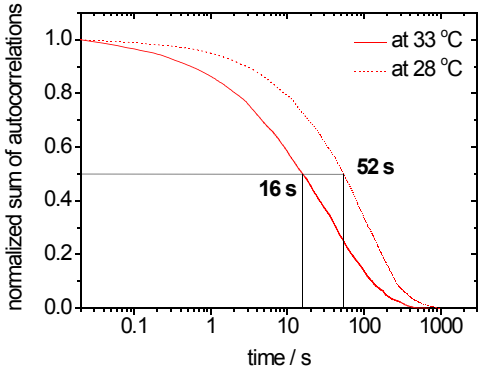


Figure 4. Sum of single molecule autocorrelation functions with indicated times at which autocorrelation drops to its half value.

intensity trajectories. Only a fraction of ca. 15% of the individual probes had a relatively smooth and narrow distribution of fluorescence intensities (before irreversible bleaching).

Besides dynamic fluctuations of fluorescence intensities due to rotation, long off periods of the fluorescence ranging from several tens of milliseconds up to several tens of seconds were observed for individual molecules of **1** at T_g (28°C) and at 33°C (Fig. 7). In general, off periods of fluorescence of individual molecules are attributed to triplet blinking [50,51], translational diffusion, spectral diffusion [51,52] or formation of transient chemical reaction products such as radicals [53-55]. The first phenomenon occurs on a microsecond timescale and so is too fast to explain the observed behavior of **1** in **pvac**. Wide field measurements showed no translational diffusion on length scales exceeding the excitation volume of confocal microscopy [56]. Also spectral measurements on individual molecules did not show spectral jumps of the probe molecules. Another explanation could relate to the use of linear polarization of the excitation light; the molecule could simply reorient in such a way that its transition moment is perpendicular to the direction of the electric field vector and the excitation efficiency drops to zero. This explanation, however, seems to be incomplete; the off periods are rather long when compared to the very dynamical reorientation of **1** in **pvac** at T_g and above. Based on our previous work, we assign the long off periods to the intramolecular photoinduced electron transfer (PET) that quenches the fluorescence. The requirement for PET is the availability of sufficient free volume around the molecule to reach

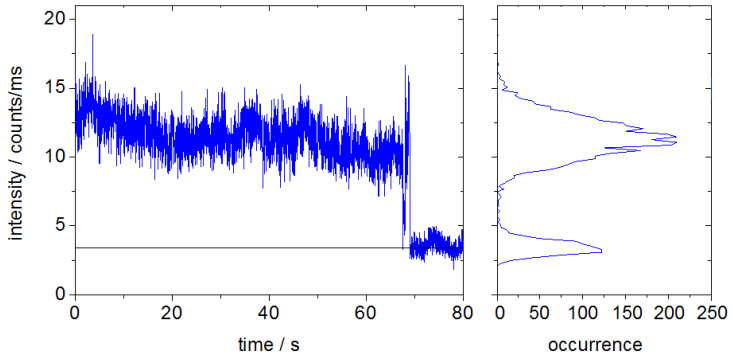


Figure 5. An example of a single molecule fluorescence trajectory with intensity histogram recorded at 21°C. The black horizontal line shows the background level.

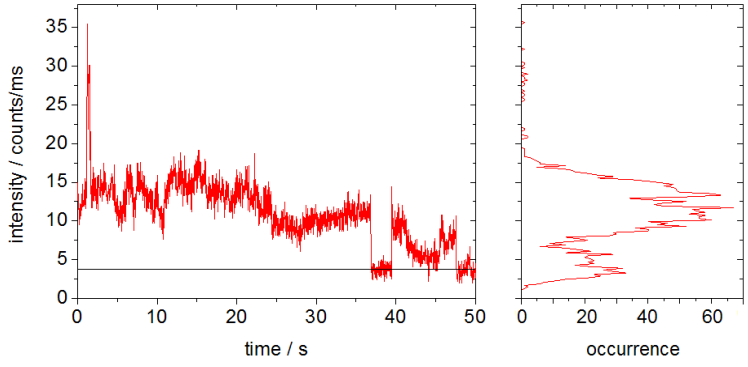


Figure 6. An example of a single molecule fluorescence trajectory with intensity histogram recorded at 33°C showing different types of dynamics. The black horizontal line shows the background level.

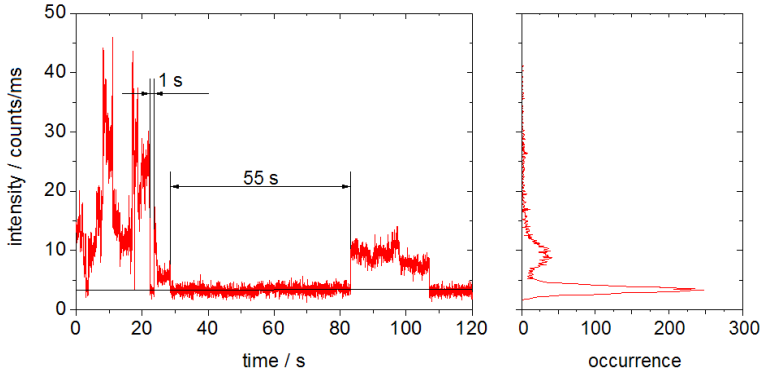


Figure 7. An example of a single molecule fluorescence trajectory and intensity histogram recorded at 33°C. The time trace is characterized by a long off periods. The black horizontal line shows the background level.

the geometry needed for efficient excited-state electron transfer.

The long off periods due to PET as well as the rotational dynamics of molecules influence their average fluorescence intensity. When molecules are fixed in the polymer matrix, the emission intensity will vary from molecule to molecule due to their different orientations with respect to the polarization of the excitation light. On the one hand, the population of molecules that have their transition moment parallel to the electric vector of the excitation light will be very efficiently excited during the time of measurement. On the other hand, the population of molecules with transition moment oriented perpendicularly to the polarization of the

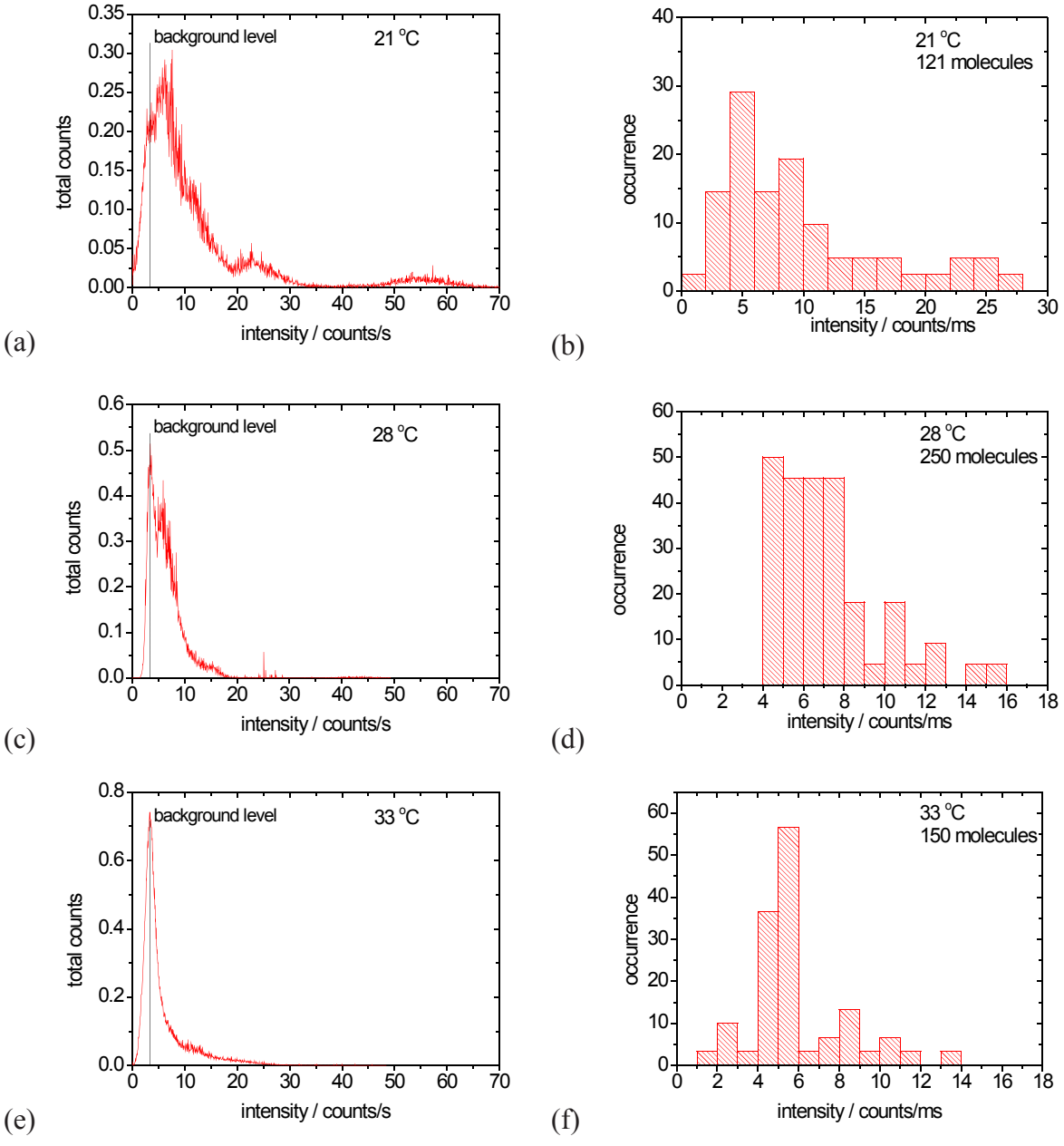


Figure 8. Average fluorescence intensities of molecules 1; (a), (c) and (e) weighted sum of the fluorescence intensity histograms of single molecules at 21°C, 28°C and 33°C respectively; (b), (d) and (f) histograms of average intensities of single molecules at 21°C, 28°C and 33°C respectively. The measurements at 21°C were performed using 10 times lower excitation power.

excitation light will not be fluorescent. As a result the distribution of average intensities in the population of molecules embedded in a polymer matrix and frozen in small, rigid voids should be very broad. In case of rotating molecules the distribution of mean intensities ought to be narrower and possible long fluorescence off periods due to PET are expected to additionally lower the mean intensity. In Fig. 8, histograms are shown of the average count rates for groups of molecules probed at 21, 28 and 33°C. The results indicate that there is indeed strong evidence for dependence of the average emission intensities of molecules on their dynamics. Figs. 8a, 8c and 8e show the weighted sum of the fluorescence intensity histograms (cf. Fig. 7) of individual molecules. The weights normalize the total number of counts in the fluorescence histogram of each molecule to assure the same influence of each molecule on the overall statistics.

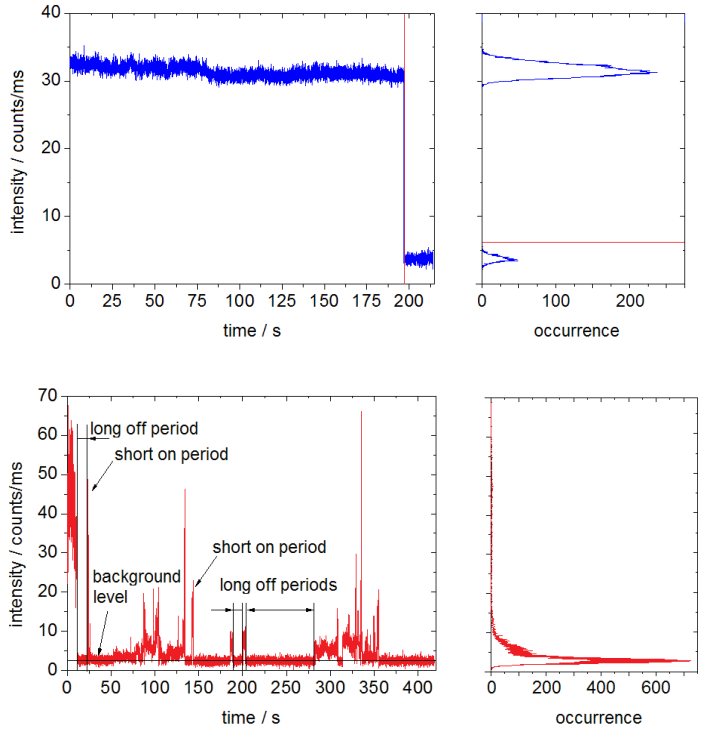


Figure 9. Examples of fluorescence time traces of **1** in **pvac** recorded at (a) 21°C and (b) 33°C.

The statistics includes only the periods of the fluorescence traces until the measured molecules photobleached. The presence of the long off periods, however, required introduction of an arbitrary parameter – the maximum waiting time - in order to distinguish photobleaching events from long off periods. The intensity of about 3 - 4 counts ms^{-1} is the background level, which is quite dominant in the fluorescence characteristics of molecules measured at high temperatures. These molecules are characterized by fluorescence time traces with many off periods and strongly fluctuating emission signals due to rotational diffusion and PET. The measurements at 21°C were performed with 10 times lower excitation intensity but despite that the intensity distribution (Fig. 8a) shows quite high emission intensities in comparison to the high temperature measurements.

The histograms in Figs. 8b, 8d and 8f were prepared by collecting the average intensities of molecules until they bleached (one value for one molecule). These characteristics also reflect the very dynamical behavior of molecules measured at high temperatures and show that average intensities of molecules decrease with increasing temperature.

In general the fluorescence time traces recorded for molecules at different temperatures show very different characters. Most of the molecules of **1** measured at 21°C showed a small fluctuation of the emission intensity and a very sharp one step bleaching (Fig. 9a) while the traces recorded at 28°C or 33°C are much more complicated (Fig. 9b).

The complete statistics on the off periods were performed on molecules measured at 21, 28 and 33°C. As shown in Fig. 10 the numbers of off periods as well as their durations are very different at different temperatures. At 21°C 41 off periods were found while measuring 151 molecules. The longest off period was about 14 s. A much higher number of 204 off periods per 250 measured molecules were found while measuring at T_g. The duration of the nonfluorescent periods of molecules increased to about half a minute. An even more drastic change was observed for measurements at 33°C. Over 170 off periods were found while only 121 molecular traces were analyzed. The duration of the off period was increased up to 100 s.

The occurrence of the one-step fluorescence bleaching of molecules, which is very distinct for molecules measured at room temperature, decreases very drastically with increasing temperature. At higher temperatures it is much more difficult to observe the photodegradation of the compound due to a very complicated character of the fluorescence time traces and existence of the long off periods. Increasing temperature causes also appearance of new features in

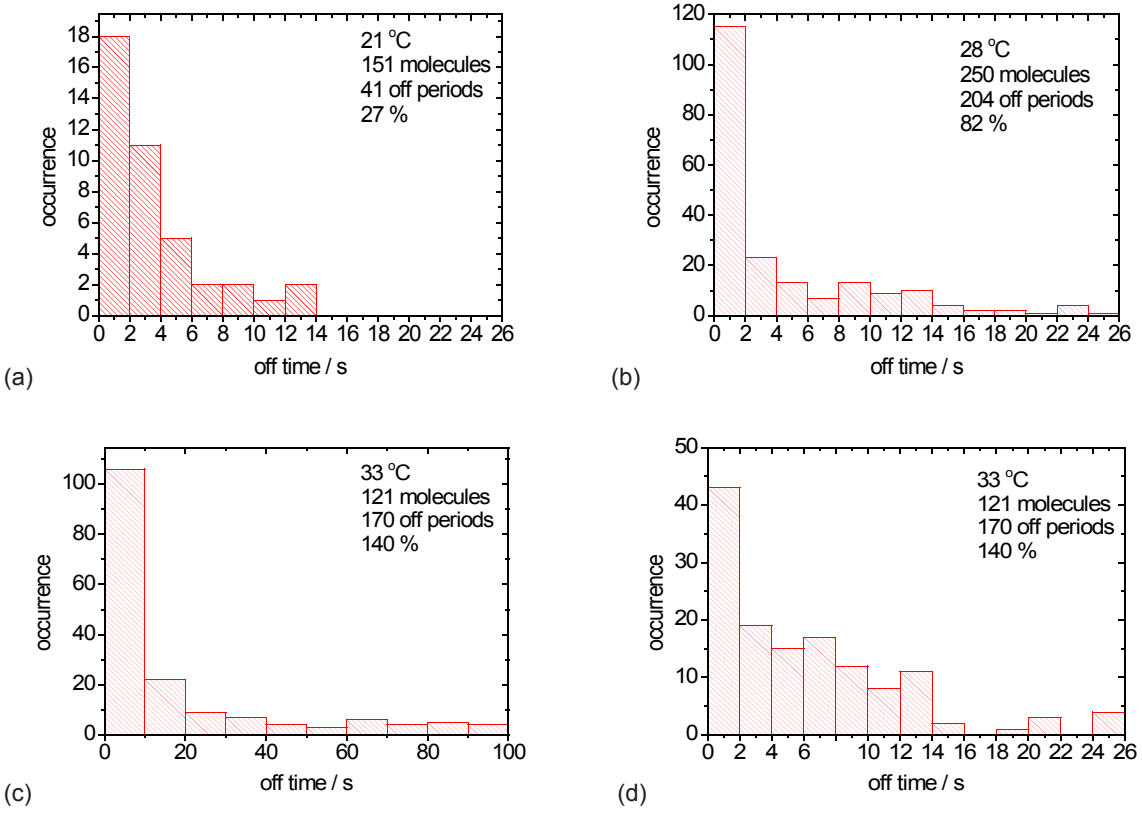


Figure 10. Histograms of single molecule off times at (a) 21°C, (b) 28°C and (c) and (d) at 33°C.

the fluorescence traces such as short on periods. There are time intervals from about several tens of miliseconds up to a few seconds in which the molecule (being in an overall off state) shows a very intense fluorescence. At each temperature there are also molecules undergoing rotational motions (tumbling) inside the voids of the polymer matrix. Such behavior, manifested in emission intensity fluctuations, makes it also very difficult to observe **1** bleaching, especially in the case of molecules with low emission intensity.

In order to characterize groups of molecules measured at different temperatures several features in their fluorescence traces were distinguished. This required applying criteria for defining these features, which necessarily introduced some arbitrariness into the analysis. One-step bleaching was assumed to happen if the fluorescence dropped to the background level within 20 ms (resolution of the traces) and stayed there for the next several tens of seconds. Traces with fluctuations of fluorescence without sudden jumps of the intensity (except one-step bleaching) were assigned to tumbling of molecules. Long off periods were assumed to be sudden intensity drops to the background level with the fluorescence recovery after several tens of milliseconds up to several tens of seconds. Very similarly were defined periods of intensive blinking with the difference that the signal drops to a certain level that was higher than the background (since with 20 ms binning the off periods in the microsecond range only lowered the signal). Periods of fluorescence surrounded by longer off periods are presented in the statistics as short on periods. The numbers characterizing the fluorescence traces of molecules **1** embedded in **pvac** matrix are shown in Fig. 11.

All features of the fluorescence trajectories mentioned here can be related to the viscoelastic properties of the polymer matrix in the close neighborhood of the probe molecule. Very intensive blinking and long off periods of fluorescence suggest that the molecule has enough free volume to undergo PET. On the other hand the molecule is not off for the whole time of the experiment, which implies that the polymer around it is also a dynamical system. In fact, the blinking dynamics are related to the dynamics of the polymer.

3.3. Wide field imaging

Wide field imaging allows recording fluorescence images of many fluorescing molecules at the same

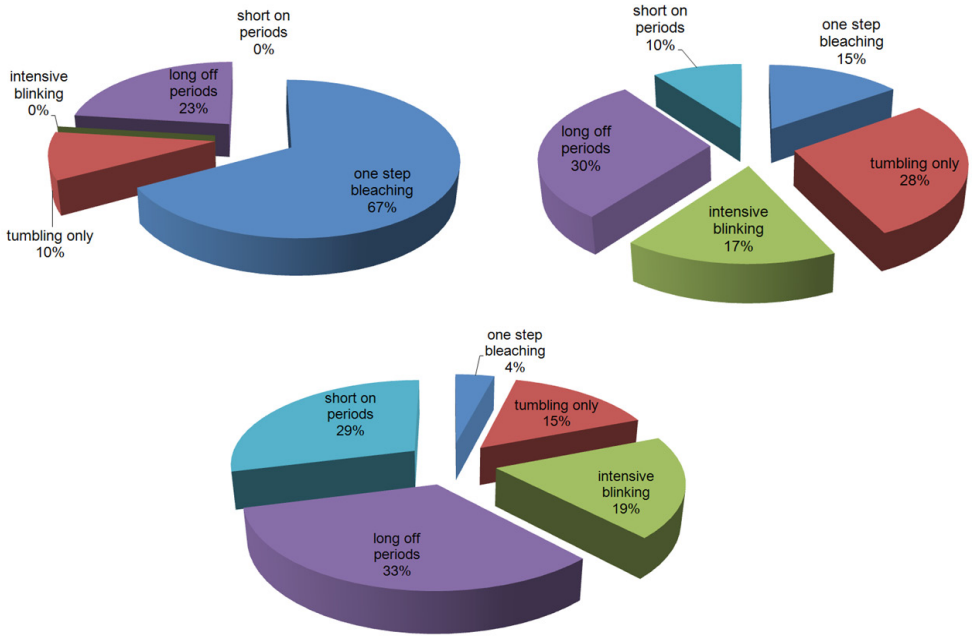


Figure 11. Characterization of the fluorescence time traces of groups of single molecules 1 embedded in pvac matrix and measured at (a) 21°C, (b) 28°C and (c) 33°C.

time. In our previous study [39] we used this technique to monitor the density of molecules **1** as a probe for sensing the changes that occur when a polymer is taken through its glass transition by heating or cooling. The molecule-counting method applied to the measurements on polymers at different temperatures gives direct information about the state of the close neighborhood of the molecule, the fluorescence of which can be either on or off for most of its measurement time. Temporal changes in the material (dynamical heterogeneities) can be monitored by following the fluorescence behavior of molecules on the same area frame by frame. The proper choice of the integration time can be crucial for observation of dynamics at different timescales. On the one hand observations of rotational motion of molecules (on a timescale of seconds or its fractions), using defocused imaging [31], requires short enough integration time. The molecule-counting method, on the other hand, should be performed using accumulation times long enough to capture fluorescence from all emitting fluorophores, also these that are rotating or have smaller active cross section for the excitation.

Here we have performed variable-temperature single molecule counting measurements on **1** embedded in **pvac**. Previous measurements on such a system showed that the number of fluorescent molecules for a given surface area as a function of temperature decreases smoothly from a constant maximum value at T < 20°C to a low background level at T > 36°C. Results of the experiment with slow temperature changes are shown in Fig. 12. The temperature of the film was decreased from 41°C to about 24°C, crossing the T_g at 28°C, and then increased again up to 41°C. Ten images of different areas of the sample were taken at each temperature. Average numbers of fluorescing molecules were obtained after equilibration for about 15 min at each temperature. The error bars show the standard deviation of the average number of molecules at the ten areas.

In these experiments the numbers of observed molecules decrease gradually to the background level at around 40°C and are very similar by cooling and heating the sample, except for small deviations that can result from spatial heterogeneity of the sample. By slow changes of temperature the polymer matrix has enough time to adjust to the applied load and little or no hysteresis was observed [57]. However, there was still a question about the local changes of the properties of the **pvac** when the temperature was changed up and down around the T_g: is the number of fluorescing molecules of compound **1** at different temperatures a matter of statistics or are always the same molecules on at a certain temperature while others are always off?

Confocal measurements on single molecules described above partially answer this question. Especially the measurements performed at 33°C show that the fluorescence time traces of individual probe molecules are very complicated, with frequent long off periods.

Recording a small area of the sample with slow temperature changing allows us to probe the dynamical changes of local properties in the polymer matrix. In our previous work [39] it was shown that switching the temperature far enough below and above the T_g of a softer polymer (poly(methyl acrylate)) caused appearance and disappearance of the same molecules [39]. The experimental conditions were, however, set in such a way that at high temperature basically all molecules were off and and at low temperature all molecules were on. Fig. 13 presents an example of a measurement in which the temperature was switched between 30°C and 24°C reversibly in two cycles. The applied temperature range allowed evaluation of the behavior of probe molecules in a much smaller window around T_g. The time between recording subsequent images was about 1 hour. The average number of molecules follows the temperature changes but deeper analysis allows one to distinguish four different groups of molecules. Examples of molecules belonging to each of these groups are marked with different-color circles in Fig. 13.

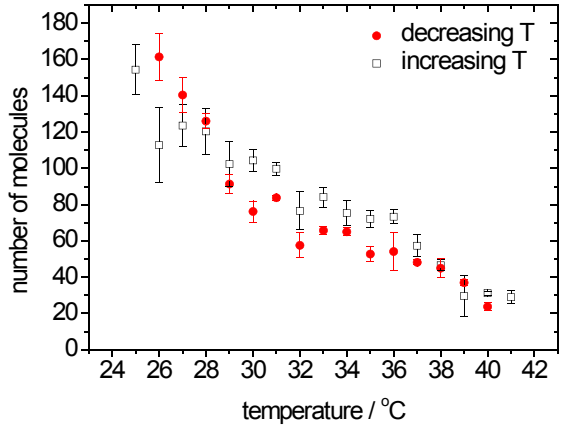


Figure 12. Average numbers of fluorescing molecules observed in a 100 nm thick **pvac** film on 10 areas of 26×26 μm at different temperatures.

The first group of molecules (green circles) shows irreversible bleaching and the fluorescence is not observed on the next images. The second group (yellow circles) is made by molecules that stay always on – temperature changes do not affect their emissive properties. The **pvac** matrix at these positions stays rigid enough to block electron transfer of **1** and thus the molecule is fluorescent. Red circles represent molecules that are off at high temperature and on at low temperature. There is also group four (blue circles), comprising **1** which behave in the opposite way to those of the previous group.

The series shown above may indicate that the dynamics of the polymer matrix is very slow; in the timescale of the cyclic changes of temperature. There is, however, a question about the behavior of probe molecules at each temperature on shorter timescale. Fig. 14 shows images recorded on a certain area of the sample at constant temperature with 1 s steps. Some molecules stay on (at least for the time of the experiment) while others get on and off many times suggesting the existence of dynamical processes taking place in the polymer matrix in the close neighborhood of such molecules.

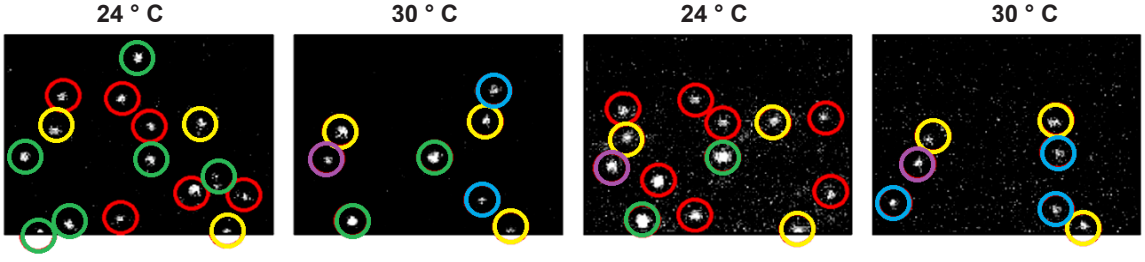


Figure 13. Sequence of wide-field images of one area of sample containing **1** embedded in a **pvac** film showing two cycles of changing temperature from 24°C and 30°C. Size of the images ca. 13×9 μm. Accumulation time: 1 s.

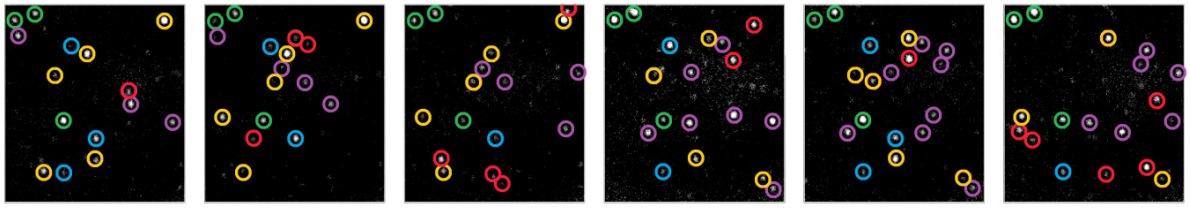


Figure 14. Sequence of wide-field images of one area of a sample containing **1** embedded in a **pvac** film recorded at 30°C. Size of the images 17×17 μm. Accumulation time of the image: 1 s.

The variety of colored circles in the series of images from Fig. 14 shows differences in fluorescent behavior of molecules. Molecules marked with green circles are on in every image indicating their positions in relatively rigid voids in the polymer. Blue circles surround molecules that are fluorescent on four out of six recorded images. The yellow, violet and finally red circles indicate decrease in the presence of individual molecules on recorded series of images. The disappearance of the fluorescence can be linked to the confocal observations of a very intensive blinking and existence of long off periods that lower the average intensities of molecules as the temperature is raised.

4. Discussion

By performing ensemble and single molecule measurements using **1** as a probe molecule we have investigated relaxation phenomena in a **pvac** matrix. Anisotropy measurements suggest that motions of probe molecules on a nanosecond timescale exist that cause a lowering of the fundamental anisotropy. A similar result has been reported for **pvac** at 25°C with time resolved emission spectroscopy using prodan (6-propionyl-2-(dimethylamino)naphthalene) as a probe molecule [5]. A decay component of several nanoseconds was attributed to relaxation of the interaction of the probe with the acetate moieties of the polymer matrix.

No temperature dependence of the anisotropy was observed in the temperature window of 14 degrees that was studied. No significant anisotropy changes were observed when passing the T_g: fast overall rotational motion of the whole molecule is restricted also well above T_g.

Confocal measurements performed on individual molecules allowed us to obtain relaxation times determined from autocorrelation functions of fluorescence trajectories. The correlation function of each molecule was distinctly unveiling differences in rotational dynamics of probes. The decays of the autocorrelations were nonexponential, as was found in other studies [58-60].

Using molecular probes embedded in the polymer for sensing relaxation processes based on their rotational dynamics is not straightforward. If the probe molecule is large in comparison to the size of the void in the polymer matrix, the rotation is slowed down and leads to an increase of the measured autocorrelation time [61,62]. Indeed, the work of Adhikari *et al*. [40] on **pvac** with T_g = 33°C revealed relaxation times of a few hundred seconds at T_g + 6.3°C up to a several tens of minutes around T_g. The decay time was assumed to be the time at which the autocorrelation function drops to zero, which is obviously much longer than the half-height value (cf. Fig. 4). Another factor that may contribute to the shorter relaxation times observed in the present work is that **1** is a larger probe molecule than rhodamine B that was used by Adhikari *et al*.

Although we used a very similar polymer the relaxation times distribution in the present study (Fig. 3) is different from the normal distribution in log scale presented in reference [40] and describing heterogeneous dynamics of the polymer. One of the possible explanations of this can be a finite trajectory length having a significant influence of the determination of the autocorrelation decay times [48,59,60,63]. Trajectories used for autocorrelation calculation that are 20 times longer than the calculated decay times can already have errors on the decay times of about 50%. The errors associated with short time traces used for autocorrelation decay estimation propagate also to other parameters characterizing the correlation function [26]. In the present study the obtained apparent decay times can be shortened since the trajectories used for determination of the correlation function decay were in some cases only twice longer than the decay time itself. The distribution of obtained decay times seems to be cut on the side of long decay times as compared to the distribution presented by Adhikari *et al*. [40].

Despite the uncertainties of the absolute decay time value, there is an obvious difference between the decay times of the intensity autocorrelation function obtained for measurements at T_g and T_g + 5°C: it is ca. three times faster at high temperature. Moreover the analysis of fluorescence trajectories unveiled the existence of long off periods of which the length and frequency of appearance is also temperature dependent. This phenomenon causes an additional shortening of the autocorrelation decay when the on or off periods are short. At high temperature (T_g + 5°C) the off periods (from several tens of milliseconds up to several tens of seconds) are observed more often and are noticeably longer than in the case of measurements at lower temperature (21°C). In the literature, several frequently encountered processes causing blinking are described. The most common source of characteristic short off periods in the intensity is the formation of the triplet state leading to a monoexponential distribution of off times [50]. Very long off periods (in the range of seconds) in the case of organic molecules were observed for example in the case of strongly coupled chromophores in dendrimers in which one chromophore being in an off state, such as the triplet state, would quench all the others [64]. Another explanation for long off periods can

be radical formation [54,65] or charge separation and charge transfer which in turn shows weak dependence on temperature [53]. In the case of compound **1**, we have assigned the blinking to the presence of photoinduced electron transfer from the electron-rich calixarene to the electron-poor perylene part of the probe molecule **1**. We proposed that intramolecular electron transfer requires a geometrical rearrangement of the molecule, which is easier to reach at higher temperature, when the polymer matrix has more free volume. The excited state electron transfer process does not require any structural reorganization of the medium leading to electrostatic stabilization. This is shown by the fact that photoinduced electron transfer occurs efficiently in nonpolar cyclohexane [39]. Possibly, the structural fluctuations of the matrix that govern the PET are related to the fluctuations that give rise to spectral jumps of other molecular probes [66].

Long off periods have a significant influence on the average fluorescence intensities of probe molecules. Here we have shown that the average intensity of molecules (until the irreversible photobleaching) depends on the temperature at which the measurements were performed. This finding indicates that the average intensities of molecules depend also on the viscoelastic properties of the polymer matrix. Wide field imaging shows the same observations from different perspective and with much lower time resolution. Disappearance of molecules by raising the temperature can be explained as switching them off for several seconds or simply decreasing their average intensities due to a very intensive blinking to the level which is not detectable using the wide field imaging method.

Due to existence of long off periods in the fluorescence trajectories of individual molecules of compound **1**, the fluorescence autocorrelation analysis is more complicated than for other molecular probes studied [12,40]. In the present case, it is difficult to separate the effect of blinking from the rotational dynamics of the molecule. Additional measurements on a similar compound, with similar dynamics but not showing PET, would shed some light on this problem. Furthermore, advanced detection techniques, *e.g.* using imaging with high frame rate and polarization sensitivity [62] may allow to turn the multi-facetted the photophysics of **1** to an unambiguous benefit for monitoring dynamics of polymers and other supercooled liquids. Such an experiment would also avoid the problem that it is difficult to locate single molecules that are mostly in the off state in a confocal scanning experiment.

5. Conclusion

The fluorescence of electron-donor-acceptor compound **1** is quenched by an intramolecular electron transfer process, which can be prevented by confinement in a matrix that provides little free volume. This phenomenon causes intensity fluctuations different from those caused by reorientation of the molecular transition dipole moment direction with respect to the polarization direction of the optical field. When a poly(vinylacetate) matrix containing **1** in very low concentration is gradually heated to above the glass transition temperature (28°C), the emissions of individual molecules are switched off one by one. This suggests a spatial heterogeneity of the polymer film, in which each molecule has a different environment. Closer inspection using single molecule intensity traces shows that molecules that appear to be off in wide field imaging detection still have short on periods, indicating that the environment fluctuates on a time scale of seconds to minutes at 33°C. At T_g the relaxation times are ca. 3 times longer, at temperatures below T_g they are not detectable because the molecules are “on” most of the time due to the constrained environment that prevents PET.

The long off periods, characteristic for **1**, may make it of potential interest for sub-diffraction-limited imaging methods [67-71]. The accurate positions of the fluorophores can be obtained from a series of imaging cycles with molecules stochastically switched on and off. In principle, the technique provides molecular-scale resolution of fluorescence images. In the present case the stochastic switching process can be observed in a medium that provides a fluctuation confinement.

Acknowledgment

This research was supported by NanoNed, a national nanotechnology program coordinated by the Dutch Ministry of Economic Affairs.

References

[1] M.L. Williams, R.F. Landel, J.D. Ferry, J. Am. Chem. Soc. 77, 3701 (1955)
[2] R.M. Kimmel, D.R. Uhlmann, J. Appl. Phys. 40, 4254 (1969)
[3] M.T. Cicerone, F.R. Blackburn, M.D. Ediger, J. Chem. Phys. 102, 471 (1995)
[4] H. Sillescu, J. Non-Cryst. Solids 243, 81 (1999)
[5] B.C. Barja, C. Chesta, T.D.Z. Atvars,

P.F. Aramendia, J. Phys. Chem. B 109, 16180 (2005)
[6] R. Svoboda, P. Pustková, J. Málek, Polymer 49, 3176 (2008)
[7] P.G. Santangelo, K.L. Ngai, C.M. Roland, Macromolecules 26, 2682 (1993)
[8] C.M. Roland, L.A. Archer, P.H. Mott, J. Rheol. 48, 395 (2004)
[9] K.L. Ngai, D.J. Plazek, A.K. Rizos, J. Polym. Sci., Part B: Polym. Phys. 35, 599 (1997)
[10] M.T. Cicerone, M.D. Ediger, J. Chem. Phys. 105, 5684 (1995)
[11] T. Xia, L. Xiao, M. Orrit, J. Phys. Chem. B 113, 15724 (2009)
[12] R. Zondervan, F. Kulzer, G.C.G. Berkhout, M. Orrit, Proc. Natl. Acad. Sci. U.S.A. 104, 12628 (2007)
[13] A. Schob, F. Cichos, J. Schuster, C. von Borczykowski, Eur. Polym. J. 40, 1019 (2004)
[14] E. Vidal Russell, N.E. Israeloff, Nature Chem. 408, 695 (2000)
[15] T. Ha, T.A. Laurence, D.S. Chemla, S. Weiss, J. Phys. Chem. B 103, 6839 (1999)
[16] F.R. Blackburn, M.T. Cicerone, M.D. Ediger, J. Polym. Sci., Polym. Phys. Ed. 32, 2595 (1994)
[17] C.Y. Wang, M.D. Ediger, J. Chem. Phys. 112, 6933 (2000)
[18] N. Tomczak, R.A.L. Valleé, E.M.H.P. van Dijk, M. García-Parajó, L. Kuipers, N.F. van Hulst, G.J. Vancso, Eur. Polym. J. 40, 1001 (2004)
[19] M. Tyagi, A. Alegría, J. Colmenero, Phys. Rev. E 75, 061805 (2007)
[20] K.L. Ngai, C.M. Roland, Polymer 43, 567 (2002)
[21] M. Tyagi, A. Alegría, J. Colmenero, J. Chem. Phys. 122, 244909 (2005)
[22] C.M. Roland, R. Casalini, Macromolecules 36, 1361 (2003)
[23] U. Tracht, M. Wilhelm, A. Heuer, H. Feng, K. Schmidt-Rohr, H.W. Spiess, Phys. Rev. Lett. 81, 2727 (1998)
[24] X. Wu, Z. Zhu, J. Phys. Chem. B 113, 11147 (2009)
[25] E. Braeken, G. De Cremer, P. Marsal, G. Pepe, K. Müllen, R.A.L. Valleé, J. Am. Chem. Soc. 131, 12201 (2009)
[26] C.Y.J. Wei, D.A. VandenBout, J. Phys. Chem. B 113, 2253 (2009)
[27] S. Nie, D.T. Chiu, R. N. Zare, Science 266, 1018 (1994)
[28] W. E. Moerner, J. Phys. Chem. B 106, 910 (2002)
[29] F. Kulzer, T. Xia, M. Orrit, Angew. Chem. Int. Ed. 49, 854 (2010)
[30] L.J. Kaufman, Annu. Rev. Phys. Chem. 64, 177 (2013)
[31] H. Uji-i, S.M. Melnikov, A. Deres, G. Bergamini, F. De Schryver, A. Herrmann, K. Müllen, J. Enderlein, J. Hofkens, Polymer 47, 2511 (2006)
[32] R.A.L. Valleé, M. Baruah, J. Hofkens, F.C. De Schryver, N. Boens, M. Van der Auweraer, D. Beljonne, J. Chem. Phys. 126, 184902 (2007)
[33] R.A.L. Vallée, M. Cotlet, M. Van Der Auweraer, J. Hofkens, K. Müllen, F.C. De Schryver, J. Am. Chem. Soc. 126, 2296 (2004)
[34] R.A.L. Valleé, N. Tomczak, G.J. Vancso, L. Kuipers, N.F. van Hulst, J. Chem. Phys. 122, 114704 (2005)
[35] J.N. Clifford, T.D.M. Bell, P. Tinnefeld, M. Heilemann, S.M. Melnikov, J. Hotta, M. Sliwa, P. Dedecker, M. Sauer, J. Hofkens, E.K.L. Yeow, J. Phys. Chem. B 111, 6987 (2007)
[36] Y.W. Hou, A.M. Bardo, C. Martinez, D.A. Higgins, J. Phys. Chem. B 104, 212 (2000)
[37] D.A. Higgins, M.M. Collinson, G. Saroja, A.M. Bardo, Chem. Mater. 14, 3734 (2002)
[38] C. Hippius, I.H.M. van Stokkum, E. Zangrando, R.M. Williams, F. Würthner, J. Phys. Chem. C 111, 13988 (2007)
[39] J.R. Siekierzycka, C. Hippius, F. Würthner, R.M. Williams, A.M. Brouwer, J. Am. Chem. Soc. 132, 1240 (2010)
[40] A.N. Adhikari, N.A. Capurso, D. Bingemann, J. Chem. Phys. 127, 114508 (2007)
[41] C. Hippius, F. Schlosser, M.O. Vysotsky, V. Böhmer, F. Würthner, J. Am. Chem. Soc. 128, 3870 (2006)
[42] B. Valeur, Molecular Fluorescence. Principles and Applications (Wiley-VCH, Weinheim, 2002)
[43] V. Veissier, J.L. Viovy, L. Monnerie, J. Phys. Chem. 93, 1709 (1989)
[44] L.B.-Å. Johansson, J. Chem. Soc., Faraday Trans. 86, 2103 (1990)
[45] D. Axelrod, Biophys. J. 26, 557 (1979)
[46] D. Axelrod, Methods in Cell Biology 30, 333 (1989)
[47] H. Nyquist, Proc. IEEE 90, 280 (2002)
[48] S. Adhikari, M. Selmke, F. Cichos, Phys. Chem. Chem. Phys. 13, 1849 (2011)
[49] A.P. Bartko, R.M. Dickson, J. Phys. Chem. B 103, 3053 (1999)
[50] J. Bernard, L. Fleury, H. Talon, M. Orrit, J. Chem. Phys. 98, 850 (1993)
[51] T. Plakhotnik, E.A. Donley, U.P. Wild, Annu. Rev. Phys. Chem. 48, 181 (1997)
[52] T. Irngartinger, A. Renn, U.P. Wild, J. Lumin. 66-67, 232 (1995)
[53] R. Zondervan, F. Kulzer, S.B. Orlinskii, M. Orrit, J. Phys. Chem. A 107, 6770 (2003)
[54] R. Zondervan, F. Kulzer, M.A. Kol'chenko, M. Orrit, J. Phys. Chem. A 108, 1657 (2004)

[55] F. Köhn, J. Hofkens, R. Gronheid, M. Van Der Auweraer, F.C. De Schryver, J. Phys. Chem. A 106, 4808 (2002)
[56] A. Deres, G.A. Floudas, K. Müllen, M. Van Der Auweraer, F. De Schryver, J. Enderlein, H. Uji-i, J. Hofkens, Macromolecules 44, 9703 (2011)
[57] M. Orrit, Angew. Chem. Int. Ed. 52, 163 (2012)
[58] L.E. Walther, N.E. Israeloff, E. Vidal Russell, H. Alvarez Gomariz, Phys. Rev. B: Condens. Matter 57, R15112 (1998)
[59] S.A. Mackowiak, T.K. Herman, L.J. Kaufman, J. Chem. Phys. 131, 244513 (2009)
[60] S.A. Mackowiak, L.J. Kaufman, J. Phys. Chem. Lett. 2, 438 (2011)
[61] L.-M. Wang, R. Richert, J. Chem. Phys. 120, 11082 (2004)
[62] L.M. Leone, L.J. Kaufman, J. Chem. Phys. 138, 12A524 (2013)
[63] D. Thomsson, H.Z. Lin, I.G. Scheblykin, ChemPhysChem 11, 897 (2010)
[64] J. Hofkens, M. Maus, T. Gensch, T. Vosch, M. Cotlet, F. Köhn, A. Herrmann, K. Müllen, F. De Schryver, J. Am. Chem. Soc. 122, 9278 (2000)
[65] M. Haase, C.G. Hübner, F. Nolde, K. Müllen, T. Basché, Phys. Chem. Chem. Phys. 13, 1776 (2011)
[66] B. Araoz, D. Täuber, C. von Borczyskowski, P.F. Aramendia, J. Phys. Chem. C 116, 7573 (2012)
[67] M.J. Rust, M. Bates, X. Zhuang, Nat. Meth. 3, 793 (2006)
[68] G. Patterson, M. Davidson, S. Manley, J. Lippincott-Schwartz, Annu. Rev. Phys. Chem. 61, 345 (2010)
[69] S.W. Hell, Nat. Meth. 6, 24 (2009)
[70] S. van de Linde, U. Endesfelder, A. Mukherjee, M. Schüttpelz, G. Wiebusch, S. Wolter, M. Heilemann, M. Sauer, Photochem. Photobiol. Sci. 8, 465 (2009)
[71] P. Dedecker, G.C. Mo, T. Dertinger, J. Zhang, Proc. Natl. Acad. Sci. U.S.A. 109, 10909 (2012)