

Fluorescence of Single Molecules in Polymer Films: Sensitivity of Blinking to Local Environment

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The single-molecule fluorescence blinking behavior of the organic dye Atto647N in various polymer matrixes such as Zeonex, PVK, and PVA as well as aqueous media was investigated. Fluorescence blinking with off-times in the millisecond to second time range is assigned to dye radical ions formed by photoinduced electron transfer reactions from or to the environment. In Zeonex and PVK, the measured off-time distributions show power law dependence, whereas, in PVA, no such dependence is observed. Rather, in this polymer, off-time distributions can be best fitted to monoexponential or stretched exponential functions. Furthermore, treatment of PVA samples to mild heating and low pressure greatly reduces the frequency of blinking events. We tentatively ascribe this to the removal of water pockets within the polymer film itself. Measurements of the dye immobilized in water in the presence of methylviologen, a strongly oxidizing agent, reveal simple exponential on- and off-time distributions. Thus, our data suggest that the blinking behavior of single organic molecules is sensitive to their immediate environment and, moreover, that fluorescence blinking on- and off-time distributions do not inherently and uniquely obey a power law.

Introduction

Recent advances in optical spectroscopy and microscopy enable us to monitor the fluorescence of individual molecules.^{1–3} This has exciting implications, as single-molecule spectroscopy (SMS) can be utilized to gain fundamental insights into processes at the molecular level which were previously hidden under the veil of ensemble measurement. Indeed, by monitoring the fluorescence of individual fluorescent probes, SMS can be employed to investigate a plethora of processes including biological fluorescence resonance energy transfer (FRET), protein folding, and (enzymatic) catalysis.^{4,5}

However, the performance of organic molecules as fluorescent probes in SMS is impaired by the “photoblinking” phenomena in which the fluorescence signal alternates between a bright “on” state and non-emissive “off” states. Photoblinking has obvious implications on the effectiveness of SMS, as any interference of the probe emission will subsequently interfere with the main process under interrogation. It has previously been explained

by triplet state dynamics (in the domain of tens to hundreds of microseconds),^{6,7} molecular reorientation,⁸ spectral diffusion,⁹ conformational changes,^{10,11} and intramolecular electron transfer.¹² More recent studies by this group and others of single molecules immobilized on glass surfaces and in polymer matrixes have suggested that photoblinking may also be due to intermolecular charge transfer between the excited state of the single molecule and traps present in the surrounding environment.^{13–18} These studies revealed long-lived dark states with durations ranging from milliseconds to hundreds of seconds and where the resulting on- and off-time distributions obey a power law function. A charge tunneling model has been employed to explain these distributions.^{19–21} A good account of the theoretical work employed to model photoblinking can be found in refs 22 and 23. The assignment of long-lived dark states responsible for photoblinking to radicals is supported in a study by Zondervan and co-workers in which radical anions of rhodamine 6G (Rh6G), suggested to be formed via electron transfer from poly(vinyl alcohol) (PVA) to Rh6G upon irradiation, were observed in an electron-spin-resonance experiment.¹⁸

It has been suggested that power law behavior of off-state distributions appears as a universal feature for single emitters undergoing fluorescence blinking.¹⁷ Herein, we question the validity of this assumption by investigating the fluorescence blinking of an organic dye, Atto647N, in various polymer

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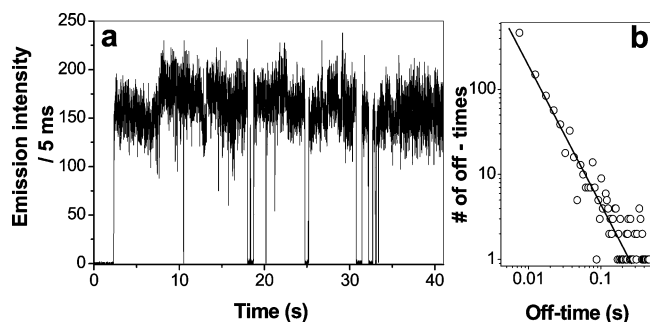


Figure 1. (a) Fluorescence intensity time trace of a single Atto647N molecule in Zeonex and (b) off-time distribution for a collection of 85 Atto647N molecules in Zeonex (with an off-time exponent (m_{off}) of 1.5).

matrixes and also immobilized under aqueous conditions. Atto647N belongs to the class of carborhodamine dyes which can be readily exploited in SMS experiments due to its brightness and high photostability. In addition, it does not undergo cis–trans isomerization which allows conformational changes to be disregarded as a source of photoblinking.

Experimental Methods

The polymers Zeonex (polynorbornene) and PVA (poly(vinyl alcohol)) were purchased from Aldrich and used without further purification. PVK (poly(vinyl carbazole)) was a gift from Prof. Klaus Müllen and Prof. Andreas Herrmann (Max Planck Institut für Polymerforschung, Mainz). Their structures are given in the Supporting Information. Atto647N was purchased from ATTO-TEC GmbH.²⁴ Samples were prepared by spin coating 0.5–1% polymer solutions of Atto647N ($\sim 10^{-10}$ M) onto clean glass coverslips. Synthesis and immobilization of dye labeled oligonucleotides under aqueous conditions is described in ref 25. Measurements in aqueous solution were carried out in phosphate-buffered saline (PBS), pH 7.4, containing 100 μM methylviologen and an oxygen scavenging system (10% (wt/vol) glucose, 12.5% (vol/vol) glycerine, 50–100 $\mu\text{g/mL}$ glucose oxidase, 100–200 $\mu\text{g/mL}$ catalase, and 200 μM phosphin) for oxygen removal. Details of the apparatus used to monitor emission intensity time traces of single molecules are described elsewhere.¹³ Atto647N was either excited at 642 nm using a picosecond diode laser (PicoQuant, 10–80 MHz) or excited at 633 nm using a continuous wave HeNe laser (Melles Griot).

Results and Discussion

Figure 1a shows a typical fluorescence intensity time trace recorded for a single molecule of Atto647N embedded in Zeonex. Fluorescence blinking occurs rather infrequently, meaning that analysis of on- and off-time distributions for individual molecules is not possible. We therefore analyze on- and off-time distributions for a population of 85 molecules of Atto647N, as has been done previously in other fluorescence intermittency studies,^{14–17} and the resulting distribution is shown in Figure 1b. The distribution when plotted log–log can be fitted well to a straight line. We can therefore describe the off-time distribution ($P(\tau_{\text{off}})$) using a power law function:

$$P(\tau_{\text{off}}) = P_0 \tau_{\text{off}}^{-m_{\text{off}}} \quad (1)$$

with the power law exponent $m_{\text{off}} = 1.5$. The on-times are also found to be power law distributed with the on-time power law exponent $m_{\text{on}} = 1.6$ (see the Supporting Information). The broad distributions for both the on- and off-time durations are

rationalized using a charge tunneling process whereby a charge is transferred between the Atto647N molecule and localized states found in the polymer matrix. Subsequent charge recombination by back charge tunneling restores the fluorescence. The dependency of the on- and off-time distributions upon power law manifests itself from the exponential distribution of charge tunneling rates which arise from the exponential distributions of both the spatial locations and energies of the traps present in the Zeonex film. The charge tunneling model^{18–21} dictates that the value of m_{off} falls between 1 and 2 and the m_{off} value of 1.5 extracted from the off-time distribution indeed falls within this range.

Figure 2a shows a typical fluorescence intensity time trace for a single molecule of Atto647N embedded in PVK. These data are the first SMS data recorded for an organic dye in PVK, a polymer which is commonly used as a hole conducting material in organic light emitting diodes (OLEDs).²⁶ The fluorescence intensity time trace reveals two distinct regions: an initial region which displays rather short-lived off-events (< 200 ms), followed by a second region which displays longer-lived off-events. The opposite scenario in which a region displaying long-lived off-events is followed by a region displaying short-lived off-events was also observed (see the Supporting Information).

Power law distributions are observed for the off-time events for an individual molecule that exhibits a sufficient number of on- and off-events ($m_{\text{off}} = 1.6$, Figure 2b) and also for a collection of 80 molecules ($m_{\text{off}} = 1.9$, Figure 2c). We can therefore ascribe a charge tunneling process from the electron-donating PVK ($E_{\text{ox}} = +1.05$ V versus SCE) to Atto647N ($E_{\text{ox}} = +1.11$; $E_{\text{red}} = -0.64$ V versus SCE) forming the dark state, in this case a neutral or zwitterionic radical species.

Further insight into the fluorescence intermittency dynamics is provided by the wide distribution of emission lifetimes (τ_{fl}) extracted from single molecules. In Figure 2, the emission lifetime distribution shows an unquenched monoexponential fluorescence lifetime τ_{fl} (≈ 3.1 ns) for the first half of the emission intensity time trace followed by a quenched monoexponential lifetime τ_{fl} (≈ 1.4 ns) for the remaining half. Other emission intensity time traces display both mono- and biexponential decays (see the Supporting Information). Such a wide distribution in the value of τ_{fl} indicates that the interaction between each molecule and its local environment varies considerably from molecule to molecule. Furthermore, the quenched monoexponential fluorescence decay observed for the molecule can be taken as proof that charge-separation reactions between Atto647N and PVK are in operation, and occur within the time resolution of the experimental setup.

A model commonly used to explain the power law behavior in quantum dots is based on a random walk of either the electron or hole before charge recombination.²⁷ This is a feasible model for PVK, since the polymer is a hole-transporting material. Upon applying this model, a power law exponent of $m_{\text{off}} = 1.5$ is expected, but no such value was recovered from the experimental data. In the case of the individual molecule (Figure 2b), a value of 1.6 for m_{off} was extracted. The disparity between the theoretical and experimental values may derive from the bias which results from performing a double logarithmic (log–log) transformation followed by least squares (LS) fitting of the off-time distribution to obtain m_{off} .²² Concerning the value of $m_{\text{off}} = 1.9$ extracted for the population of 80 molecules (Figure 2c), bias resulting from the fitting procedure cannot explain the deviation of this value from the theoretical value of 1.5. Instead, the data suggest a time-dependent diffusion constant for the hole

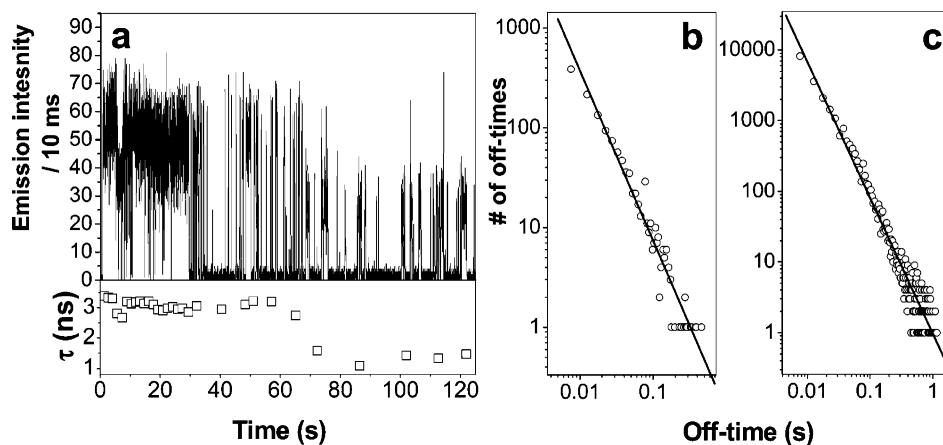


Figure 2. (a) Fluorescence intensity time trace (upper panel) and fluorescence lifetimes (lower panel) of a single molecule of Atto647N in PVK and off-time distributions for (b) a single molecule of Atto647N and (c) a collection of 80 molecules of Atto647N in PVK. The off-time exponents (m_{off}) are 1.6 (b) and 1.9 (c).

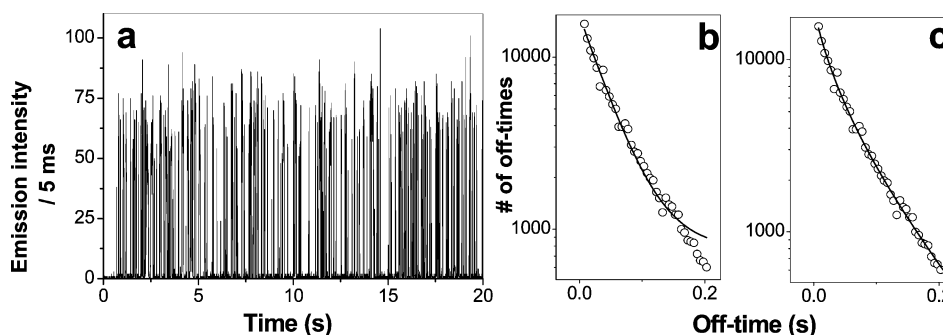


Figure 3. (a) Fluorescence intensity time trace of a single molecule of Atto647N in PVA and an off-time distribution of a collection of 48 molecules of Atto647N embedded in PVA fitted to (b) a monoexponential and (c) stretched exponential function (where $n = 0.7$ and $\tau_{\text{SE}} = 29.3$ ms).

created in PVK after charge separation. The hopping rate of the hole between different sites in PVK most likely varies with time and local environment. A theoretical description of anomalous charge diffusion and its effects on fluorescence blinking in quantum dots has recently been discussed by Tang and Marcus.²⁸ It is important to bear in mind that while the fluorescence blinking behavior of Atto647N in the polymer matrixes discussed hitherto reveals off-time distributions which are power law dependent, we propose that the origins of the long-lived dark states are quite different. In the case of Zeonex, the long-lived states we believe are due to charge tunneling between the exponentially distributed trap states in the matrix and the dye, whereas, in the case of PVK, we suggest that the hole created after electron transfer undergoes anomalous diffusion in the polymer matrix resulting in the long off-time events prior to charge recombination.

The fluorescence of single molecules of Atto647N in PVA falls into two distinct groups determined by the frequency of fluorescence blinking events: (i) those that display *infrequent* long off-time blinking events (see the Supporting Information), similar to those discussed for Zeonex, and (ii) those that display *frequent* long off-time blinking events, as demonstrated by the trace in Figure 3a. The combined off-time distribution of 48 blinking molecules was collected and when analyzed on a log–log plot could not be fitted well with a straight line, indicating that the distribution does not follow a power law function. Instead, when the distribution is plotted on a log–lin plot (Figure 3b and c) and fitted with a monoexponential and

stretched exponential function (eq 2), the latter could be seen to provide an excellent fit:

$$P(\tau_{\text{off}}) = P_0 \left[-\left(\frac{t}{\tau_{\text{SE}}} \right)^n \right] \quad (2)$$

with a time constant of $\tau_{\text{SE}} = 29.3$ ms and a stretching exponent of $n = 0.7$. Similarly, off-time distributions of individual molecules do not obey power law functions and can be fitted to either monoexponential or stretched exponential functions. The range of values for n and τ_{SE} measured from a population of 48 molecules was found to be rather large ($0.53 \leq n \leq 1$ and $15 \text{ ms} \leq \tau_{\text{SE}} \leq 156 \text{ ms}$). This demonstrates the level of heterogeneity within the PVA film.

To investigate the extent to which residual water in the PVA film contributes to fluorescence blinking, experiments were also conducted on samples which were dried following a pretreatment of mild heating (50 °C) and vacuum pumping. Out of a population of 50 molecules, the proportion that display frequent blinking events such as those in Figure 3a decreases from 32% for an untreated sample to 4% for a pretreated sample. This demonstrates the importance of residual water in the local environment of individual Atto647N molecules on the dynamics of the observed photoblinking events.

Possible sources for the fluorescence blinking of Atto647N in PVA were investigated. Blinking caused by triplet state dynamics was eliminated when triplet state quenchers were found to have little effect upon blinking behavior. Furthermore, defocused wide-field experiments eliminated molecular reori-

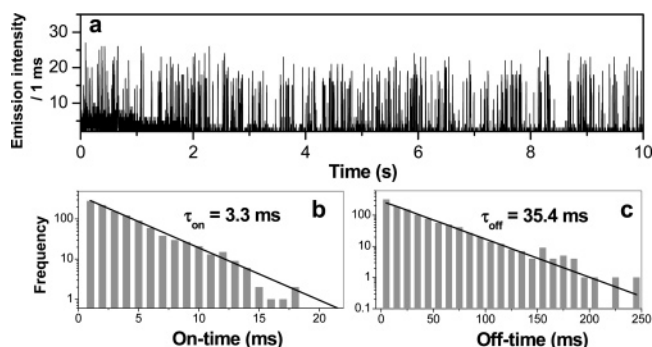


Figure 4. (a) Fluorescence intensity time trace of a single molecule of Atto647N in buffer solution containing methylviologen. On-time and off-time distributions of the same molecule and monoexponential fits are shown in parts b and c, respectively.

entation as a source. Finally, experiments conducted in water and deuterated water indicated that proton transfer from water to the dye does not play a primary role in effecting fluorescence blinking. All of this strongly suggests that charge transfer is the primary source of the long off-time blinking observed for Atto647N in PVA, a similar conclusion reached previously by Zondervan et al.¹⁸ in the case of a rhodamine/PVA system.

The absence of power law dependence and the stretched exponential nature of the off-time distribution in Figure 3c suggests that the electron tunneling process occurs over a very narrow distance range (and that tunneling barrier heights are rather restricted also). The presence of the water pockets within the PVA could explain this, with electron tunneling occurring across the water/polymer interface between Atto647N in the PVA and traps in the pocket. Such an interface would be expected to impose a narrow distribution of distances and barrier heights upon electron tunneling, thus resulting in a stretched exponential off-time distribution. As there is no such interface present in the cases of Zeonex, PVK, and indeed dry PVA, where traps are exponentially distributed throughout the polymer matrix, the off-time distributions instead show power law dependence. More precise details of the mechanism require further experiments and shall be discussed elsewhere in the future.

To demonstrate that electron transfer can indeed generate fluorescence blinking, single molecules of Atto647N were immobilized in buffer using bovine serum albumin (BSA)–biotin/streptavidin chemistry. Under these conditions, all Atto647N molecules have very similar nanoenvironments so that the influence of the environment on blinking can be directly probed. Figure 4a displays a fluorescence intensity time trace recorded for a single Atto647N labeled oligonucleotide immobilized in aqueous buffer. To frequently generate dye radical cations, the oxidant methylviologen ($E_{\text{red}} = -0.69$ V versus SCE in aqueous solution) was added to the deoxygenated solution. Under these conditions, rhodamine derivatives are known to form long-lived radical cations.²⁹ It is clear that the time trace in Figure 4a bears a striking resemblance to the time trace in Figure 3a, indicating that electron transfer is the source of blinking of Atto647N in PVA. Parts b and c of Figure 4 show on- and off-time histograms, respectively, from an individual Atto647N molecule with sufficient statistical weight so that averaging over many molecules is avoided. The histograms show neat single exponential behavior with $\tau_{\text{off}} = 35.4$ ms and $\tau_{\text{on}} = 3.3$ ms. The value recorded for τ_{off} (35.4 ms) is quite similar to the value of τ_{off} recorded for the dye in PVA (29.3 ms), suggesting indeed that electron transfer is the source of blinking of Atto647N in PVA. The data directly demonstrate that radical cations are

formed by reduction of methylviologen according to classical electron transfer theory,³⁰ and subsequent charge recombination prepares the dye in its ground state ready for further excitation emission cycles. The kinetics of this process are clearly monoexponential, as electron transfer rates are primarily dominated by contact formation rates of the reactants.

Conclusions

The fluorescence blinking of an organic molecule, Atto647N, has been probed in several polymer films, namely, Zeonex, PVK, and PVA, as well as in aqueous media, and it has been shown that the blinking behavior can change depending on the environment. In Zeonex, a dependency of the on- and off-time distributions upon power law is as a result of the exponential distributions of charge tunneling rates arising from the exponential distributions of both the spatial locations and energies of the traps present in these polymer films. While PVK also shows an on- and off-time dependence upon power law, the long-lived dark state is not necessarily based on a simple charge tunneling model. In this case, we propose that the hole created in the polymer matrix following electron transfer to Atto647N undergoes anomalous diffusion in the polymer matrix giving rise to the observation of long-lived dark states. The off-time distribution of Atto647N in PVA shows no power law dependency but instead displays monoexponential and stretched exponential dependency. Finally, measurements in water in the presence of a strong oxidizing agent demonstrate simple exponential on- and off-time distributions as expected for homogeneously distributed electron-accepting traps with similar energies.

An important conclusion of this work is that, as simple chemical intuition would suggest, power law distributed blinking is not necessarily a universal feature for single organic molecules undergoing electron transfer reactions, and we demonstrate the transition from power law, over stretched exponential to monoexponential behavior by manipulation of the heterogeneity imposed by the direct environment of the chromophore. It is, therefore, pertinent to assess each dye molecule in its particular environment individually when information regarding fluorescence intermittency is needed.

Supporting Information Available: Scheme showing the structures of Zeonex, PVK, and PVA and figures showing fluorescence intensity time traces and fluorescence lifetimes. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References and Notes

- Moerner, W. E.; Orrit, M. *Science* **1999**, *283*, 1670.
- Kulzer, F.; Orrit, M. *Annu. Rev. Phys. Chem.* **2004**, *55*, 585.
- Tinnefeld, P.; Sauer, M. *Angew. Chem., Int. Ed.* **2005**, *44*, 2642.
- Michalet, X.; Weiss, S.; Jager, M. *Chem. Rev.* **2006**, *106*, 1785.
- Roelfaers, M. B. J.; Sels, B. F.; Uji-i, H.; De Schryver, F. C.; Jacobs, P. A.; De Vos, D. E.; Hofkens, J. *Nature* **2006**, *439*, 572.
- Yip, W. T.; Hu, D. H.; Yu, J.; Vanden Bout, D. A.; Barbara, P. F. *J. Phys. Chem. A* **1998**, *102*, 7564.
- Köhn, F.; Hofkens, J.; Gronheid, R.; van der Auweraer, M.; De Schryver, F. C. *J. Phys. Chem. A* **2002**, *106*, 4808.
- Ambrose, W. P.; Goodwin, P. M.; Martin, J. C.; Keller, R. A. *Phys. Rev. Lett.* **1994**, *72*, 160.
- Lu, H. P.; Xie, X. S. *Nature* **1997**, *385*, 143.
- Weston, K. D.; Carson, P. J.; Metiu, H.; Buratto, S. K. *J. Chem. Phys.* **1998**, *109*, 7474.
- Weston, K. D.; Buratto, S. K. *J. Phys. Chem. A* **1998**, *102*, 3635.
- Bell, T. D. M.; Stefan, A.; Masuo, S.; Vosch, T.; Lor, M.; Cotlet, M.; Hofkens, J.; Bernhardt, S.; Mullen, K.; van der Auweraer, M.; Verhoeven, J. W.; De Schryver, F. C. *ChemPhysChem* **2005**, *6*, 942.

- (13) Yeow, E. K. L.; Melnikov, S. M.; Bell, T. D. M.; De Schryver, F. C.; Hofkens, J. *J. Phys. Chem. A* **2006**, *110*, 1726.
- (14) Haase, M.; Hubner, C. G.; Reuther, E.; Herrmann, A.; Mullen, K.; Basche, T. *J. Phys. Chem. B* **2004**, *108*, 10445.
- (15) Schuster, J.; Cichos, F.; von Borczyskowski, C. *Opt. Spectrosc.* **2005**, *98*, 712.
- (16) Schuster, J.; Cichos, F.; von Borczyskowski, C. *Appl. Phys. Lett.* **2005**, *87*, 051915.
- (17) Hoogenboom, J. P.; van Dijk, E. M. H. P.; Hernando, J.; van Hulst, N. F.; García-Parajó, M. F. *Phys. Rev. Lett.* **2005**, *95*, 097401.
- (18) Zondervan, R.; Kulzer, F.; Orlinskii, S. B.; Orrit, M. *J. Phys. Chem. A* **2003**, *107*, 6770.
- (19) Tachiya, M.; Mozumder, A. *Chem. Phys. Lett.* **1975**, *34*, 77.
- (20) Kuno, M.; Fromm, D. P.; Johnson, S. T.; Gallaher, A.; Nesbitt, D. *J. Phys. Rev. B* **2003**, *67*, 125304.
- (21) Verberk, R.; van Oijen, A. M.; Orrit, M. *Phys. Rev. B* **2002**, *66*, 233202.
- (22) Hoogenboom, J. P.; den Otter, W. K.; Offerhaus, H. L. *J. Chem. Phys.* **2006**, *125*, 204713.
- (23) Hoogenboom, J. P.; Hernando, J.; van Dijk, E. M. H. P.; van Hulst, N. F.; García-Parajó, M. F. *ChemPhysChem* **2007**, *8*, 823.
- (24) Product information on Atto647N available from Atto-Tec GmbH (<http://www.atto-tec.com/>).
- (25) Heilemann, M.; Margeat, E.; Kasper, R.; Sauer, M.; Tinnefeld, P. *J. Am. Chem. Soc.* **2005**, *127*, 3801.
- (26) Hung, L. S.; Chen, C. H. *Mater. Sci. Eng., R* **2002**, *39*, 143.
- (27) Shimizu, K. T.; Neuhauser, R. G.; Leatherdale, C. A.; Empedocles, S. A.; Woo, W. K.; Bawendi, M. G. *Phys. Rev. B* **2001**, *63*, 205316.
- (28) Tang, J.; Marcus, R. A. *Phys. Rev. Lett.* **2005**, *95*, 107401.
- (29) Korobov, V. E.; Chibisov, A. K. *J. Photochem.* **1978**, *9*, 411.
- (30) Marcus, R. A. *J. Chem. Phys.* **1956**, *24*, 966.