Single-Molecule Chemistry

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Distinguishing Alternative Reaction Pathways by Single-Molecule Fluorescence Spectroscopy**

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Over the past two decades, single-molecule fluorescence spectroscopy (SMFS) has been employed to resolve heterogeneities in solid-state physics, biophysics, and for localization studies with high spatial resolution.[1–7] Although the notation "molecule" is inherently connected to chemistry, applications of SMFS to reaction dynamics were rare, despite the early report of observed self-sensitized photo-oxidation of single terrylene molecules hosted in a *p*-terphenyl crystal.[8] So far, single-molecule methods are mostly used to investigate catalytic turnover of enzymes.[9–11] Only recently, SMFS has been applied to study heterogeneous catalysis. The cleavage of fluorogenic esters was used to investigate and map the spatial distribution of active catalytic sites for ester hydrolysis on crystal particles[12,13] and to characterize the catalytic behavior of individual enzymes.[14] With a similar approach, catalytic redox reaction of fluorogenic substrates have been studied at the surface of gold nanoparticles[15] and titanosilicate zeolites.[16] Aiming at progress in studying heterogeneous organometallic reactions, the kinetics of ligand exchange at platinum has been studied on the molecular level.[17] Recently, the high spatial localization accuracy of SMFS allowed homogeneous catalysis to be distinguished from surface reactions.[18] All of the mentioned single-molecule studies share the same experimental approach in which individual chemical reactions are indicated by the occurrence of single fluorescent spots that are, for example, due to the conversion of ubiquitous leuko-dyes or quenched substrates into brightly fluorescing product molecules. Here, light emission appears during the last event in a succession of elementary reaction steps. We have shown earlier that a reversible complexation of metal ions can be characterized with SMFS by immobilizing a fluorescently labeled ligand.[19] Binding of a metal ion leads to specific fluorescence quenching of the ligand such that the forward chemical process (complexation) and the reverse reaction (dissociation) can be quantitatively studied by recording fluorescence trajectories of single ligand molecules. A similar approach was used to observe the reversible redox reaction of immobilized perylene diimide dyes.[20] In the present work we expand this concept to the more general case of irreversible reactions. To observe the initial and final states, we had to make sure that the reactive group was part of the chromophoric structure.[21] More specifically, we were interested in following the conversion of a single substrate molecule along its reaction pathway to the final product during the well-known epoxidation reaction on a double bond with *m*-chloroperbenzoic acid (*m*CPBA).[22]

Here, the course of the reaction is embedded by the disappearance of the fluorescent substrate and the appearance of the fluorescent product. The experimentally most convenient and reliable indicator of a completed reaction is the change of the emission color.[8,23] Apart from the design of an appropriate probe molecule for epoxidation, where we could rely on previous experiments with BODIPY dyes,[23,24] other challenges have to be overcome.

For maintenance of single-molecule conditions during the whole reaction, the fluorescent substrate is less abundant by several orders of magnitude than in experiments with fluorogenic substrates, but the concentration of the co-reagent prevails in a large excess. Continuous observation until reactive collision takes place can be achieved using less mobile or even immobile substrates. Finally, the substrate must fluoresce until the reaction proceeds hence demanding a photostable fluorophore without addition of stabilizers. In the following, we present a system which fulfills the mentioned requirements.

To probe the epoxidation reaction with SMFS, we designed the fluorescent probe oxyallyl BDP **1** (1-methyl-(*E*)-3-(2-allyloxy)styryl-4,4′-difluoro-bora-3a,4a-diaza-(s)-indacene) consisting of a BODIPY core expanded by an oxyallyl styryl unit (Figure 1a). Its synthesis followed the conventional Knoevenagel-like condensation.[25,26] The previously introduced styryl-BODIPY system has already shown promising results in epoxidation reactions,[24] while here the additional tagging with an oxyallylic residue serves as linker for covalent surface binding (Figure 2a). This latter moiety does not expand the chromophoric π-system, resulting in almost indistinguishable spectral properties compared to the

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Supporting information for this article, including details of reagents used, instruments, and analytical data, including spectroscopic characterization, is available on the WWW under http://dx.doi.org/10.1002/anie.201300100.

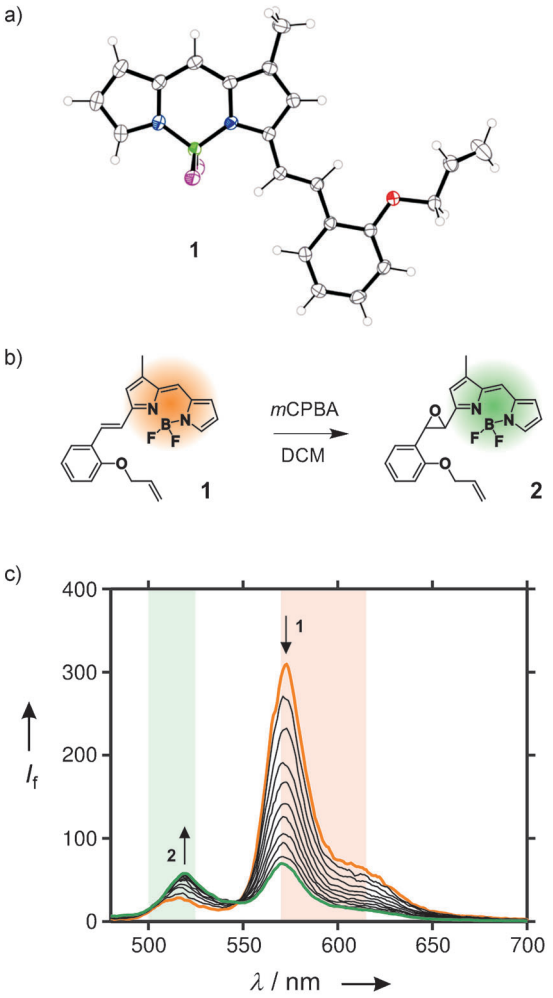

Figure 1. Epoxidation of **1** with a 200-fold excess of *m*CPBA yields **2** with significantly changed emission properties from **1**. a) Crystal structure of the substrate oxyallyl BDP **1**. b,c) Epoxidation of **1** (red line) with *m*CPBA to the product **2** (green line) can be followed as a spectral shift in fluorescence emission. The green and red shaded areas signify the wavelength range of the emission filters used in single-molecule experiments for product and substrate detection, respectively. Emission spectra were recorded after addition of a 200-fold excess of *m*CPBA (ca. 0.2 mM) in CH_2Cl_2 over 6 h with an excitation wavelength of 470 nm.

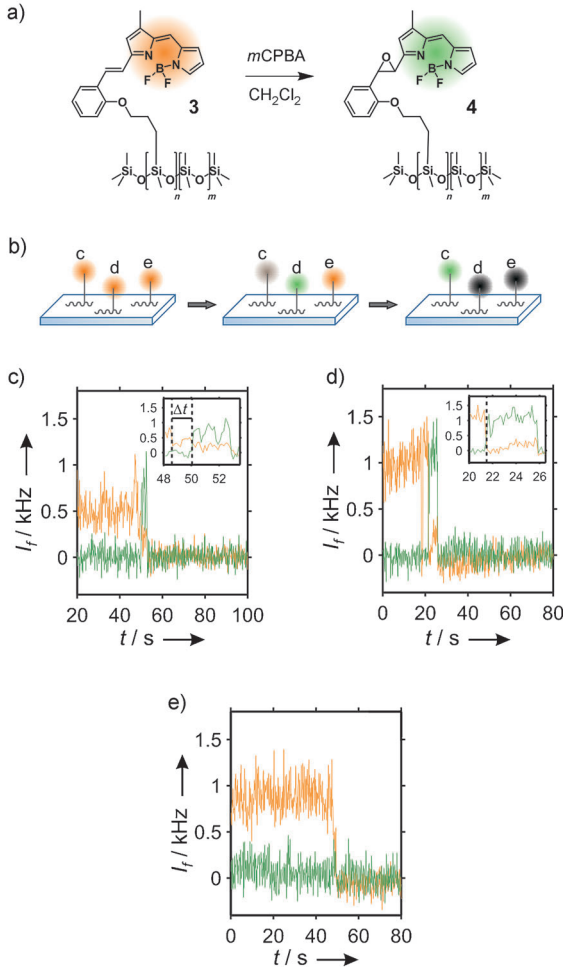

Figure 2. Proposed reaction of immobilized **3** and characteristic signatures of the different reactions and reaction pathways observed in time-resolved single-molecule experiments. a) Reaction process of the epoxidation of immobilized **3** to **4**. b) Representation of the three different conversions observed in single-molecule experiments. c)–e) Single-molecule traces of substrate (570–615 nm, red line) and product emission (500–525 nm, green line) recorded at 7.4 Hz show specific features that are characteristic for substrate conversion via a dim intermediate state (c), immediate conversion (d), and photobleaching of **3** (e).

formerly studied system. Furthermore, the missing conjugation ensures that surface binding minimizes influences on the electronic properties of the substrate.

Epoxidation of the yellow fluorescent BODIPY moiety ($\lambda_{em} = 572$ nm) with *m*CPBA in excess leads to formation of **2** with green fluorescence ($\lambda_{em} = 518$ nm) owing to shortening of the chromophoric unit (Figure 1b).[23,24] The blue shift of the emission by roughly 50 nm as a result of the transformation provides the required color change by which the initial substrate **1** and the reaction product **2** can be unambiguously assigned in a two-channel detection process. The overall performance remains unchanged when the dye is immobilized.

For single-molecule experiments, **1** was first covalently attached to polysiloxane polymer HMPS (hydridomethyldimethylpolysiloxane) by hydrosilylation of the allylic double bond.[27–29] The resulting labeled polymer was immobilized on a glass cover slide. We tested different solvents for their suitability in SMFS and found dichloromethane (CH_2Cl_2) to be the most suitable for the epoxidation reaction, whereas toluene and ethylacetate showed a rather high fluorescence background. Reactions of individual substrate molecules **3** with *m*CPBA in CH_2Cl_2 to oxidized product **4** were recorded on a TIRF microscope equipped with simultaneous laser excitation at 532 and 488 nm (Figure 2a,b). Dual-color detection in the wavelength ranges of 570–615 nm for the yellow and 500–525 nm for the green fluorescence (Figure 1c) was realized by an emCCD camera. The emCCD was operated at a frame rate of 7.4 Hz; that is, 135 ms per frame to optimize the signal-to-noise ratio and at the same time to minimize photo-induced blinking and bleaching. The immo-

bilized probes exhibit a photostability sufficient for extended observation periods (up to one minute; Figure 2e). Moreover, the marginal hydrogenation of the double bond during preparation leading to initially green fluorescent spots was used to establish perfect alignment of the two detection channels.

Epoxidation was initiated by addition of *m*CPBA (0.2 mM) in CH_2Cl_2. Immediately after sealing with a second cover slide, the sample was placed on the microscope for image acquisition (ca. 2 min). In total, we acquired 1595 single-molecule traces, which were manually inspected and categorized in three relevant classes (Figure 2b). The traces in Figure 2c–e show the background corrected fluorescence emission recorded in the substrate (red line) and the product channel (green line). More than 90% of single-molecule traces showed a loss of substrate emission in a single frame without change to product emission. We attribute this behavior to photobleaching of individual substrate molecules or oxidative destruction of the chromophoric system as a side-reaction. The latter is also observed in ensemble experiments (Figure 1c). However, a relatively high percentage of traces (115 traces, that is, ca. 7%) exhibit a clear spectral shift of the emission from substrate to product channel (Figure 1c). Most of the transformations occur in direct manner while few of them exhibit a dark intermediate state.

Again, we observed that product emission is always terminated in a single frame owing to photobleaching (Figure 2c,d), clearly indicating the single-molecule nature of the investigated system. We emphasize at this point the well-known benefits of single-molecule techniques for investigating chemical reactions in which significant trajectories can be selected specifically. Design of our experiments and spectral detection ranges strongly suggest that the spectral shift observed in single-molecule experiments signifies conversion of the double bond in **3** to an epoxide in **4** (Figure 2c,d). To support this interpretation, we carried out control experiments with immobilized **3** in the absence of *m*CPBA (Supporting Information, Figure S17). Here, most of the traces indicate photobleaching of the immobilized substrate, but only a minor part of the traces ($< 0.4\%$ out of > 1200 traces) show a similar spectral shift. The more than 20-fold increased probability is a clear indication that the observed spectral shift in presence of *m*CPBA occurs owing to epoxidation of the exocylic double bond, leaving the chromophoric BODIPY core intact, in good agreement with previous findings.[23,24]

Epoxidation by *m*CPBA is assumed to proceed most probably in a concerted reaction[22] via a single transition state within a few picoseconds and thus far below the time resolution of the image acquisition. Most trajectories obeyed this expected immediate conversion from yellow to green fluorescence. However, long-lasting dim states were frequently observed during conversion (Figure 2c). To characterize these transitions we collected the lag time Δt (Figure 2c inset) of the dim states in a histogram. Figure 3a shows a semilogarithmic plot of the probability densities, which we obtained by normalization of the histogram (Supporting Information, Figures S14, S15).

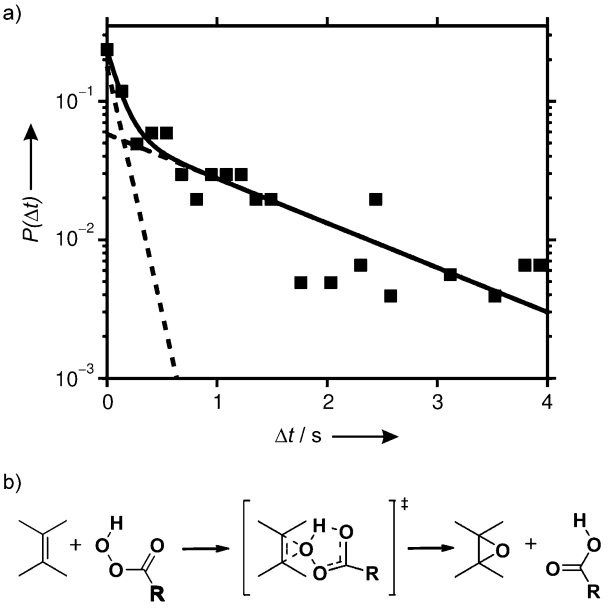

Figure 3. a) Normalized probability histogram of the lag time Δt that is fitted best by a double exponential model with time constants of 120 ms (75%) and 1.4 s (25%). b) Reaction process of the concerted epoxidation mechanism as proposed by Bartlett.[22]

The simplest model that fitted the data with equally distributed fit residuals was a bi-exponential function that yielded two time constants of 120 ms and 1.4 s and amplitudes of 75% and 25%, respectively. This corroborates that the two time constants reflect two distinguishable reaction pathways, yielding the same product **4**. The more abundant reaction path, which can be attributed to the lifetime below our instrumental response time, is compatible with the widely accepted concerted reaction mechanism (Figure 3b).

The intermediate state with the lifetime of roughly 1 s demands, however, an alternative explanation. Experiments at higher excitation intensities do not support any contribution of a light-driven process (Supporting Information, Figure S16). We also examined whether the lag time Δt depends on the reaction time starting from the initiation of the experiment and found that subsequently taken movies show very similar probability density distributions for Δt (Supporting Information, Figure S15). Thus, the lifetime of the intermediate state does not depend on the overall progress of the reaction in the ensemble. It is therefore unlikely that reaction pathways are caused by side-products, which would accumulate with time. Control experiments did not provide any indication of further, equally abundant fluorescent side-products (Supporting Information, Figure S4).

The shorter pathway is below the time resolution of our instrument and corresponds to the well-known concerted mechanism occurring on picosecond timescale. The slower pathway strongly indicates a transient intermediate formed upon epoxidation of the conjugated double bond with *m*CPBA. Although our current data allows distinguishing the two pathways, we can only speculate on its nature.

We could exclude light-driven dim states by additional experiments at higher excitation intensity, which gave similar

results. Thus, possible explanations for the intermediate state could be alternative reaction pathways. A radical intermediate is improbable as those are usually not associated with fluorescence emission and might not explain the weak emission we observe. Another option, a possible fluorescence quenching caused by formation of a π-complex of the substrate with *m*CPBA, could not be confirmed by titration experiments with the inert derivative *m*-chlorobenzoic acid (Supporting Information, Figure S18). Alternatively, an extended mechanism involving protonated epoxide as intermediate has been proposed and discussed already by different authors in the 1990s.[30] This pathway was also supported recently by quantum chemical calculations.[31] The transient nature of the proposed intermediate, however, imposes practical limitations for its experimental verification so far and might be fully understood with support of theoretical calculations estimating the energetic profiles of reaction pathways and intermediates.

In conclusion, we visualized the irreversible conversion of a substrate into a product by means of single-molecule detection. We provide a versatile experimental setup comprising probe design, immobilization and experimental conditions for single-molecule experiments that have high potential to study the wealth of organic reactions at double bonds. Interpretation of our experiments is based on the correlation between the spectral shifts observed in bulk and at single-molecule level indicative for a reaction of the double bond. Reaction details will be fully understood with additional chemical analysis. We further envision design of similar fluorescent probes where different specific reactions occurring can be directly observed and studied by means of single-molecule techniques. Such a method has high potential, opening new perspectives on observing, understanding, and ultimately controlling chemical reactions.[32] Beyond that, direct observation of the stochastic nature of chemical reactions provides a new way for perceiving, conceiving, and realizing chemistry.

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