Polarized Spectroscopy Studies of Single Molecules of Porphycenes: Tautomerism and Orientation[†]

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Received: November 7, 2008; Revised Manuscript Received: January 2, 2009

Using ambient atmosphere instead of pure nitrogen environment enabled efficient recording of room temperature fluorescence from single molecules of porphycenes, chromophores with a high triplet formation efficiency. Double hydrogen transfer between two chemically identical trans tautomers has been demonstrated for parent porphycene and three alkyl derivatives by the analysis of spatial patterns of the emission obtained after raster scanning the sample excited with an appropriately polarized laser beam. Because of tautomerization, fluorescence in porphycenes is due to two nearly orthogonal transition dipole moments. This property allows the spatial orientation of the single molecule chromophores to be determined using radially and azimuthally polarized laser beams as excitation sources.

1. Introduction

Diverse techniques of single molecule spectroscopy (SMS) have reached a stage of going beyond spectral and spatial detection and characterization of various chromophores. More and more works are being devoted to chemistry occurring on a single molecule level. This fascinating area includes, e.g., studies of electron and energy transfer,[1−10] conformational effects,[11−14] photochromism and optical switching,[15−21] enzymatic activity,[22−25] radical polymerization,[26] and single molecule catalysis.[27,28]

Using optical microscopy in combination with higher order laser modes,[29−42] we have been able to detect tautomerization in a single molecule.[43] This was done for porphycene (**1**, Scheme 1),[44] a constitutional isomer of porphyrin, the first obtained in a series that now includes corrphycene,[45] hemiporphycene,[46] isoporphycene,[47] and an "inverted" (or "confused") porphyrin.[48,49] The tautomerization involves double intramolecular hydrogen transfer between trans tautomers, with the substrate and product being chemically equivalent.[50,51] The two species differ, however, in the absolute direction of the transition moments (Figure 1). Using azimuthally polarized light to excite molecules immobilized in polymer matrices at room temperature, spatial patterns were observed which could only be explained by the presence of two transition moments forming an angle of 72 ± 3°. This value was in perfect agreement with the results obtained from fluorescence anisotropy studies for bulk porphycene embedded in polymeric films at 293 K[52] and in organic glasses at low temperatures.[53] Recently, the same value was independently confirmed by measurements of the anisotropy of transient linear dichroism in the pico- and femtosecond range.[54]

In this work, we first focus on the optimization of conditions which enable efficient recording of single molecule fluorescence of porphycene and its derivatives. Porphycene is known to have a large triplet formation efficiency (0.4)[55−57] and thus could be expected to be a poor candidate for single molecule studies.

We demonstrate that a controlled quenching of the triplet population results in photon counting rates sufficient for single chromophore detection.

Using SMS, we then look for spectral signatures of tautomerization in three derivatives of **1**: 2,7,12,17-tetra-*tert*-butylporphycene (**2**), 2,7-di-*tert*-butyl-10,19-dimethylporphycene (**3**), and 2,7,12,17-tetra-triphenylmethylporphycene (**4**) (Scheme 1). The spatial patterns of the emission from single chromophores prove the occurrence of trans−trans conversion involving double hydrogen transfer in all four molecules.

Finally, we show how the finding that porphycenes are, in fact, double-dipole emitters with nearly, but not exactly, orthogonal S_0−S_1 transition dipoles can be exploited for orientational imaging. Comparison of fluorescence patterns obtained from samples excited using azimuthal and radial polarization allows to distinguish between chromophores of which the planes are parallel to the support surface and the molecules with their planes perpendicular to the support. For the latter population, it is also possible to make a distinction between the molecules "standing" on the edges and on the vertices.

2. Experimental Section

All of the investigated porphycene derivatives were synthesized in our group, **1** and **2** according to published procedures.[58] 2,7-Di-*tert*-butyl-10,19-dimethylporphycene (**3**) was synthesized by a McMurry coupling reaction between 5,5′-diacetyl-2,2′-bipyrrole and 5,5′-diformyl-4,4′-di-*t*-butyl-2,2′-bipyrrole using a titanium reagent (TiCl$_4$/Zn); the yield after isolation by column chromatography and crystallization was 7%. 2,7,12,17-Tetra-triphenylmethylporphycene (**4**) was synthesized by a McMurry coupling reaction from 5,5′-diformyl-4,4′-ditriphenylmethyl-2,2′-bipyrrole using a titanium reagent (TiCl$_4$/Zn) in 3% yield after isolation by column chromatography and crystallization. Detailed reports on the syntheses of **3** and **4** will be presented in separate publications.

For spectral studies, the samples used were polymer films spin-coated onto glass microscope cover slides from a cosolution
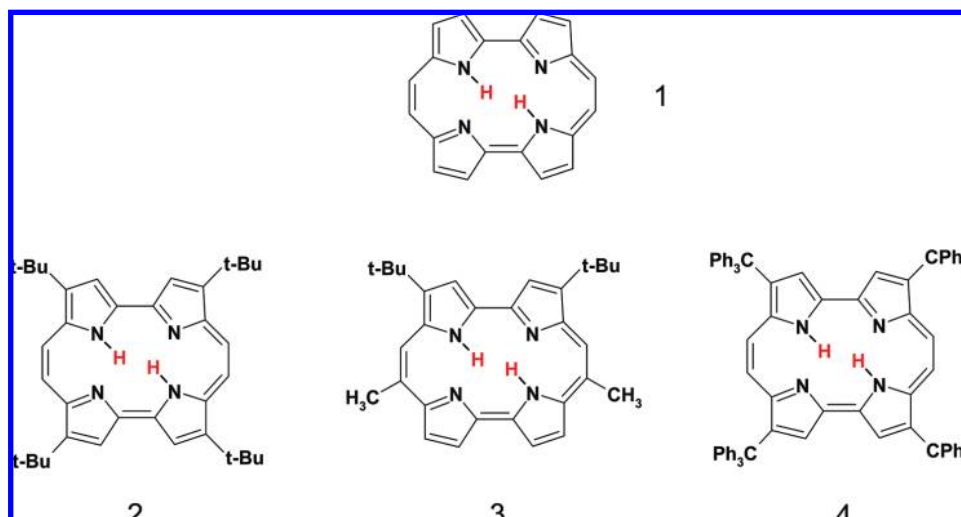

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SCHEME 1: Derivatives of Porphycene Investigated in the Single Molecule Regime

of poly(methyl methacrylate) (PMMA, 10 mg/ml) and the appropriate porphycene (5×10^{-10} to 10^{-7} M) in toluene. The thickness of such samples, estimated using AFM and ellipsometry, was 30 ± 20 nm.

The optical setup for bulk measurements resembles a low-resolution inverted microscope. The sample was excited with the 632.8 nm He−Ne laser (Coherent, 31−2041−000). The photon flux at the sample was adjusted with a variable optical density metallic filter (Newport). The laser beam passed, consecutively, through an acousto-optic modulator, (TEM-85−2, Brimrose), through a laser divergence correction lens, and through a circular aperture. Next, the beam was reflected by a dichroic laser beam splitter (XF 2021, Omega Optical), and finally focused on a sample with an immersion microscopic objective (either $10\times$, $40\times$, or $100\times$ magnification). To ensure the homogeneous illumination of molecules in the image plane, care was taken to illuminate the aperture as uniformly as possible. Fluorescence collected by the objective was transmitted through a beam splitter and then through a filter (XR3003−640 ALP, Omega Optical) which blocked the back-scattered laser light. The fluorescence was detected by a photomultiplier tube (Hamamatsu R-955) coupled with a digital oscilloscope (Tektronix TDS 3240). Bulk samples under study were subjected to a continuous flow (100 mL/h) of the gas (nitrogen and/or oxygen) above the sample surface.

Single-molecule experiments were performed in Tuebingen on a home-built inverted confocal microscope optimized for single-molecule detection and imaging. A collimated linearly polarized laser beam provided by a He−Ne laser (1137P, Uniphase) is modified in its polarization properties by passing through a mode converter (MC) described earlier,[37] which is the key element of the setup. In short, the MC consists of a four-quadrant $\lambda/2$-retardation plate. The optical axis of each quadrant is oriented in such a way that the polarization of the transmitted field is rotated by 90° in adjacent segments. The MC was placed in the path of the excitation beam, transforming the linearly polarized Gaussian mode into higher order laser modes, i.e., an azimuthally polarized doughnut mode (Figure 2, right) or a radially polarized doughnut mode (Figure 2, left). One can switch between a radially and an azimuthally doughnut beams by rotating the MC by 90°. The mode converter was followed by a spatial filter which served to suppress higher spatial modes. The collimated beam was reflected by a nonpolarizing beam splitter and focused on the sample with an immersion objective (CP-Achromat $100\times$/NA = 1.25, Zeiss).

Raster scanning of the sample in x and y directions with respect to the diffraction-limited spot of the fixed microscope objective was done using a feedback-controlled sample stage (P-527, Physik Instrumente). The collected fluorescence signal was separated from the reflected laser light by a holographic notch filter (λ_{center} = 633 nm, Kaiser). Successively, the light transmitted through the notch filter was focused on a single photon counting avalanche photodiode (SPCM-AQR-14, Perkin-Elmer), serving as a detector. Fluorescence spectra were acquired with a grating spectrograph (SpectraPro 300i, Acton Research) in combination with a thermoelectric cooled CCD camera (Roper Scientific, OE).

Theoretical modeling of the spatial patterns of the emission collected from single chromophores was performed using a Matlab code. In the first step, the focal fields $E(x, y)$ generated by the different laser modes were calculated analytically following ref 32. In the second step, the resulting fluorescence intensity maps were determined for a given transition dipole orientation **u** according to $F(x, y) \sim |\mathbf{u}^*E(x, y)|^2$ This procedure allowed the fluorescence intensity to be simulated due to one or two electronic transition moments. For the latter case, the angle between the transition dipoles could be varied at will. Also the orientation of the transition moments in the laboratory system of coordinates could be changed. For each particular orientation, the calculations have been performed for both azimuthal and radial excitation modes. The results of simulations were compared with the experimentally obtained fluorescence patterns. The same value of the angle between the $S_0−S_1$ transition moment directions as in parent porphycene, was assumed for **2−4**.

3. Results and Discussion

3.1. Optimization of Conditions for Single Molecule Detection. In order to obtain a satisfactory signal-to-noise ratio in an SMS experiment, the number of photons emitted by the
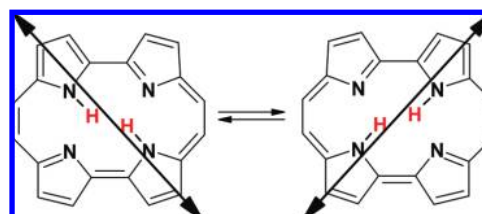

Figure 1. Tautomerization in porphycene and the accompanying change in the direction of the $S_0−S_1$ electronic transition moment.
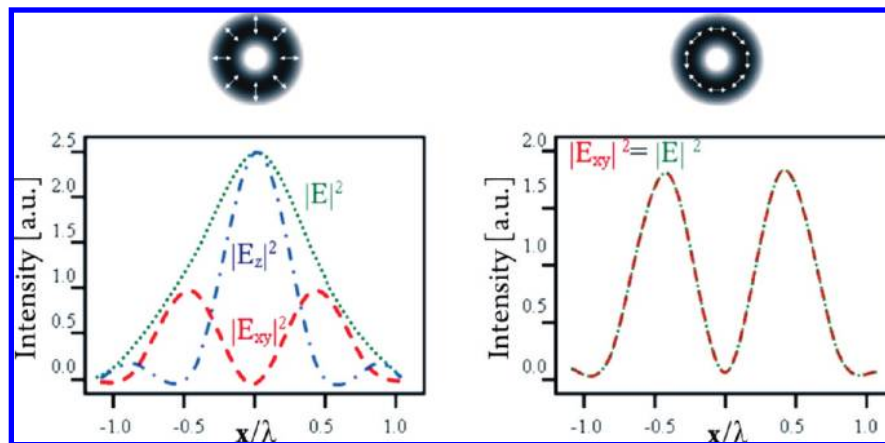
Figure 2. (Top) Field distributions of the transverse components of the radially (left) and azimuthally (right) polarized laser beams. (Bottom) Total, transversal, and longitudinal field strengths in the focus of a high numerical aperture objective.
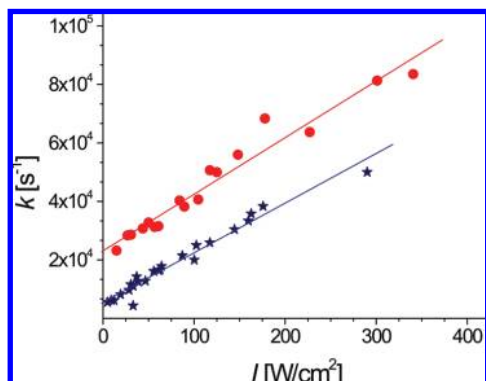

Figure 3. Emissivity decay rate k as a function of the excitation intensity for a bulk sample of porphycene in PMMA film at 293 K. The sample under study was subjected to a continuous flow of gaseous nitrogen (blue stars) and oxygen (red dots) above the sample surface.

Figure 4. Confocal images of single porphycene molecules showing typical fluorescence patterns observed in the experiment: samples of **2** (middle), **3** (left), and **4** (right). Scan range: $5.0 \times 5.0 \ \mu m^2$.

excited chromophore must be sufficiently high. Such conditions are more easily achieved for molecules with low triplet yields. If the triplet formation efficiency is high, intensive blinking occurs, making it difficult to detect the appropriate number of photons per time unit. Moreover, the triplet states can also act as doorways to other dark states which, in turn, can lead to even longer "off" times or to bleaching. Such is the case, e.g., of rhodamine 6G in poly(vinyl alcohol).[59,60] It is therefore crucial, especially in the case of chromophores exhibiting high triplet yield, to minimize the triplet lifetime. However, to avoid lower photon collection efficiency, such a procedure must be done without introducing photobleaching.

In order to optimize the conditions for effective detection of single porphycene molecules, we first carried out experiments on molecular ensembles. The methodology was similar to that used recently by Orrit and co-workers.[60] In the bulk-fluorescence experiment, the emissivity decay of the ensemble (fluorescence normalized by the excitation intensity) was monitored as a function of the illumination time for various excitation intensities (Figure 3). In practical terms, a reversible decay of the fluorescence signal, observed immediately after the onset of the continuous laser excitation was recorded in the time range of 10^{-4} to 10^{-2} s. The samples of porphycene in PMMA were kept either in the atmosphere of pure nitrogen or in oxygen. For low excitation intensity, the emissivity decay rate is approximately equal to the triplet deactivation rate. Figure 3 shows that using oxygen led to the shortening of the triplet lifetime by a factor of 5. A compromise had to be sought
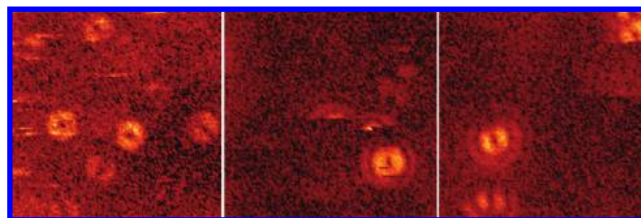
between, on one hand, the highest possible count rates and, on the other hand, possibly low bleaching efficiency. Therefore, for single molecule experiments the samples were kept under ambient atmosphere: such arrangement resulted in a sufficient photon count rate, without leading to significant decrease in lifetime of single chromophores. In principle, further improvement would be possible by optimizing the oxygen concentration separately for each porphycene derivative. Such a procedure was recently applied for single molecules of indocarbocyanine in PMMA: it was demonstrated that the best conditions for signal detection corresponded to 2% of oxygen in an inert atmosphere.[61] In another work, improvement of photostability in the presence of oxygen was reported for ionic dyes.[62]

3.2. Detection of Double Hydrogen Transfer in Single Molecules. Figure 4 presents the images obtained for the porphycene derivatives **2**−**4** after raster-scanning the PMMA films containing the three chromophores. Azimuthal mode was used for excitation, which resulted in a better contrast than while using radially polarized beam. For the latter case, the nonzero longitudinal component of the electric field leads to an increased background emission, whereas it is not absorbed by chromophores whose planes are oriented parallel to the substrate surface (it may be recalled that porphycenes are planar aromatic chromophores in which the lowest transitions, of $\pi\pi^*$ nature, are purely in-plane polarized). However, for more detailed studies of the orientation, described below, the same molecules were investigated using both polarization modes.

The images reveal characteristic blinking and bleaching patterns, typical of single chromophores. Moreover, fluorescence spectra agree well with those obtained from ensemble measurements. We checked that the fluorescence of aggregates of porphycenes, formed, e.g., in nonpolar solutions at high concentrations, is different from the monomer emission. Moreover, it only becomes intense at low temperatures. Therefore,
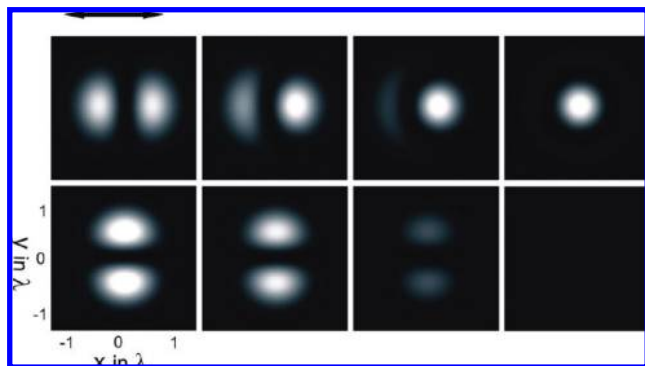
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Figure 5. Simulated patterns for a single emitting dipole excited with radially (top) and azimuthally (bottom) polarized beams. The sample surface corresponds to the *xy* plane. The dipole, initially oriented along the horizontal *x* axis is rotated by 30, 60, and 90° about the *y* axis.
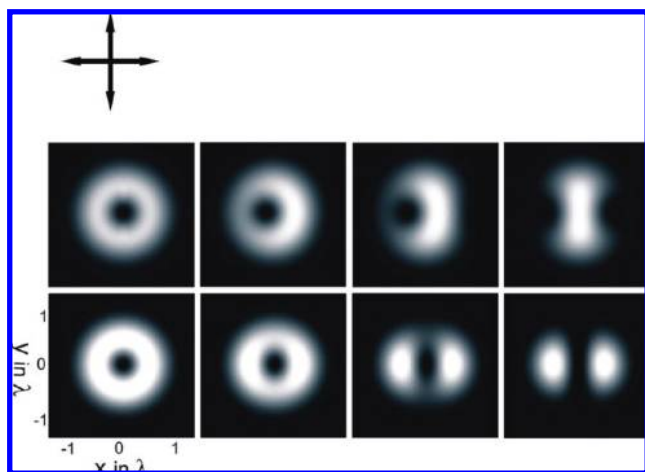

Figure 6. Simulated patterns for two orthogonal emitting dipoles excited with radially (top) and azimuthally (bottom) polarized beams. The sample surface corresponds to the *xy* plane. The dipoles are initially oriented along the *x* and *y* axes. Left from right, rotation by 0, 30, 60, and 90° about the vertical *y* axis.

Figure 7. Simulated patterns for two emitting dipoles forming an angle of 72° excited with radially (top) and azimuthally (bottom) polarized beams. The sample surface corresponds to the *xy* plane. The first dipole is initially oriented along the *x* axis, the second lies in the *xy* plane, forming an angle of 72° with *x*. Left from right, rotation by 0, 30, 60, and 90° about the vertical *y* axis.

the observed images can be assigned to the fluorescence originating from single molecules of porphycenes.

Among various observed spatial patterns, the most intriguing ones are those corresponding to the ring or quasi-ring shape. Such a pattern cannot be caused by a single transition dipole, no matter what its orientation is (Figure 5). On the other hand, it is readily simulated assuming a presence of two transition moments of equal magnitude (Figures 6 and 7). The angle between the transition moments can be treated as a parameter in the numerical simulation procedure. The angle between the transition moments obtained by fitting, 72 ± 3°, is practically the same as that determined for bulk samples independently by different techniques, fluorescence anisotropy[52,53] and the anisotropy of transient absorption.[54] It can be therefore concluded that the ring shapes obtained for all four porphycenes studied in the single molecule regime are manifestation of tautomerization involving two trans tautomeric forms, chemically equivalent, but differing in the absolute orientation of the transition moments (Figure 1). Expressed differently, porphycene is a "dual transition dipole" or a "dual emitter" molecule, with a well-determined angle between the two transition moments. It is important to note that the two dipoles are close to being orthogonal, but not exactly so. This fact has important implications for the determination of absolute molecular orientation, as discussed below.
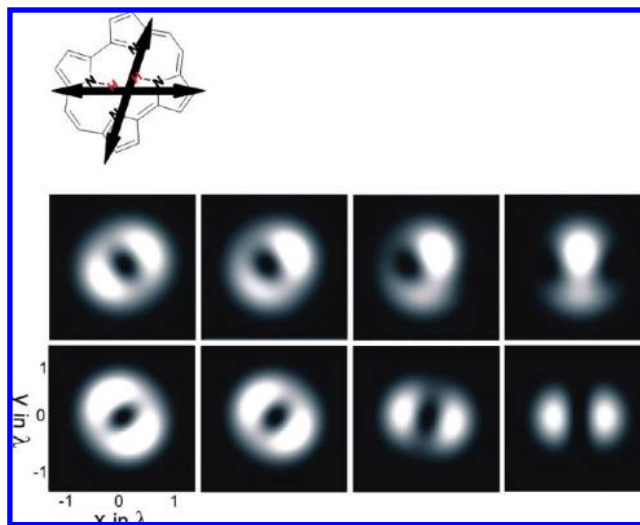
In addition to ring-like shapes, an often-encountered motif is that of a double lobe. This can be explained in two ways. First, one could assume that for certain molecules the tautomerization is hindered. As a result, the molecule behaves as a single emitting dipole, for which a double-lobe pattern is expected (cf. Figure 5). Such an assumption, however, seems rather improbable. Tautomerization in porphycene has been shown to occur in both ground and excited electronic states,[50,51] with the rates approaching 1 ps^{-1} in solutions and polymeric films at 293 K. In crystalline porphycene, much lower rates have been reported;[63,64] still, the reaction occurs in nano- or microseconds. It is therefore highly unlikely that the reaction be frozen on the time scale of our experiments, which collect photons in millisecond intervals over a period of a few seconds.

The second explanation for a double-lobe pattern is that it is also due to molecules which fluoresce as "dual emitters", but whose orientations are close to perpendicular to the substrate. For such orientation, the overall transition dipole can have in-plane and out-of-plane components for both single and dual-dipole chromophores. Since the longitudinal field strength is zero for the azimuthally polarized beam, the out-of-plane components will not be detected in any case. However, they are observed using radially polarized mode. Most important, comparison of emission patterns obtained for the two polarizations enables, in most cases, the single and dual dipole cases to de distinguished and the 3D orientation of the chromophore to be determined.

3.3. Orientation of Single Molecules. Figure 8 shows three different single molecules of **2**, each observed using both radial and azimuthal polarization excitation modes. Simulations of the observed patterns using two transition dipoles forming an angle of 72° leads to a satisfactory agreement with experiment. The ring-like pattern obtained for the molecule presented on the left implies that two transition moments have to be present. However, this is not the case with the other two molecules observed only with azimuthal mode. As stated above, the double-lobe pattern could be due either to a single dipole or to two transition moments lying in the plane perpendicular to the sample surface. Comparison with the patterns observed while using radially polarized excitation leaves no doubt that the
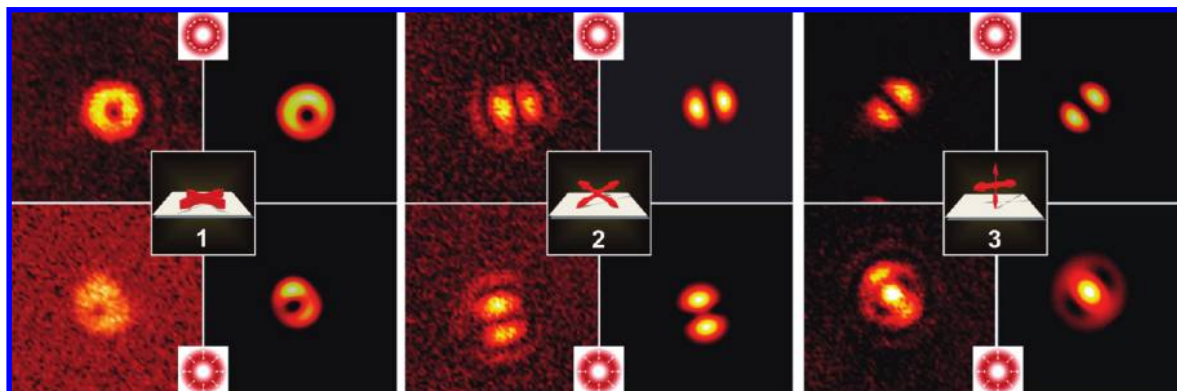
Figure 8. Confocal fluorescence images observed for **2** excited either by azimuthally (top) or radially (bottom) polarized beam, showing three different orientations of the chromophore. (1) A molecule lying flat on the surface; (2 and 3) molecules with perpendicular orientation of molecular plane with respect to the sample surface. In 2, two dipoles form the same angle with the surface plane, whereas in 3 one dipole is perpendicular to the surface and the other is almost parallel to it. Left, experimental, right, simulated patterns.

emitting molecule contains two dipoles. This is particularly clear for the case 3 in Figure 8. For a single dipole emitter, switching from azimuthal to radial mode would not be accompanied by a change in the shape of the emission pattern but only by its in-plane rotation by 90°, preserving a double-lobe structure. This is evidently not observed. On the other hand, the simulation which assumes the presence of two dipoles nicely reproduces different experimental shapes observed for the two modes.

Interestingly, simulations can clearly differentiate between the two extreme cases of perpendicular orientation: first, with the two dipoles forming the same angle with the surface, and the second, with the first and second dipole approximately parallel and perpendicular to the surface, respectively. Such distinction would not have been possible for the case of two exactly orthogonal transition moments, for which identical in-plane and out-of-plane components should be obtained for any perpendicular orientation. The deviation from orthogonality by 18° is more than sufficient to distinguish between the two cases, illustrating the power of single molecule spectroscopy in orientational imaging. It seems worthwhile to mention in this context that a recent study of single gold nanorods allowed determining their orientation with an accuracy better than 5°.[29]

4. Summary

Our results demonstrate that the basic chemical reaction, hydrogen transfer, can be monitored on a single molecule level. The implications for future works are obvious and include such possibilities as kinetic studies, dependence on the environment, "locking" the protons in one well of a double minimum potential, etc.

Second, it was shown that the high yield of triplet formation, approaching 50% for porphycenes, does not preclude an efficient recording of fluorescence from a single chromophore. Actually, judicious choice of triplet quencher concentration can strongly enhance the signal. We plan to optimize the conditions for single molecule fluorescence collection in selected porphycenes using not only oxygen, but also heavy rare gases.

Dual-emitter character of porphycenes provides methods for accurate determination of the orientation and/or transition moment directions. We intend to pursue these studies on those porphycenes which reveal the presence of cis tautomers.[65] For symmetry reasons, the transition moment direction in the latter bisects the angle formed by the transition dipoles in the trans forms. This may either enhance or diminish the inequality between the in-plane and out-of-plane components of molecules

oriented perpendicularly to the surface. In the former case, one could expect to be able to detect the presence of rare cis forms. Such presence should also be manifested by distortions from ideal ring patterns for molecules lying flat on the surface.

It seems worthwhile to stress the merits of combined use of both azimuthal and radial modes of light to excite fluorescence from single molecules. Using azimuthal polarization results in a better signal-to-noise ratio. In turn, emission patterns obtained with radial polarization are much more sensitive to the direction of the emitting dipole and therefore this mode is more appropriate for orientation studies. Of course, it is preferable to apply both modes to each single chromophore: in practical terms, it just requires rotating the mode converter by 90°.

Finally, the possibility of observing fluorescence and orientation from single porphycene molecules can find application in observing the phenomena related to photodynamic therapy, such as penetration of the chromophore into the cell and its localization. Actually, various porphycenes are known to be very good candidates to become efficient phototherapeutic agents.[66]

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