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Published in:
Single Molecules

Single Mol. **2** (2001) 1, 35-44

Polarisation Sensitive Single Molecule Fluorescence Detection with Linear Polarised Excitation Light and Modulated Polarisation Direction Applied to Multichromophoric Entities

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submitted 19 Jan 2001
published 06 Apr 2001

Abstract

Recently, investigations of the fluorescence properties of a multichromophoric dendritic entity at the single molecule level have revealed multiple fluorescence levels, collective off-states, variations of the polarisation, large shifts in the spectral position and changes in the fluorescence decay time (Gensch et al. (1999), Hofkens et al. (2000)). In order to further elucidate the multiple processes taking place in this entity, measurements were done in which the polarisation direction of the linear polarised excitation light was modulated. The detection was sensitive for the s- and p-components of the emitted light. The patterns of modulation and relative intensity in the acquired traces reflect the energy transfer processes occurring in this multichromophoric molecule. In-phase modulation and no modulation are the typical modulation patterns that were observed. Simulations involving several models for energy transfer between the chromophores have been carried out taking into account identical conditions as for the performed measurements. The comparison of the modulation patterns and polarisation histograms to the measured data rules out certain models and refines the photophysical model for the multichromophoric entity.

Introduction

Biologically relevant multichromophoric systems like the light harvesting systems from the photosynthetic apparatus have become prominent investigation objects for single molecule fluorescence spectroscopy (SMS) in the recent past. In these systems tens of identical or similar chromophores bound to a supramolecular protein skeleton reside in close proximity. Many interactions including energy transfer and exciton formation occur among the chromophores leading to a complex time-, excitation and emission wavelength-dependent fluorescence behaviour. It is often described by a sum of exponential functions. The decay times and wavelength-dependent amplitudes, however, have no direct physical meaning unless a detailed kinetic model is established. Especially in the case of multichromophoric units with many similar chromophores

and possible interactions between all of them, the choice of a model is not completely unequivocal. SMS measurements can help to support or to disprove certain assumptions made in the model.

A particular SMS technique detects the fluorescence intensity time course of a single multichromophoric entity upon excitation with linear polarised light for which the polarisation direction is modulated with a few Hz. This is combined with a polarisation sensitive detection of the s and p polarised components of the emitted light by means of two independent detectors. In the following such a data set is named MFIT (Modulated Fluorescence Intensity Trace). The method gives information about the number of chromophores, their orientation, arrangement and interactions. This technique was applied to discriminate whether single spots in a SMS fluorescence image consisted of one or more chromophores [1]. For multiple chromophores the signal in the two detection channels will not be in phase and the intensity does not reach the background level (see Figure 3 in reference [1]). An alternative way for the determination of single chromophore orientation has been developed by Weston and co-workers [2].

In some recent articles Weiss and co-workers have pointed out the use of polarisation in excitation and detection in the field of SMS [3,4,5]. They suggested that the mobility of single chromophores bound directly to surfaces or to immobilised proteins and to DNA could be characterised upon excitation with a sweeping excitation polarisation. Applications in single molecule fluorescence resonance energy transfer (FRET) studies - pioneered in the group of Weiss [6] - with a four detector scheme simultaneously recording donor and acceptor fluorescence resolved in s and p polarisation would increase the accuracy of the distance determination in FRET studies due to the exact knowledge about the donor and acceptor orientation [7]. Seidel and co-workers recently reported the use of such an instrumental set-up [8].

A conjugated polymer (MEH-PPV) has become an interesting study object among single multichromophoric entities. Barbara and co-workers have shown that non-radiative trap states exist, which lead to collective off-states [9]. They also concluded that efficient energy transfer occurred. Recently anisotropy [10] and polarisation measurements on single MEH-PPVs [11] were used to determine the energy transfer processes and the structure of MEH-PPV in different environments. Furthermore, Hu et al. applied a similar modulation technique as used for this study but detected only the total fluorescence intensity [12]. They generated sets of MEH-PPV based on certain polymer structure models and simulated MFIT measurements. The comparison of the histograms of simulated and measured MFIT modulation depths enabled the authors to rule out the existence of some of the structure models.

SMS studies on multichromophoric entities are not only important for FRET measurements or for polymer research but also relevant for biological systems. The photosynthetic apparatus is probably the most important multichromophoric system in nature consisting of many tens or even hundreds of identical and similar chromophores. Photosystem 1 [13] and several of the light harvesting subunits have been investigated in the recent past. Historically, B-phycoerythrobilin (B-PE), a pigment-protein complex from the phycobilisome, the antenna system of cyanobacteria and red algae, has been the first molecule in room temperature single molecule spectroscopy [14,15]. It has a huge absorption coefficient (34 tetrapyrrole chromophores) and a high fluorescence quantum yield. Keller and co-workers concluded from fluorescence burst and photon pair correlation measurements on B-PE in a flow stream that the photodestruction of B-PE occurs in a single step and that the 34 independent chromophores behave like a single quantum system [16]. The observation time of those measurements was limited in time to 0.5 - 40 ms. In a recent study on B-PE in polyvinylalcohol films, for which much longer observation times were achievable, further indications were found for one-step transitions from a high level state to an off-state. In addition, multiple fluorescence intensity levels and reversible off-states were observed and contradicting results concerning the single step photobleaching were obtained [17].

Until now the light harvesting complex LH2 of the photosynthetic system of purple bacteria is the most studied antenna complex in SMS, because an X-ray crystal structure with atomic resolution is available [18]. The supramolecular disk-like complex consists of bacteriochlorophylls with different spectral properties anchored to dipeptides and arranged in two rings with three-fold rotation symmetry. Hochstrasser, Cogdell and co-workers found also for this multichromophoric entity collective off-states as well as multiple fluorescence intensity levels each having a characteristic fluorescence decay time [19]. Use of polarisation sensitive detection and excitation polarisation orientation modulation in a second study revealed that one of the rings deviates from the ideal ring structure and can be described as an elliptical absorber and emitter (with a small ellipticity, though) [20]. This non-ideal behaviour was explained by structural distortions at one or more of the chromophores. Sudden discrete changes in the phase of the MFITs occurred commonly in steps of $2\pi/9$, that is the angle between two neighbouring dipeptides. The latter result was interpreted as a hopping of the structural distortion from one chromophore to a neighbour chromophore. For PS1 similar studies are currently under way [21].

Ying and Xie studied the photophysics of allophycocyanin (APC) another pigment-protein complex from the phycobilisome with SMS techniques [22]. APC has a trimeric structure with six phycocyanobilin chromophores organised in three strongly interacting pairs. In contrast to B-PE and LH2 APC does not exhibit any collective off-states. The off-state is populated after photobleaching of all six

chromophores, exclusively. This proves a very weak coupling between the three chromophore pairs. Often three intensity levels were observed which the authors assigned to one, two or three emitting chromophore pairs. This was explained by quenching of the emission within a single pair by photoinduced exciton traps as proposed for MEH-PPV and LH2 [9,19]. Ying and Xie also measured fluorescence intensity traces with modulation of the excitation polarisation modulation but without polarisation sensitive detection (similar to Hu et al. [12]). Due to the high symmetry of the APC structure they could predict modulation patterns for one, two and three emitting pairs and found those back in the fluorescence intensity traces.

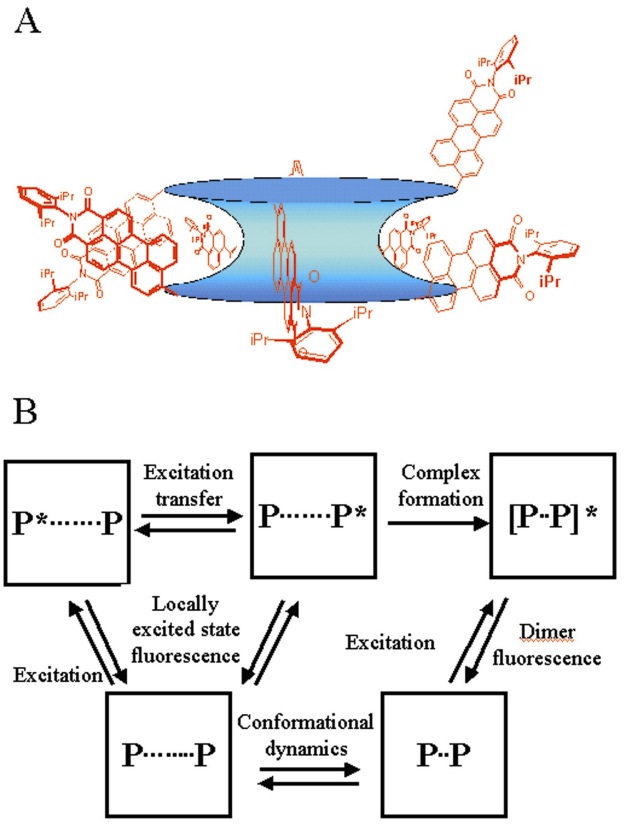

Fig. 1. *3-dimensional structure and photophysical scheme of compound 1 (adapted from [24]) is schematically represened in figure* **1a***, the 8 chromophores are drawn and the dendritic core is represented by the double cone like structure. So the whole cartoon represents figure 1.*

A multichromophoric model system based on a polyphenylene dendrimer family decorated with perylenecarboxyimides (PCI) has been studied extensively [23-29]. The dendrimer construction principle offers a systematic control over the number of chromophores and their interactions. The photostability of this model system is higher compared to some of the biological antenna complexes. It shows collective off-states and changes of fluorescence spectrum, decay and polarisation. Therefore, it is a good model for biological antenna systems as B-PE and APC. We decided to study the information content in SMS fluorescence traces obtained with linear excitation polarisation with modulated polarisation (MFIT) on a 2nd generation polyphenylene dendrimer with eight PCI chromophores (compound 1, Figure 1A). The MFITs were characterised by their modulation pattern, polarisation and total fluorescence intensity. Due to a huge variety of behaviours that were found no direct conclusions about the photophysics of compound 1 could be drawn. Instead, sets of MFITs (100 each) have been simulated for a number of photophysical models for the energy transfer and emission processes in compound 1. A comparison of the results of measured and simulated data based on similar statistics provided new insight into the energy transfer and emission properties of compound 1. A similar comparison of measured and simulated data was performed by Hu et al. [12]. While in their contribution the creation of sets of multichromophoric molecules was based on physical interactions between the building blocks of the polymer we have used the geometry information given by the chemical synthesis of compound 1.

Materials and Methods

Experimentals

The synthesis of compound 1 – a 2nd generation polyphenylene dendrimer decorated with eight perylenecarboxyimides (PCI, see Fig. 1) - and of compound 2 - a single chromophore model compound (hexaphenyl-perylenecarboxyimide) - has been already described in detail [23,30]. Samples for the single molecule measurements on the dendrimer were prepared by spin-coating solutions of compound 1 in chloroform ($5x10^{-10}$ M) containing 3 mg/ml polyvinylbutyral (PVB) on a cover glass at 4000 rpm to yield thin polymer films (20 - 40 nm as measured by AFM) containing on average 0.2 molecules per μm^2. The sample preparation included careful cleaning of the glassware used for sample preparation as well as a subsequent cleaning of the cover glasses by sonification in acetone, sodium hydroxide (10 %) solution and MilliQ water.

The fluorescence of single molecules was detected using a confocal microscope (Diaphot 200, Nikon) with an oil immersion lens (NA 1.4) equipped with two avalanche photodiodes (APD) in single photon counting mode (SPCM AQ15, EG&G) as the detector. The emitted light was split with a polarising beam splitter cube (Newport 05FC16PB.3) and detection of s and p polarised components of the fluorescence light was carried out with two independent detectors. Suitable filters were placed in the detection path to suppress remaining excitation light. The fluorescence intensity traces were recorded with a dwell time of 2 ms per data point. The monochromatic excitation source was an

Argon ion laser (Spectra Physics Stabilite 2017) with an attenuated power of 450 nW at 488 nm (~ 300 W cm^{-2}). Modulation of the excitation polarisation was obtained by guiding linear polarised laser light through a $\lambda/2$ plate which was rotating with a stable frequency. The modulation frequency implied on the excitation light polarisation direction was 20 Hz. This frequency was chosen in order to obtain data which were sensitive for changes of the modulation pattern in less than 100 ms.

The measured and simulated MFITs (see below) were inspected for a number of properties, namely the modulation pattern, the total fluorescence intensity and the polarisation. The latter is defined as the ratio of difference and sum of the intensity detected in the s and p detection channel (I_p, I_s, see equation (1)). The background signals were measured in dark areas of the sample ($I_{p,\,bg}$, $I_{s,\,bg}$). The detection sensitivity in the s and p detection channel is not equal due to non-ideal optical elements, detectors and alignment. The ratio of detection efficiency in the two detection channels (so-called g-factor, $g = I_p/I_s$) was determined by measuring a sample with isotropic emission [31]:

$$P(t) = \frac{\left[\left(I_p(t) - I_{p,bg}(t)\right) - g * \left(I_s(t) - I_{s,bg}(t)\right)\right]}{\left[\left(I_p(t) - I_{p,bg}(t)\right) + g * \left(I_s(t) - I_{s,bg}(t)\right)\right]} \quad (1)$$

The modulation pattern of the MFITs was classified into five major groups: in-phase modulation down to the background signal (IPM), in-phase modulation with off-set (IPMO), out-of-phase modulation (OPM), no modulation in two channels (NM2) and modulation in one, no modulation in the other channel (NM1). For a number of MFITs a $\cos^2$ function was fitted to the data and 20 Hz was exclusively found as modulation frequency.

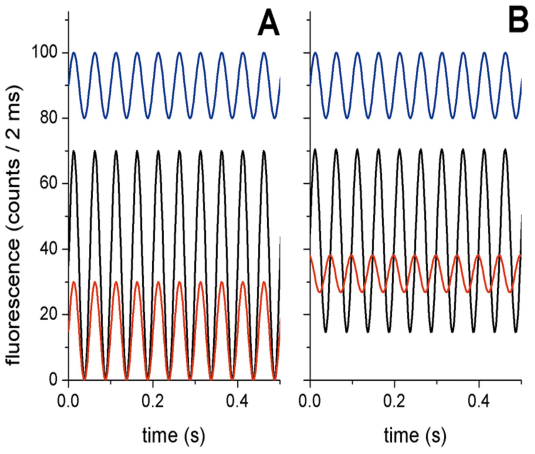

Fig. 2. *Simulated MFITs (black: p polarisation channel, red: s polarisation channel, blue: excitation modulation) of one* **(A)** *and two non-interacting chromophores* **(B)**.

The prediction for the modulation pattern of a single chromophoric molecule is very simple. It will show an in-phase modulation with a high modulation depth down to 0 (IPM, see Figure 2A). For single molecules of the model compound 2 only such behaviour was found exclusively [24]. In general, if two chromophores without interaction are excited their absorption/emission dipole moments will be not parallel. This will result in an out-of-phase modulation (OPM) as depicted in Figure 2B. If there were an efficient energy transfer forth and back among the two chromophores, the modulation pattern would alter. No modulation in both channels (NM2), modulation in one and no modulation in the other (NM1) or in-phase modulation with offset (IPMO) would occur (see Figure 4).

Simulations

Although modulation pattern and polarisation contain much information, it is not possible to determine the exact photophysical behaviour of a molecule with eight chromophores and possible energy transfer among them from its MFIT. The imperfection in isotropic excitation and isotropic emission collection in the commonly used SMS set-ups contributes significantly to this fact. Neither excitation nor emission collection can be currently performed with the same probability for x-, y- and z-direction. The occurrence probabilities for certain behaviours of those properties, however, will be characteristic. Therefore we decided to simulate MFITs for molecules of compound 1 with distinct photophysical behaviour to compare their occurrence probabilities to those for the traces of the measured molecules. We produced 100 MFITs for each model to have a similar statistical accuracy as for the measured data (165 molecules). The simulations of MFITs were performed with the help of Microcal Origin 6.1 using scripts written in Microcal Labtalk (Origin). As a first step, 100 individual molecules of compound 1 were "created" for each model for the energy transfer and emission properties of compound 1. For gas-phase free energy minimisation calculations a tendency was found for an equatorial arrangement of the PCIs rather than a complete 3-dimensional isotropic situation (see Figure 1A) [24]. This might be elicited due to attractive interactions between the highly aromatic PCI chromophores. From this we derived the following model: each chromophore of compound 1 has its own octant in the x, y plane and $\pm\pi/4$ relative to the equatorial plane of the dendrimer.

A photophysical scheme was derived based on time-resolved fs-ns absorption and fluorescence ensemble measurements and single molecule measurement on the dendrimer family 1 (Figure 1B) [23-29]. Evidence for an excited state, dimer-like fluorescence as well as several modes of energy transfer on a sub-ns time scale have been found. Part of the latter can be explained by FRET (R_0 of PCI (38 Å). Since the absorption spectrum of compound 1 is almost identical to the one of a model compound 2 (one PCI

linked to a hexaphenylene), one can assume that the eight PCIs act as individual absorbers. The coupling of the PCIs to the dendrimer branch ends can occur to two chemically identical meta positions. Due to the non-symmetric substitution and a certain degree of flexibility in the C-C bond of the biphenyl core many different structural isomers exist. There is also some flexibility in the position of the dendrimer arms. Therefore the distance between neighbouring PCIs is not a discrete value but a distance distribution.

Based on this photophysical knowledge we simulated MFITs for the following models:

- SIM1) No energy transfer among the eight PCIs
- SIM2A) Energy transfer among the eight PCIs funnelled to one emitting state (static)
- SIM2B) Energy transfer among the eight PCIs funnelled to two emitting states (static)
- SIM3A) Energy transfer among the eight PCIs funnelled to one emitting state (dynamic, all PCIs populate the energy receiving state within the bin time of the measurements)
- SIM3B) Energy transfer among the eight PCIs funnelled to one emitting state (dynamic, all PCIs populate the receiver state within the bin time of the measurements); dendrimer equatorial plane perpendicular to the light propagation

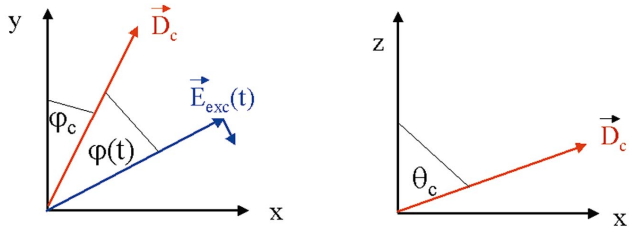

Fig. 3. *Geometry of the excitation light vector and the absorption dipole moment of one representative chromophore from compound 1 (the light propagates along the z-axis).*

SIM3 is identical to a situation, for which effective energy transfer between all eight iso-energetic PCIs occurs. The eight PCIs were arranged in the "dendrimer arrangement", i.e. every PCI with an arbitrary orientation in its λ/4 sector (x,y-plane, φ_c), $-\pi/4<\Theta_c<\pi/4$ (relative to the dendrimer "equator") and a free orientation of the "dendrimer"-plane in space (φ_{Den}, Θ_{Den}) (see Figure 3). The fluorescence quantum yield of compound 1 is 20 % lower compared to that of compound 2. Therefore, energy transfer efficiencies between 1 and 0.8 were tested in the simulations. The modulation pattern was not affected. Only the absolute values of the fluorescence intensity decreased for lower energy transfer efficiencies. Following the arguments of Ha et al. [3] the high NA (1.4) of the microscopic lens will lead to a negligible excitation probability for the z-component of the absorption dipole moment (< 5 %) while the contribution of the emission dipole z-component is significant (~21 %). Ip and Is for a single PCI were calculated with the following formulas with some necessary modifications for the models SIM1-SIM3:

$$I_p(t) = \cos^2(\varphi(t)) \cdot \left\{0.79 \sin^2(\Theta_c)\cos^2(\varphi_c) + 0.21\cos^2(\Theta_c)\right\}\sin^2(\Theta c) \quad (2)$$

$$I_s(t) = \cos^2(\varphi(t)) \cdot \left\{0.79 \sin^2(\Theta_c)\sin^2(\varphi_c) + 0.21\cos^2(\Theta c)\right\}\sin^2(\Theta c) \quad (3)$$

$$\varphi(t) = \varphi_{exc}(t) - \varphi_c = \frac{ft}{(2\pi)} - \varphi_c \quad (4)$$

Afterwards a Poissonian distributed noise was added to I_p and I_s and the sums of all eight I_p and I_s, respectively, were calculated. Each set of 100 MFITs was analysed in an identical way as the 165 measured MFITs. The properties were histogrammed and the histograms were compared with those of the measured data.

Results and Discussion

165 modulated fluorescence intensity traces (MFIT) of compound 1 were measured for 60 s. Four different modulation patterns were observed. Typical examples are depicted in Figure 4.

Only 2 % of the molecules showed the same modulation pattern, polarisation and fluorescence intensity in both detection channels for 60 s. Characteristic for a multichromophoric entity is that the modulation is only partial, i.e. not down to the background level (compare with Fig. 2). The rest of the MFITs showed rich dynamical behaviour in many fluorescence properties like the total fluorescence intensity (95 % of the molecules), the polarisation (55 %), the modulation pattern (45 %) and periods of no fluorescence or "off-states" (80 %, with off times from 10 ms to 30 s). The times a molecule showed certain MFIT behaviour varied from 10 ms up to 40 s (time resolution determined by the 2 ms dwell time, the 20 Hz modulation frequency and the total observation time). Only in a few cases a stepwise unidirectional decrease in emission intensity was observed, as it would occur for subsequent irreversible photobleaching of the eight PCIs.

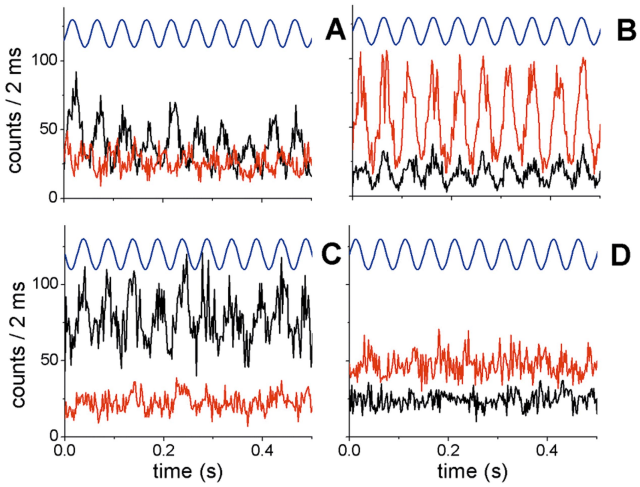

Fig. 4. *Measured MFITs (black: p polarisation channel, red: s polarisation channel, blue: excitation modulation) representing the four observable modulation patterns for compound 1:* **A)** *out of phase modulation (OPM),* **B)** *in phase modulation with off-set (IPMO),* **C)** *one channel modulated and no modulation in the other channel (NM1), and* **D)** *no modulation in both channels (NM2).*

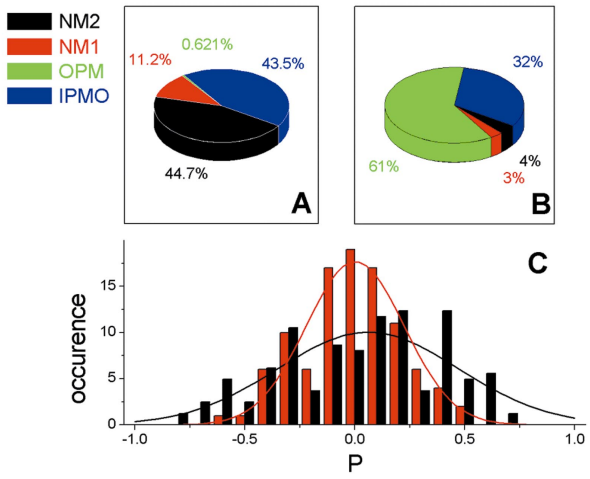

Fig. 5. *Modulation pattern distribution of the measured MFITs* **(A)** *and 100 simulated MFITs with eight chromophores in dendrimer arrangement but no energy transfer (SIM1,* **B)**. *Panel* **(C)** *shows histograms of the MFIT polarisations (black: measured, red: SIM1). The data were fitted with a Gaussian function yielding FWHMs of 0.82 (measured data) and 0.45 (SIM1).*

The first period of an MFIT is defined as the time period until the total fluorescence intensity, polarisation or modulation pattern is changing. This first period of the MFITs is obviously of highest interest since the probability for photoinduced changes in the photophysical properties of the eight-chromophoric molecule compound 1 is minimal. The average time of the first period is nearly 3.5 s. However this value is misleading since a few molecules with very long lasting first periods dominate the mean statistics. More information can be gained from the analysis of the first period duration histogram (not shown). It can be fitted by a biexponential function with characteristic times of 150 ms and 1.8 s (amplitude ratio of 4:1). Before the MFITs can be measured the position of the molecules first was identified by recording a fluorescence image. It is important to assure that the light applied during imaging is not leading to photoinduced changes of the distributions of the single molecule properties. From the scan speed and resolution we can calculate that the molecules have been excited for about 40 ms during imaging. This is only one fourth of the short characteristic time. Therefore the MFITs represent to a large extent the “real” first period behaviour.

In Figure 5A the distribution of the modulation pattern of the MFIT first periods is shown. In-phase modulation with an offset (IPMO) and non-modulation in both detection channels (NM2) have equal probability and account for almost 90 % of the molecules. NM1 occurs in about 10 % of the molecules and out-of-phase modulation is scarcely found. The fact that no first periods with modulation to 0 counts have been found can be explained by the multichromophoric nature of compound 1.

It proves, however, that the eight chromophores interact only weakly in the ground state as it was already suggested on the basis of the absorption spectra of the multichromophoric compound 1 and the single chromophore model compound 2 [25]. As a next step we compare the measured modulation pattern to the results from simulated MFITs on molecules of compound 1 for which energy transfer among the PCIs is not allowed (SIM1). Figure 5B depicts the modulation distribution pattern obtained from SIM1. The distribution displays altered contributions with the out-of-phase modulation as the dominating fraction (60 %). IPMO is observed for 30 % while NM1 and NM2 are unlikely patterns. In Figure 5C the distribution of the polarisation in the first period MFIT and of the SIM1 data, respectively, are plotted. Clearly the measured data show a much broader polarisation distribution compared to SIM1 (FWHM 0.82 vs. 0.45).

In a recent SMS study, simultaneous acquisitions of fluorescence spectra and fluorescence decays have been performed for compound 1 [24]. It was found that compound 1 shows two different modes of fluorescence with very distinct fluorescence properties: i) mode A is characterised by a fluorescence maximum at 560 nm, a vibronic structure and a decay time of approximately 5 ns very similar to the single chromophore model compound 2. ii) mode B has a red-shifted fluorescence maximum at 585 nm, no vibronic fine structure and a decay time of about 9 ns [24]. It was furthermore shown that individual molecules could switch back and forth between mode A and mode B fluorescence behaviour [23,24]. Taking into account these results and additionally, results of extensive ensemble

measurements in solution [25-29] mode B fluorescence was assigned to an excimer like state which acts as an energy transfer trap for the other chromophores of compound 1. The switch of the fluorescence behaviour from mode A to mode B reflects therefore the formation of the excimer like state. About mode A we know that the fluorescence spectrum and decay are identical to that of the model compound 2. Whether the eight chromophores show independent isolated fluorescence behaviour in mode A or energy transfer among the isoenergetic PCIs occurs prior to emission is still a point of discussion. The results presented in Figure 5A and 5B, however, give a clear answer. SIM1 simulates mode A type fluorescence with no energy transfer among the chromophores. Since SIM1 fails to explain the measured modulation pattern, mode A type fluorescence must involve several energy transfer processes among the eight PCIs before the photon is emitted.

Maus et al. found strong support for this conclusion in a recent time-resolved fluorescence spectroscopy study [28]. They investigated a 1st generation polyphenylene dendrimer series with one, two, three or four PCI chromophores attached. In contrast to compound 1 that dendrimer has a rigid tetrahedral structure, which leads to a more symmetrical arrangement of the chromophores. Consequently, the excimer like state contributes only to a very minor extent to the fluorescence even in the compound with four PCIs. In the time-resolved anisotropy decays of the three compounds with more than one PCI, however, a 100 - 200 ps component was found. No counterpart in the magic angle fluorescence decay was detected pointing towards a Förster energy transfer process among isoenergetic chromophores (energy hopping). The rate constant of the energy hopping process was determined as about 5×10^9 s^{-1} [28], i.e. during the excited state lifetime several (on the order of ten) energy hopping processes can occur before photon emission. These results obtained on a strongly related multichromophoric dendrimer family are in full agreement with the results presented here. A similar anisotropy decay component has been established in detailed time-resolved fluorescence spectroscopy investigations on compound 1 [29] with superior time-resolution compared to an earlier study [25].

In Figure 6A-C the modulation pattern distributions for data from three simulations based on the assumption of efficient energy transfer (SIM2A, SIM3A and SIM3B, see also in Materials and Methods) are presented. In SIM2A the emission occurs from a fixed dipole between two neighboured PCIs (similar to mode B), in SIM3A and SIM3B the emission occurs from all eight PCIs after extensive energy transfer in the excited state (similar to mode A). A common feature of all three distributions is the complete absence of OPM behaviour. This supports the conclusion drawn above about the nature of fluorescence mode A, namely an efficient energy transfer among energetically similar PCIs. Since the Förster radius of compound 2 is of the order of 38 Å energy transfer among all PCIs in compound 1 is possible. In time-resolved fluorescence anisotropy measurements on fs-ns time scale several depolarisation processes have been identified, which in part have been attributed to energy transfer. But for these bulk solution measurements it is more difficult to separate the nature of the energy transfer due to contributions of both fluorescence modes A and B at the same time.

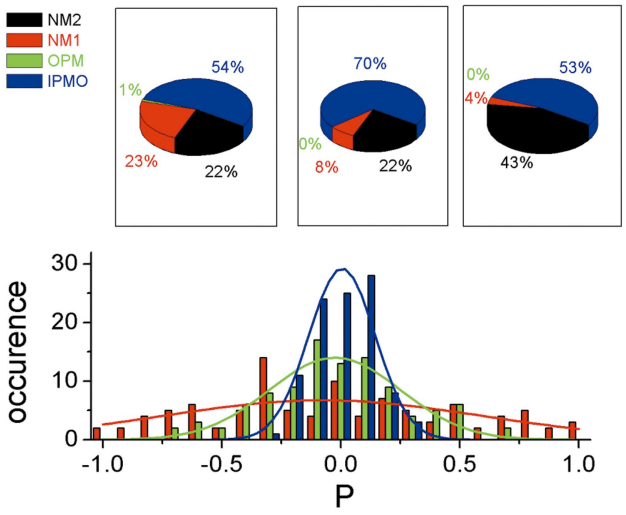

Fig. 6. *Histograms of the modulation pattern of 100 simulated MFITs with three different simulation models: energy transfer from all eight chromophores to one emitting chromophore (SIM2A,* **A)***, energy transfer between and emission from all eight chromophores (SIM3A,* **B)***, and the same as SIM3 but with the dendrimer equatorial plane parallel to the x,y-plane of the microscope (SIM3B,* **C)***. Panel (C) shows histograms of the MFIT polarisation (red: SIM2A, green: SIM3A, blue: SIM3B). The data were fitted with a Gaussian function yielding FWHMs of1.34 (SIM2A), 0.55 (SIM3A) and 0.28 (SIM3B).*

The modulation pattern distribution of SIM2A shows almost equal probabilities for NM2 and NM1, while SIM3A shows distinctively less NM1 behaviour. In both simulations IPMO is the dominating fraction. SIM2B leads to very similar results as SIM2A with a little smaller contribution of NM1. Qualitatively SIM3A gives a modulation pattern more similar to the one measured. The modulation pattern is almost identical with the measured one, if we fix the equatorial plane of the dendrimer perpendicular to the light propagation direction (SIM3B). Only NM1 behaviour is hardly observed in contrast to the measured data (Figure 6C and 5A). If compound 1 has indeed a non-spherical shape a non-isotropic spatial orientation distribution, preferentially parallel to the glass surface, might occur due to the spincoating process. The FWHMs of the polarisation

distributions (SIM2A: 1.34, SIM3A: 0.55, SIM3B: 0.28) are quite different. The value of the FWHM of the observed polarisation distribution lies in between the ones for SIM2A and SIM3A/SIM3B. The simulations have clearly shown that efficient energy transfer occurs in both fluorescence modes A and B. The observed modulation pattern and polarisation distribution appear to be a mixture of SIM2A and SIM3A/Sim3B, i.e. mode A and mode B. This is in line with a previous SMS investigation where the probability for mode A and mode B behaviour was determined as 64 % and 36 %, respectively (see Figure 14 in reference [24]). The remaining discrepancy in the modulation pattern distribution could be caused by small differences between the real geometrical arrangement of the chromophores and the one assumed for the simulations.

To learn more about dynamical changes, we investigated the modulation pattern after 60 s (an arbitrarily chosen time point). After 60 s 65 % of the molecules still emitted fluorescence light with intensities as high as in the first period. Of course the mean intensity was lower. 62 % of all molecules had the highest total fluorescence intensity in the first period. The modulation pattern of the molecules at 60 s results in a distribution (Figure 7A) almost identical to the distribution in the first period (Figure 5A). From this we

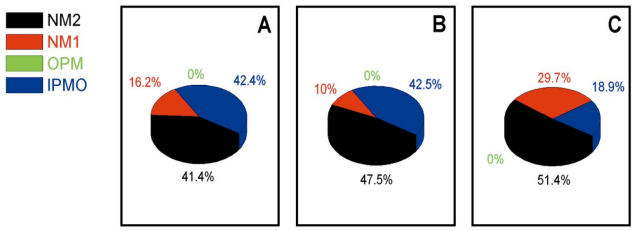

Fig. 7. *Modulation pattern distribution of subsets of the measured molecules:* **A)** *molecules still emitting after 60 s,* **B)** *molecules with non-zero intensity in the MFIT (more then one chromophore) after 60 s, and* **C)** *molecules which change their modulation pattern from the first to the second period.*

can conclude that the molecules with NM2 and IPMO behaviour have a similar photostability. If we select from the molecules that have survived the initial 60 s only those, which do not reach the background level at 60 s (75 %), we obtain the distribution shown in Figure 7B. This class of molecules must have more than one PCI intact. We again obtain a modulation pattern distribution identical to the measured one without any OPM behaviour. Even though some PCIs might be photodestructed after 60 s illumination, the energy transfer processes and the relative contributions of the two fluorescence modes must be very similar to the starting situation. Among the remaining 25 % of the molecules fluorescing at 60 s, which reach the back-

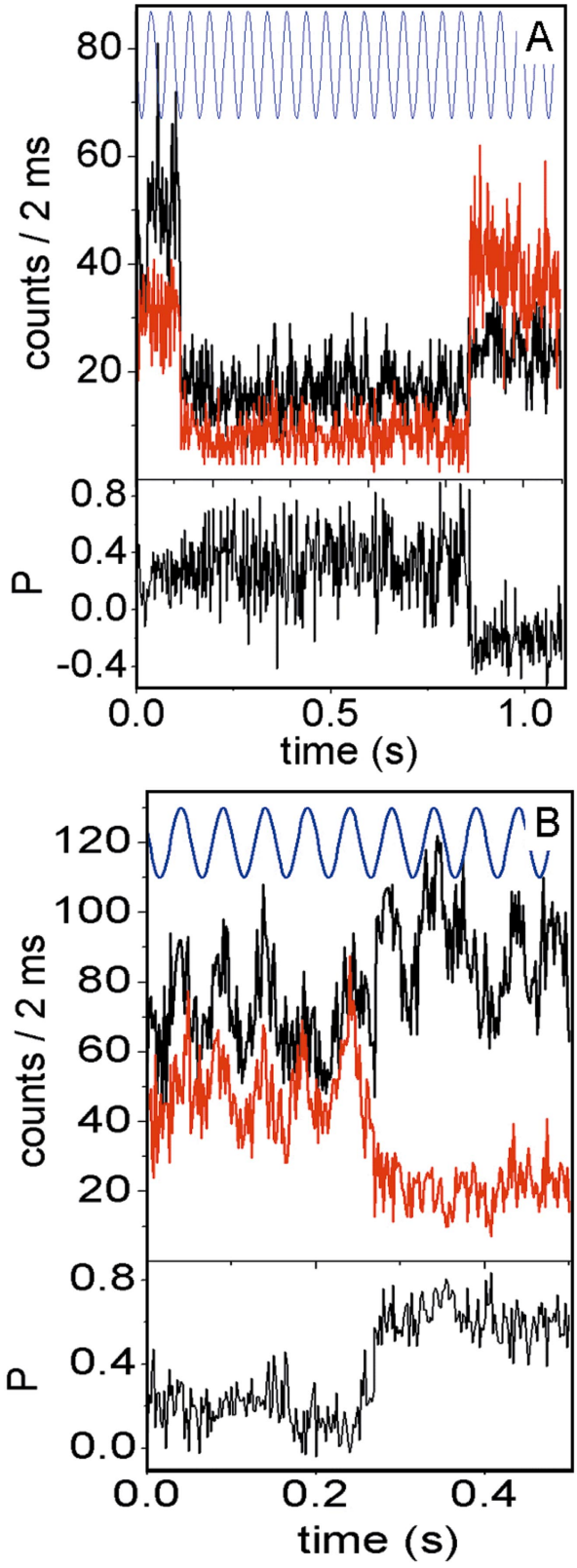

Fig. 8. *Measured MFITs (black: p polarisation channel, red: s polarisation channel, blue: excitation modulation) depicting changes of polarisation P* **(A)** *and changes of the modulation pattern (here from IPMO to NM1) and the polarisation P* **(B)**

ground level at 60 s, will be molecules with only one active chromophore. Therefore we would anticipate a modulation pattern distribution of enhanced IPM behaviour.

The distribution, however, shows enhanced NM1 and OPM patterns (not shown), i.e. most of the molecules in this class still have more than one active chromophore.

The photostability of compound 1 and the model chromophore 2 is remarkable. From the known molecular absorption coefficients, the used excitation power, the polarisation conditions one can calculate that compound 1 undergoes 270000 excitation-emission cycles in 1 s, i.e 1.6 10^8 excitations in 60 s. About 50 % of the molecules seem to be intact after this large amount of excitation-emission-cycles as we can learn from Figure 6B with the majority of the PCI chromophores still intact. From this we can, although not directly measured here, derive a photodestruction quantum yield for compound 2 and the PCI chromophore < 10^{-7}. This outstanding photostability qualifies compound 1 and related multichromophoric dendrimers as important and useful model compounds for the establishment of single molecule spectroscopy on biological relevant multichromophoric systems. Those are usually based on tetrapyrrole chromophores with relatively high triplet yields leading to a fast destruction of the original interactions between the chromophores. We can also estimate the detection efficiency of the apparatus. Assuming that in the first period all PCI chromophores are intact, we obtain η_{eff} equal to 0.12 using the rate of excitations per second, the average total count rate in the first period and the fluorescence quantum yield from ensemble measurements in solution.

The last MFIT property investigated is dealing with the changes in the step from the first to the second MFIT period. Only 10 % of the molecules increase their total fluorescence intensity in that first step. 22 % of the molecules go to an off or very low level state, i.e. an efficient trap state with non-radiative deactivation is formed at the end of the first period. 25 % of the molecules change their polarisation. Figure 8A shows such a molecule with two drastic changes in the polarisation (at 115 and 870 ms) although the modulation pattern (NM2) stays the same. The total fluorescence intensity in the first and third period is nearly the same but the polarisation is changed. Most probably in both periods the molecule shows mode B type fluorescence. All eight chromophores absorb and the energy transfer is efficient but the excimer like state, which is energetically the lowest, is situated at different chromophores in period 1 than in period 3. The resulting change in orientation of the emission transition dipole moment causes the observed polarisation difference between period 1 and 3. The second period with its much lower total fluorescence intensity indicates the existence of an additional trap site, which is relaxing radiationless to the ground state. This trap state is most probably a triplet state located on one PCI [17]. More interesting, however, is the change of the modulation pattern. 24 % of the molecules alter their modulation pattern during the step from the first to the second period. The modulation pattern distribution of those molecules (Figure 7C) is remarkably different from the measured distribution on all molecules (Figure 5A). Molecules with NM1 and NM2 behaviour change their modulation pattern with a much higher probability than the molecules with IPMO. The non-modulation behaviour is obviously more affected by distortins of the energy transfer process. Figure 8B shows such a case, in which a molecule switches from IPMO to NM1 behaviour. The total fluorescence intensity before and after the change (at 270 ms) is nearly the same while the polarisation has changed. The first period might be mode A or mode B but the second period with NM1 is typical for a single emitting species among the eight chromophores. Therefore the event at 270 ms might be either a relocation of the excimer state (mode B $\rightarrow$ mode B) or the creation of the excimer state (mode A $\rightarrow$ mode B).

Conclusion

In this study excitation polarisation modulated fluorescence intensity traces (MFIT) on a multichromophoric model entity (1) have been measured. Several different energy transfer models have been used to simulate MFITs. The analysis of the modulation pattern in the measured and simulated data have revealed that in nearly all molecules of compound 1 several energy transfer processes occur before the photon emission. The modulation pattern could be explained by a combination of two energy transfer models corresponding to two previously characterised fluorescence modes of compound 1. In both efficient energy transfer occurs among the chromophores. The difference between the two consists in the presence (mode B) or absence of an excimer state (mode A). The excimer state is energetically lower and acts therefore as an energy trap. Here and in previous work it was found that the excimer state is not a static but dynamic property of compound 1.

The results presented here promise that MFIT measurements and modulation pattern analysis will be a useful tool in the future for SMS investigations on biologically relevant multichromophoric entities. MFIT measurements allow to estimate the degree of energy transfer and therefore to screen for static or slow dynamic heterogeneity of this property. It can also help to confirm or disprove certain photophysical models.

Acknowledgment: The authors gratefully acknowledge the FWO, the Flemish Ministry of Education for the support through GOA/1/96, the EC through the TMR Sisitomas and TMR Marie Curie, the VW Stiftung and the support of DWTC (Belgium) through IUAP-IV-11. J.H. thanks the FWO for a

post-doctoral fellowship. The European Science Foundation through SMARTON is thanked for financial support.

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