Optically probing structure and organization : single-molecule spectroscopy on polyethylene films and a resonance Raman study of a carotenoid

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1 Introduction

The work presented in this thesis covers two research topics, the investigation of the nanoscale organization of polyethylene by means of single-molecule spectroscopy, and quantum-chemical calculations of the resonance Raman spectra of spheroidene in the reaction center of *Rhodobacter sphaeroides*, a photosynthetic bacterium. The calculations serve to determine the correct structure of the carotenoid spheroidene in the reaction center. In the sections 1.1 and 1.3 of this introduction I will elaborate on both of the aforementioned subjects and place them in their scientific context. In section 1.2 I will examine a third topic, the design of a single-molecular switch. At the beginning of my PhD research, I spent 1.5 years trying to realize such a switch, which could be turned off and on by irradiating with light. I will introduce the concept of a typical photochromic molecular switch and elaborate on a recent example from literature, where it was claimed that single-molecular switching had been achieved.

1.1 Single-Molecule Optics: Local Probes

Since the first single-molecule spectroscopy experiments in 1989 and 1990 [1,2], the field of single-molecule optics has expanded to cover the disciplines of physics, chemistry, biology and even materials science. The detection of either the absorption or the red-shifted fluorescence signal from an individual chromophore, allows the observation of a single quantum system. In conventional ensemble spectroscopy, information on the system is averaged out. Photons emitted by single molecules, however, can reveal properties of the fluorophores or their environments. The spectra, directions of emission or polarization of fluorescence photons may be recorded [3–9]. Performing statistics on the arrival times of photons originating from a single emitter, can enhance the understanding of the (photo)physical dynamics involved in emission [10–12]. Access to the quantum world of molecules at the individual level can reveal, among others, what lies beneath ensemble spectra [2, 13, 14], how histograms are constituted or what goes on in a molecule's immediate environment.

In many experiments, not the fluorophores themselves are the objects of study, but their close surroundings are. As nano-objects, single guest

molecules offer a unique opportunity to observe both dynamics and structure of the host at nanometer scales. Such a closer look at a dopant molecule's environment can of course only be meaningfully performed if the probe molecule itself is well characterized, both in its photophysics and its photochemistry. Only then can one address them as local reporters and observe how they are being affected by changes in their environment. Such changes might entail the rearrangement of host atoms or molecules [15–22], or fluctuations of the interchromophore separation [7,23]. These processes may be classified as dynamics of the host. There may also be local changes in the presence or absence of a particular order [24,25]. In that case one can study the structure and organization of the host. Also movement of the individual probe molecules themselves may be monitored in living cells, for example [26–30]. With single-molecule optics, the scientist has an extremely sensitive and ingenious tool available, a true nano-probe.

The principle of nano-probing was exploited by Bloeß et al. when they investigated the so-called Shpol'skii effect [24, 25]. A host/guest system of n-tetradecane (or other n-alkanes) and aromatic hydrocarbons displays this effect when rapidly cooled down to cryogenic temperatures. The electronic transitions of the embedded chromophores are significantly narrower than for the same guest molecules in conventional organic glasses [31]. Single-molecule probing of the Shpol'skii matrices yielded an upper limit of several hundreds of nanometers for the sizes of crystalline domains, which posed a direct challenge to existing explanations attributing the Shpol'skii effect to the poly-crystalline structure of the host. For organic glasses and polymers, single-molecular probes provided the first direct evidence of the existence of two-level-systems (TLS), more than 30 years after these had been proposed [19]. These TLS are defects in a glass structure at low temperatures that are associated with two metastable configurations of one or several atoms. The two conformations may switch from the one to the other by means of phonon-assisted tunneling. Such switching is the cause of most residual dynamics in glasses at cryogenic temperatures. Orrit and co-workers have investigated such dynamics in polyethylene [19,20,32] and have also been able to show deviations from TLS behavior. Fluctuations in the local density, resulting from chain dynamics in amorphous host polymers, were demonstrated by Vallée et al. to give rise to excursions towards longer fluorescence lifetimes of single guest molecules [33, 34]. As such, the fluorescence lifetimes of reporter molecules may be used to probe such fluctuations as a function of temperature.

Even protein dynamics may be probed by means of single-molecule optics. The most commonly applied method at room temperature is the well-known technique of single-molecule fluorescence resonance energy transfer (FRET)

[23]. This technique relies on labeling proteins with two (or more) different fluorophores at separate sites. One such label (the donor) absorbs light further to the blue side of the visible spectrum than the other (the acceptor), such that the latter's absorption spectrum and the former's emission spectrum overlap. Depending on the acceptor's proximity, the donor may fluoresce when excited, or transfer its excitation energy to the acceptor, which in turn will fluoresce at a different wavelength. Also naturally present pigments can be used to probe dynamics in a protein. Köhler and co-workers studied structural fluctuations of the light-harvesting complex 2 (LH2) protein backbone, by monitoring the spectral positions of the embedded chromophores [35].

Not only rearrangements in the structure of a host material have been studied with single-molecule optics. Attempts at detecting the flow of charges on a solid have also been made. It is known that electric fields can influence the electronic excitation energies of single molecules, as was demonstrated by measuring the Stark effect [36, 37]. Caruge et al. were the first to report the effect of electric currents on resonance energies of single molecules close to a semiconductor film at low temperatures [38]. Recently, Hofmann et al. have proposed a low-temperature scheme to study the conduction of electrons in molecular crystals [39], by means of local impurity molecules.

The research described in Chapters 2, 3 and 4 concerns the use of local probes in polyethylene (PE). Parts of the semi-crystalline PE polymer matrix are highly ordered or crystalline. The crystalline domains are interspersed in areas of no order whatsoever, called amorphous regions. Initially, our interest in using single molecules to probe PE, lay in determining if local probes might reveal the size range of the crystalline regions. In essence, our approach is similar to that of Bloeß et al. for the Shpol'skii systems. The use of dopant molecules to report PE crystal sizes requires the determination of both the positions and the orientations of many individual chromophores. The orientations are determined by means of single-molecule spectroscopy and the lateral positions with single-molecule microscopy. The method we used does not offer any resolution in the axial (depth) direction. In this experiment, one can build a two-dimensional map of chain PE orientations in the sample. In crystalline regions, dopant molecules will reside on the crystal surfaces and their orientations will display a similar order to that of the surface chains. Amorphous regions on the other hand, will not induce the chromophores to adopt a particular alignment.

Unfortunately polyethylene tends to form rather opaque films, when processed in its pure form. Interference from the host material compromises both the orientation and the position measurements. For this reason, it was neces-

sary to minimize host scattering and make the PE samples very clear indeed. On top of that, the investigated PE films needed to be as thin as possible. In this way, one can minimize background from scattering and confine observed effects to a smaller confocal volume. As it turned out, making clear, thin films of pure PE was no trivial matter. We needed to develop a technique to spincoat such films from solution at high temperatures (about 100 °C). This method enabled us to prepare perfectly clear films of a mere 200 nm thickness. Chapter 2 is devoted to this technique of making such films and the demonstration of the feasibility of both localizing single dopant molecules and determining their in-plane orientation. In Chapter 3 we report our surprising findings for the aforementioned samples.

In Chapter 4 the process of aligning single dopant molecules by stretching polyethylene films is discussed. Already in the last century, it was noted that by embedding organic molecules in PE films and subsequently stretching these films, a high degree of alignment could be produced [40, 41]. Molecules that are flat and oblong in shape become most efficiently oriented along the stretching direction. This is a convenient effect if one is interested in studying the orientation of molecular properties such as transition-dipole moments or the magnetic g-tensor. Since PE is a cheap and inert host material, the technique of stretching films to align guest molecules is quite common and has been studied at length [42]. It is known that by stretching, all PE chains become gradually better oriented along the stretching direction. Our aim was to reveal what happens locally during the stretching process. Do dopant molecules gradually get oriented, or does the system need to overcome certain barriers? Do molecules become oriented by PE chains making up the amorphous regions or are only chromophores adsorbed on the crystal surfaces aligned?

The methods used were similar to those mentioned for the ultra-thin PE films. Our stretched samples were thicker and not transparent, however, and we left out the microscopy part. By probing the process of molecular alignment using single-molecule spectroscopy, we were able to show that, perhaps contrary to one's expectations, dopant molecules do not become ever better aligned, as the film is stretched further. Instead, orientation of a chromophore occurs rapidly by a sudden rearrangement of its PE environment.

Chapters 2, 3, and 4 describe the use of single molecules as nano-probes. In the next section I will discuss how single fluorophores could be made to work as nano-bits.

1.2 Optical Switching at the Single-Molecule Level

What if it were possible to reversibly switch the emission from a single fluorophore off and on at will? Given a stable chromophore, and the means of quenching its fluorescence on command, it would be possible to build a single molecular switch. Such a system would be the ultimate step in the ongoing process of miniaturization of storage bits, for example. Such a 'nanobit', if possible, could dramatically increase the density of optical data storage, given the means of addressing each switch individually. Something like optical switching has been realized for the first time in a reversible way by Kulzer et al. for terrylene embedded in sublimated crystals of *p*-terphenyl at 1.2 K [43]. The authors were able to optically induce reversible frequency jumps for a certain part of the embedded molecules. At room temperature, optical switching was realized using a mutant of green fluorescent protein [44]. Prolonged excitation of the protein induces a reversible photochemical reaction to a non-fluorescent state. This dark state can be converted back to the fluorescent one by excitation at a shorter wavelength. Unfortunately GFP and its mutants all exhibit considerable photobleaching, severely limiting the duration of their use. In that respect, the recent publication by the group of Hofkens in Leuven of a stable mutant of GFP, which can be reversibly switched on and off, is a promising development towards stable, durable single-molecular optical switches [45, 46].

The initial goal of my PhD research was to develop a molecular switch. The project was based around the concept of durable photochromic switching. A photochromic molecule is one that has two stable forms, which may be interconverted when irradiated with light of certain wavelength, through a reversible photochemical reaction. The molecule in question needs to be able to cycle between the two forms an infinite number of times. An irreversible photochemical reaction with oxygen for example, would destroy its capacity to switch. Such a switch itself need not be fluorescent. It suffices to act as a means of quenching the fluorescence of an attached chromophore. Consider the simplified representation of a molecular switching scheme in Figure 1.1. This switch is based on a photochromic bisthienylcyclopentene derivative, and is representative of the compounds with which we attempted to demonstrate photochromic switching.

Upon irradiation with UV light, the open-ring form (A) reacts to form the closed-ring form (B). The open-ring form can be restored by intense irradiation with visible light. The thermostable bisthienylcyclopentene molecules have been shown to also be extraordinarily photostable and will cycle between the two states over 10^4 times without photodestruction occurring [47, 48]. The

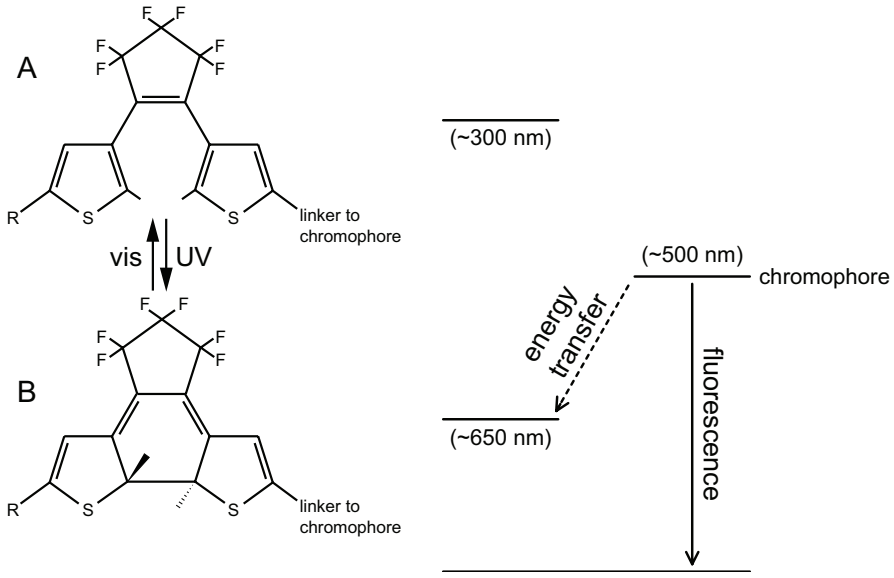

Figure 1.1: Simplified representation of a molecular switching scheme. Excitation of the chromophore (not shown here) leads to fluorescence (solid arrow), unless excitation energy is transferred. The closed-ring form (B) acts as an efficient acceptor for the excited-state energy of the chromophore (dashed arrow), thereby quenching fluorescence. In closed-ring form the bisthienylcyclopentene unit mainly relaxes by radiationless decay, but occasionally exciting form B leads to ring opening.

closed-ring form has a lower excited state energy than the open-ring form. If a chromophore that absorbs at a wavelength between that of the open and closed-ring forms, is attached and linked in such a way that energy transfer to B can occur efficiently, the optical switch will work. Under continuous excitation of the chromophore, fluorescence will be visible if the switching unit is in the A form. Irradiation with UV light will then isomerize the bisthienylcyclopentene unit and the B form will quench the fluorescence. Prolonged energy transfer from the chromophore to B will eventually return the switch to the open-ring form, so ring opening must be less likely to occur than ring closure, for there to be a visible quenching effect.

Working photochromic switches have been synthesized and demonstrated in ensemble experiments [47, 49–52]. For such a switch to work at the single-molecule level, however, stricter criteria apply for both the chromophore and the switching unit. Custom-building a single-molecular switch boils down to fine-tuning all relevant properties of the system. The fluorophore should be photochemically stable and have a high quantum yield of fluorescence

($\Phi_{fl} \approx 1$). The energy transfer to the closed-ring form of the bisthienylcyclopentene unit needs to be very efficient and, moreover, should not immediately lead to ring-opening. The precise excited-state energies of the switching unit can be tuned relative to the chromophore absorption using substituents to the switch moiety. Our project involved a collaboration with the synthetic chemistry group of Feringa and Van Esch at the University of Groningen. Ultimately, no compounds were synthesized, however, with which we were able to demonstrate switching at the single-molecular level. Unfortunately, this collaboration was discontinued and the project abandoned after one year and three months of my participation in the project, when a publication appeared by the group of Irie at Kyushu University in Japan [53].

In retrospect, we have doubts concerning the validity of the results presented in this and subsequent articles by the group of Irie concerning single-molecular switching. The rest of this section is devoted to a discussion of the results in said publications. In the original letter, it was claimed that photochromic switching at a single-molecular level had been achieved with a system based on a diarylethene (equivalent to bisthienylcyclopentene) switch linked to a fluorescent anthracene derivative, hitherto unknown as a suitable single-molecule fluorophore [53]. Although this brief communication naturally reveals few details about the single-molecule statistics involved, it does feature a surprising distribution of on-times, which—contrary to expectation—is Gaussian in shape. I will elaborate on the expected shape of such a distribution below. In 2004, Fukaminato et al. published their detailed characterization of the single-molecular switch in question [54]. In this paper the authors attempt to demonstrate a dependency of the on-times of single switches on the intensity of UV-irradiation and on the intensity of 488 nm irradiation for the off-times. The article contains both images of fluorescent spots and time traces of fluorescence.

Regrettably, the article does not provide a detailed characterization of the fluorophore itself, such as photochemical stability, inter-system crossing yield etc.. Nor are any fluorescence time traces of single fluorophore moieties given, to demonstrate the feasibility of detecting individual chromophores. Moreover, the time traces in this article appear atypical of single-molecule fluorescence time traces. Fukaminato et al. show three traces of about 30 s length, which each display two or three on-time events. The regularity and uniformity of the displayed on-times are rather unexpected. Figure 1.2 shows a reproduction of the figure in [54]. The on-times of single photochromic switches are determined by first-order chemical kinetics, and should be exponentially distributed. The reason for this is that the on-times depend only on the switching (ring closure) and subsequent quenching processes of the diarylethene unit, as a result of

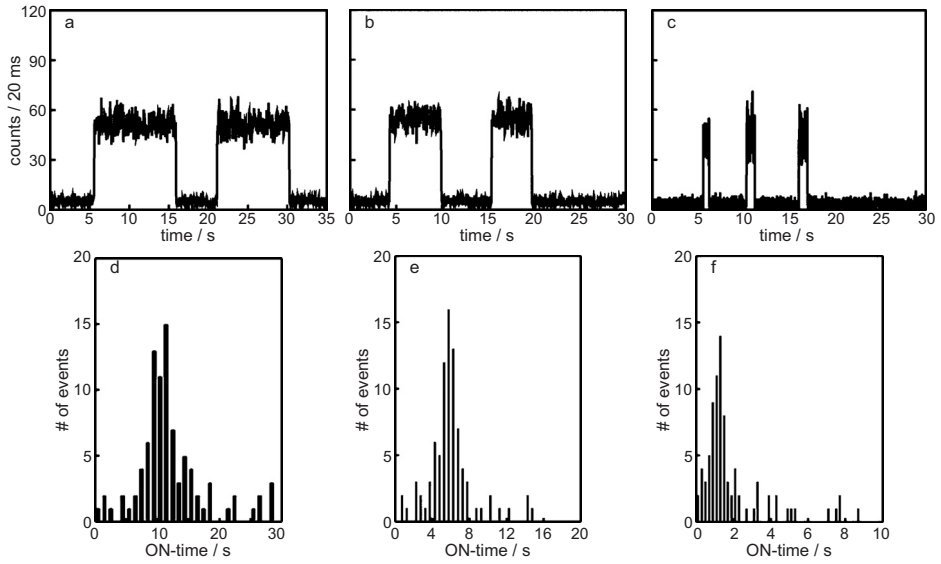

Figure 1.2: Figure reproduced from [54]. Figures (a), (b), and (c) represent fluorescence time traces of the photochromic switch compound embedded in a Zeonex film. The sample is continuously irradiated with a $100\,\mathrm{Wcm^{-2}}$ 488 nm laser, to excite the chromophore, and weak UV (325 nm) light at variable power: (a,d) $0.027\,\mathrm{mWcm^{-2}}$, (b,e) $0.054\,\mathrm{mWcm^{-2}}$, and (c,f) $0.27\,\mathrm{mWcm^{-2}}$. See remarks in main text on how histograms were constructed. Average on-times: (d) 11 s (73 molecules, 92 events), (e) 5.7 s (94 molecules, 87 events), and (f) 1.2 s (80 molecules, 84 events).

absorbing a UV photon. The paper states that ring closure has a quantum yield of $\Phi_{rc} = 0.12$. Once switching has occurred, chromophore fluorescence energy is rapidly transferred to the switch moiety with a near unity quantum yield ($\Phi_{et} = 0.99$). The on-times are therefore essentially determined by the switching step. An exponential distribution of on-times means there should be many more short on-times in a time trace than long ones. Moreover, the traces are not expected to show any considerable regularity, but rather to represent the stochastic nature of the switching process. The traces displayed in Figure 1.2(a), (b), and (c) are therefore rather uncommon and unlikely examples of single-molecule fluorescence time traces, since they show two or three comparably long, consecutive on-times and no short on-times at all. Perhaps a selection was made from hundreds of fluorescence traces in order to find these three regular ones. The same argument goes for the off-times (not displayed here), which appear equally regular in the publication.

If we turn our attention to the histograms of the on-times in Figures 1.2(d),

(e), and (f), we note that, surprisingly enough, the on-times seem to have a Gaussian distribution. The caption to the Figure reproduced in Figure 1.2 notes that "the distributions were constructed by collecting on-times of 1–3 events for each molecule." [54]. What precisely is meant by this is unclear. Were histograms of the average on-times built by taking time traces showing 1–3 events and averaging the events for each trace? In that case the number of events in each histogram should be equal to the number of molecules measured. If instead each measured event is plotted in the histograms, we expect the number of events measured to be roughly equal to twice the number of molecules measured. Adding up the total number of events in each histogram (for Figure 1.2(a) 92, (b) 87 and (c) 84) reveals that neither of these two cases are true. For Figure 1.2(e), strangely enough the number of molecules stated in the caption is even larger than the number of events plotted in the histogram. We cannot assume the distributions were constructed by simply taking the measured on-times at each of the three UV intensities. These distributions should then be described by three single-exponential decays. The characteristic decay times of these should depend on the intensity of UV irradiation. The displayed histograms, therefore, cannot correspond to histograms of the on-times of individual molecular switches. There is a possibility that due to environmental fluctuations the switching rate varies. In this case the resulting on-time distributions should look like stretched exponentials, however, and those would appear even less Gaussian. Let us consider what the on-time distributions should look like, if we interpret the explanation in the caption of Figure 1.2 to mean that averages were taken of 1–3 events for each trace.

If we assume Fukaminato et al. averaged the on-times for 1–3 events for each molecule, and assume a constant switching rate, we can demonstrate that the obtained distributions could never resemble those shown in Figure 1.2. In order to obtain the distribution of the average on-time per trace, we proceed as follows[1]: The on-time t is a random variable, so the probability of t, P(t) looks like:

$$P(t) = ae^{-at}, \tag{1.1}$$

where $\frac{1}{a}$ is the characteristic decay time. If $\langle t \rangle_2$ is the average on-time of two events as in $\langle t \rangle_2 = \frac{1}{2}(t_1 + t_2)$, then the probability $P(\langle t \rangle_N)$ of finding the average of N events $\langle t \rangle_N$, can be shown (by Laplace transformation and the

[1]The author is indebted to discussions with prof. dr. M. Orrit. Simulations courtesy of dr. R. Zondervan.

convolution theorem) to be:

$$P(\langle t\rangle_N) = Na\frac{(aN\langle t\rangle_N)^{N-1}}{(N-1)!}e^{-aN\langle t\rangle_N} \tag{1.2}$$

Equation 1.2 gives the probability distribution of obtaining $\langle t\rangle_N$ for each trace. This probability is maximum when: $\frac{dP(\langle t\rangle_N)}{d\langle t\rangle_N} = 0$, so:

$$aN(N-1)[aN\langle t\rangle_N]^{N-2}e^{-aN\langle t\rangle_N} - aN[aN\langle t\rangle_N]^{N-1}e^{-aN\langle t\rangle_N} = 0 \tag{1.3}$$

and eliminating the common factor:

$$\langle t\rangle_{N,max} = \frac{1}{a}\left(1 - \frac{1}{N}\right) \tag{1.4}$$

A histogram of average on-times could look like the probability distributions given by Equation 1.2 for a large number of time traces/molecules. We can plot the probability distribution of $\langle t\rangle_N$ for a value of $\frac{1}{a}$ of 10 s, as a function of the number of events N used to obtain the averages. Equation 1.4 tells us how the maximum of each curve depends on N. In Figure 1.3 we see what the probability distributions of Equation 1.2 look like when averaging $N = 1$, 2, 3, 4 or 10 events per trace. Note that even if we take 10 events per trace the

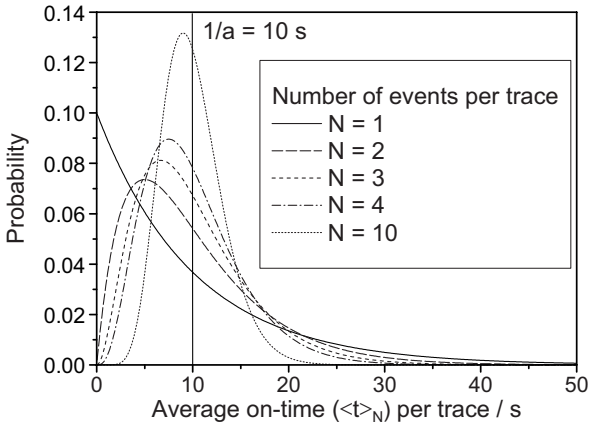

Figure 1.3: Probability distributions of average on-times per trace. Each curve corresponds to a different number of events per trace, taken to obtain the average on-time $\langle t\rangle_N$ as indicated in the legend.

resulting distribution has a FWHM that is considerably larger than that displayed in the Gaussian-like distribution in Figure 1.2(d), for which the number of events per trace is said to be 1–3. The correspondence to the theoretical curves for $N = 1$–3 in Figure 1.3 is even poorer. In fact, only if we were to take averages of traces with approximately $N = 40$ events, we might obtain something resembling the histograms of on-times presented by Fukaminato et al.. We are forced to conclude that these histograms of on-times do not show the distribution of single-molecule on-times or even of the averages of on-times for many traces. As mentioned earlier, the same argument applies to the time traces and histograms for the off-times in the article. In passing we note that the same distribution as shown in Figure 1.2(d) featured in the original letter in *Nature* [53]. As such, the authors have not proved that they have managed to produce a working single-molecular switch. A later paper by Fukaminato et al. features a molecular-switch compound containing a more common fluorophore in single-molecule optics (perylenediimide) [55]. The time traces displayed in this article appear more like genuine single-molecule fluorescence traces. Unfortunately, this paper does not contain any (attempt at) proof that switching is related to the intensity of the UV irradiation.

1.3 Structure Determination by DFT Analysis of resonance Raman spectra

The previous paragraphs described various applications of low-temperature single-molecule spectroscopy. Conventional techniques that sample an ensemble of molecules also provide powerful tools for studying molecular structures. Resonance Raman spectroscopy is particularly sensitive to the structural properties of a compound. In Chapter 5, we describe our efforts to reproduce the resonance Raman spectrum of the carotenoid spheroidene ($C_{41}H_{60}O_1$) in a bacterial photosynthetic reaction center (RC), by means of Density Functional Theory (DFT) calculations. We do so, in order to address a long-standing issue concerning the structure of spheroidene in the RC. Resonance Raman spectra reveal certain normal-mode vibrations of a molecular compound, whose composition depends strongly on the symmetry of the molecule. In order to calculate a molecule's vibrational normal-modes, one first needs to find the correct structure. Variations in the calculated structure lead to changes in the calculated normal-mode compositions.

The interpretation of the resonance Raman spectrum of a large molecule like spheroidene presents quite a challenge. The advent of relatively cheap computer power has enabled spectroscopists to go beyond 'mere' measurements

of the spectroscopic properties of molecular materials. Using sophisticated models that take into account the quantum-mechanical nature of reality at the nanometer length-scale, theoreticians can predict with increasing accuracy some of the properties previously only available to experimentalists. The added value of this reverse approach is that if one can correctly predict a certain property with a model and a computer, one is another step closer to understanding the system studied. In other words, if we can calculate and interpret the resonance Raman spectrum of spheroidene in the *Rhodobacter sphaeroides* RC, we have obtained strong clues concerning its structure.

To give an impression of the difficulties in interpreting the resonance Raman spectrum of a large molecule like spheroidene, let us consider the numbers. This molecule has $N = 102$ atoms, and therefore it has $3N - 6 = 300$ independent degrees of freedom, or normal-mode vibrations. Fortunately, the experimental method helps to eliminate some of those. A resonance Raman measurement is performed by scattering laser light that is (nearly) resonant with an electronic transition of the sample being studied. Only transitions pertaining to parts of the molecule that undergo a deformation upon transition to the excited state in question are enhanced and visible in the spectrum. This limits the number of normal modes one needs to assign, but in the case of spheroidene ($\pi^* \leftarrow \pi$ excitation), we still are still left with about 180 calculated normal modes. Not all of those, however, will be visible in the spectrum.

The research presented in Chapter 5 concerns the structure of spheroidene in the RC. In literature, the precise stereoisomer of spheroidene present in the RC has been a topic of some discussion. Investigations by means of NMR, resonance Raman, or X-ray scattering have tended to converge towards a consensus that spheroidene occurs as a 15,15'-cis structure [56, 57]. As was mentioned before, the normal modes are sensitive to changes in geometry of a molecule. Isotope-labeling also has an effect on their compositions. Introducing more massive atoms into a structure causes the local vibrational modes that make up a normal mode to shift in frequency. For complex molecules like spheroidene the influence of such mass effects on the normal-mode compositions can only be revealed by quantum-chemical calculations. We have calculated the spectra of 19 isotopomers of spheroidene and compared these to experimental spectra of spheroidene in the RC. Only if one can reproduce the spectra of all isotopomers, can one be certain to have found the correct molecular geometry. By calculating the spectra for, among others, non-planar 13,14-cis, 15,15'-cis-10,11-12,13-double-s-cis, 13,14-15,15'-13',14'-triple-cis, and 15,15'-cis spheroidene structures, we have been able to demonstrate that spheroidene in the RC occurs in at least two forms, one of which is the 15,15'-cis form. The second form is likely to be the 13,14-cis stereoisomer.