1

Nonlinear Absorption of Light in Materials with Long-lived Excited States

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1. Introduction

The absorption of light is an important phenomenon which has many applications in all the natural sciences. One can say that all the chemical elements, molecules, complex substances, and even galaxies, have their own "fingerprint" in the light absorption spectrum, as a consequence of the allowed transitions between all electronic and vibronic levels.
The UV-Visible (UV-Vis) light (200-800 nm) has an energy comparable to that typical of the transitions between the electrons in the outer shells or in molecular orbitals. Each atom has a fixed number of atomic levels, and therefore those spectra are composed of narrow lines, corresponding to the transitions between these levels. When molecules and macromolecules are considered, the absorption spectrum is no longer characterised by thin lines but by wide absorption bands. This is due to the fact that the electronic levels are split in many vibrational and rotational sub-levels, which increase in number with the increasing complexity of the molecules. IR spectroscopy is often used to investigate these lower energy modes, but for very complex biological molecules not even this technique can resolve each line precisely because the energy split between the various levels is too small. One possible way to obtain higher resolution spectra is to lower the sample temperature, in order to suppress many of the vibrational and rotational modes. For biological molecules, though, lowering the temperature can be a problem if one wants to study, for example, the activity of enzimes, which only work at physiological temperatures. One of the advantages of absorption spectroscopy (IR and UV-Vis) is to be a non-disruptive technique, also for "delicate" molecules like polymers and biomolecules.
In the process of light absorption by molecules, once a photon with the right energy is absorbed, the molecule goes into an excited state at higher energy [Born and Wolf 1999, Dunning & Hulet 1996]. Eventually, it spontaneously returns to the ground state, but it can relax following several mechanisms. When excited, the molecule reaches, in general, one of the sub-levels of a higher electronic state. The first process is then, generally, a relaxation to the lower energy state of that electronic level (schematised in figure 1). This process is usually very fast (in the femtosecond scale) and not radiative. From this level, there are several pathways to dissipate the energy: a radiative transition from the lower level of the excited state to the ground state (fluorescence), accompanied by the emission of a photon at lower energy than the absorbed one; a flip of the electronic spin, which leads to a transition between singlet and triplet state (intersystem crossing), often associated with another

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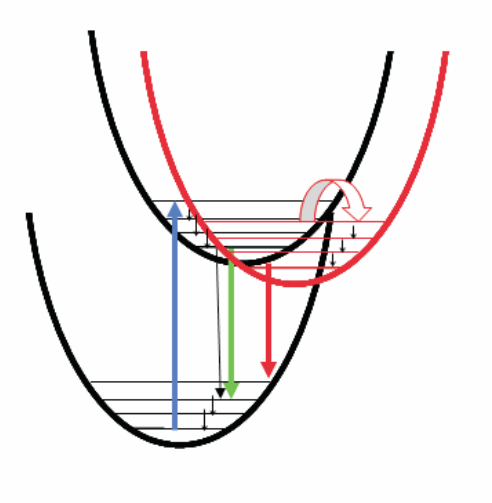


Fig. 1. A scheme representing some possibility of excitation/disexcitation of a molecule. Each electronic level is split into many vibrational and rotational sub-levels. The blue arrow describes the absorption of a photon, the green arrow the emission of a photon from the lower energy level of the excited state (fluorescence), while the black arrows indicate all the nonradiative energy dissipation mechanisms, which can be alternative to fluorescence. The intersystem crossing is another mechanism of disexcitation: the triplet state is represented with the red curve, and the transition with the thick arrow. The molecule can relax over long time to the ground state either with a nonradiative process or via phosphorescence (red arrow).

radiative process (phosphorescence); a non radiative decay where the energy is released by heat dissipation. In some molecules the relaxation pathway following the excitation is more complex, and it can involve interaction with other molecules. In such cases the energy can be transferred to other molecules via radiative or non radiative processes: azobenzene, for example, is a photosensitive molecule which, after excitation, undergoes a conformational change; a more common molecule, like chlorophyll in plant cell chloroplasts, transfers the excitation to the neighbouring molecules until the energy reaches the photosynthetic complex where the photosynthesis takes place.

The common characteristic shared by fluorescent molecules, molecules with a triplet state and photosensitive molecules like azobenzene, is that the lifetime of the excited state is long compared to the time it takes for the excitation to occur. This brings us to the subject of this chapter, which deals with a phenomenon, closely associated with the lifetime of the excited state, which we called "dynamic photobleaching". In general usage, the term "photobleaching" has been taken to refer to permanent damaging of a chemical, generally due to prolongued exposure to light. Here, we will not consider this, but rather a reversible phenomenon whereby the number of molecules in the ground state is depleted as a consequence of the long lifetime of the excited state.

This effect has important consequences for UV-Visible spectroscopy measurements. In practical use, UVVis light absorption experiments are simple and straightforward: a collimated beam of light is sent onto a sample, the transmitted light is collected by a

spectrometer and the ratio between the incident and the transmitted light is measured. Its simplicity means that this technique is widely used in many areas of science. The information one can get from these measurements concerns the allowed electronic transitions. On the other hand, once the electronic structure of a substance is known, computer simulations are able to reproduce absorption spectra.

A very common use of UV-vis spectroscopy is to measure the concentration of substances, and this requires the celebrated Lambert-Beer (LB) law. This semi-empirical law states that the light propagating in a thick absorbing sample is attenuated at a constant rate, that is, every layer absorbs the same proportion of light [Jaffe & Orchin 1962]. This can be expressed simply as the remaining light intensity at a depth x into the sample is: $I(x) = I_0 \exp(-x/D)$ where I_0 is the incident intensity and D is a characteristic length which is called the "penetration depth" of a given material. If an absorbing dye is dispersed in a solution (or in an isotropic solid matrix) this penetration depth is inversely proportional to the dye concentration. In this way it is possible to determine a dye concentration c by experimentally measuring the absorbance, defined as the logarithm of intensity ratio

$$A = \log\left(\frac{I_0}{I}\right) = \ln 10 \ln\left(\frac{I_0}{I}\right) = \frac{x}{D} = x\frac{c}{\delta} \tag{1}$$

where x is the thickness of the sample (the light path length), D is the penetration depth, c the concentration of the chromophore, and δ the universal length scale characteristic of a specific molecule/solvent. One should note that in chemistry and biology one often uses base-10 logarithm in defining the Absorbance, rather than the more intuitive natural logarithm. If c is in molar units, the constant of proportionality ε is the "molar absorption coefficient" and it is inversely proportional to the characteristic length δ defined above.

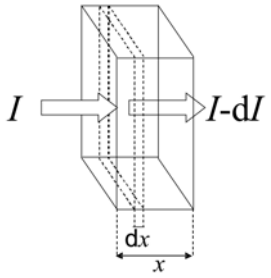


Fig. 2. Schematic diagram of a typical measurement of light absorption. The amount of absorbed light dI across the layer dx is proportional to the number of chromophores in that volume.

The derivation of this empirical law is straightforward. It assumes that the fraction of light absorbed by a thin layer of sample (thickness dx) is proportional to the number of molecules it contains (see figure 2), expressed as the volume fraction n times the volume of the thin layer ($Area \cdot dx$)

$$\frac{dI}{I} \propto n \cdot Area \cdot dx$$

where I is the intensity of the incident light. Introducing the cross section σ, which is a measure of the probability of a photon being absorbed by a chromophore, the differential equation becomes

$$\frac{dI}{I} = \sigma n dx$$

Solving the equation from 0 to x (total thickness of the sample), with a light I_0 incident on the front of the sample, one has

$$\ln(I/I_0) = -A \ln 10 = -\sigma n x$$

and we obtain equation 1 (rearranging the units opportunely).

Thanks to the Lambert-Beer law, UV-visible absorption spectroscopy is a useful and practical tool in many areas of science [Serdyuk et al. 2007]. The technique is widely used in organic chemistry and biology, as macromolecules often have a characteristic absorption in the UV and, more rarely, in the visible region of the EM spectrum. For example, all proteins have a characteristic absorption band around 190nm, due to the molecular orbital formed by the peptide bond, and another band around 280nm due to the aromatic side chains of aminoacids. Usually, this band is used to determine the concentration of proteins in a compound. Nucleic acids also absorb in the UV region and have a strong absorption band at 260 nm. The ratio between the absorption peak at 260 and 280 nm can give information about the relative quantity of DNA and protein in a biological complex, like ribosome. In atmospheric sciences, absorption spectroscopy is used to identify the composition of the air [Heard 2006]. Because the concentration of the species is very low, the light path must be very big to yield a detectable signal. Because L is so large and the concentration can change over the long range, a generalised Lambert-Beer law is preferred:

$$\ln\left(\frac{I_0}{I}\right) = \int_0^L \sigma_i c_i dx$$

where σ_i is the absorption cross section of each species i. Visible absorption can even be applied as a diagnostic tool. In medicine, for example, it is used to measure microvascular hemoglobin oxygen saturation (StO2) in small, thin tissue volumes (like small capillaries in the mouth) to identify ischemia and hypoxemia [Benaron et al. 2005].

All these applications rely on the validity of the LB law. However, this empirical law has limitations, and deviations are observed due to aggregation phenomena or electrostatic interactions between particles. The simpler form of the LB law also fails to describe the two-photon absorption and the excited state absorption process, and it must be substituted by a generalised Lambert-Beer law [Nathan et al. 1985]. These phenomena are usually present only at very high incident light intensity. Also, highly scattering media, very relevant for the medical and geological applications, produce large deviations from LB law.

This chapter addresses the topic of deviations from the LB law occurring in photosensitive media due to self-induced transparency, or photobleaching [McCall & Hahn 1967, Armstrong 1965]. This effect has been reported in a number of different biological systems such as rhodopsin [Merbs & Nathans 1992], green fluorescent protein [Henderson et al. 2007] and light harvesting complexes [Bopp et al. 1997] stimulated with strong laser radiation.

In figure 1, we showed how the excitation/disexcitation of a molecule is essentially a 3-state (or more!) process. Some of the energy loss, however, occurs very quickly and only involves vibrational levels. Considering the different time scales, one can simplify this into a 2-state model: an excitation process which promotes the molecule into a long-lived metastable state and its relaxation to the ground state. The origin of the long life of the metastable state depends on the particular system under study. In the case of spin flip of the excited electron, the physical reason underlying the stability of the triplet state is to be found in the selection rules, which practically forbid the transition between two different spin states (excited triplet state- ground singlet state). This process has raised a vivid interest in the scientific community in the last few decades, because triplet state is often a big problem in organic semiconductor devices [Wohlgenannt & Vardeny 2003]. Alternatively, the molecule, excited by light, gets "trapped" in a metastable state, separated from the ground state by an energy barrier. This is the case for azobenzene, a small molecule which exists in two different forms (isomers *trans* and *cis*). The transition between the two isomers requires breaking a double bond. UV light with a certain energy induces this double-bond breakage and lets the molecule rotate around its axis; with a certain probability, the bond will reform when the molecule is in a metastable *cis* isomer. The relaxation to the ground (lower energy) state can only happen if there is enough energy to break the double bond again. This can occur if the molecule is excited with a light at a different wavelength, or if the thermal fluctuations provide the molecule with enough energy to overcome the energy barrier and return to the ground state. The thermal relaxation is very slow and the characteristic lifetime depends on the nature of the chromophore and of the surrounding environment. This is a classical Kramers problem of overcoming an energy barrier (the breakage of the double bond) between the metastable and the ground state. In the case of this simple molecule, the Lambert-Beer law is no longer accurate because of a phenomenon which we call here "dynamic photobleaching" or saturable absorption. It means that the photons which shine on a sample are absorbed by the chromophores in the first layers. If these molecules don't return to their ground state immediately, when new photons fall on the sample they can't be absorbed anymore in the initial layers and therefore propagate through the sample with a sub-exponential law. So, the effective photo-bleaching of the first layers allows a further propagation of light into the sample and this leads to nonlinear phenomena which are interesting both from the theoretical [Andorn 1971, Berglund 2004, Statman & Janossi 2003, Corbett & Warner 2007] and from the experimental point of view [Meitzner & Fischer 2002, Barrett et al. 2007, Van Oosten et al. 2005, Van Oosten et al. 2007].
The aim of this chapter is to explore the effect that this phenomenon has on the typical absorption measurements which are commonly performed on these kinds of molecules. We will propose a new theory which can mathematically describe this effect and then we will give experimental evidence of its validity both on azobenzene, a molecule with a very long-lived excited state and whose kinetics of transition can be followed, and on more common fluorescent molecules, like chlorophyll, focussing on the absorption of light at equilibrium.

2. Materials and methods

2.1 Azobenzene

The molecule 4'-hexyloxy-4-((acryloyloxyhexyloxy)azobenzene (abbreviated as AC_6AzoC_6) was synthesized in our lab by Dr. A.R. Tajbakhsh. Its molecular structure is shown in figure 3 and its synthesis is described in [Serra & Terentjev 2008a]. All azobenzene-based

molecules exist in two isomers, *trans* and *cis*: the transition between the trans isomer, more stable, to the cis isomer, metastable, is stimulated by UV light, while the opposite reaction can be spontaneous. The isomers of the described molecule are shown in fig. 3

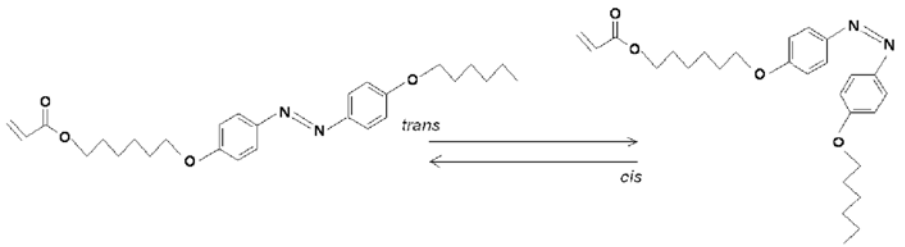


Fig. 3. The monomer used AC_6AzoC_6 has an acrylate head group followed by a carbon chain where the azo-group is attached. It is schematised here in its two isomers, *trans* and *cis*.

The two isomers of this molecule absorb light at different wavelengths: the trans isomer has a peak around 365 nm, while the cis isomer absorbs at 440 nm. It is thus possible to monitor the kinetics of *trans-cis* transition.

Monitoring a conversion process in real-time presents difficulties for a traditional spectrophotometer, because measurements over the whole spectrum of wavelengths take a long time, and moreover it is often difficult to access the sample in order to provide the UV illumination for isomerisation. For this reason, we chose a spectrometer equipped with a CCD camera, which is able to collect signal across the whole visible spectrum simultaneously. This technique works by illuminating the sample with white light; a system of gratings then splits the transmitted light into its various spectral components, whose intensity is measured by an array of photodiodes. This type of spectroscope does not require a fixed or enclosed sample holder, therefore placing another source of illumination close to the sample is easy.

For the measurements of light absorbtion a Thermo-Oriel MS260i (focal length 260mm) spectrometer was used. The apparatus consists of a quartz probe lamp with an adjustable slit, a quartz cuvette with 1cm optical path, an optical liquid lightguide to conduct the light from the cuvette to the spectrometer, a 50 μm slit at the entrance of the spectrometer, and the Andor linear-array CCD camera connected to a computer. The simultaneous measurement of all spectral frequencies allows for a response as fast as 0.021 s and the possibility of reducing noise by averaging over many measurements. Before every absorption spectrum, a background and a reference spectrum were collected: the background is the spectrum collected without the illumination from the probe lamp, and the reference was the spectrum collected with the probe lamp illuminating the cuvette filled with solvent (without the chromophore dye). The absorbance was then calculated from the counts of the detector as:

$$A = \log\left(\frac{\text{Reference} - \text{Background}}{\text{Signal} - \text{Background}}\right).$$

For all the experiments, it was important to verify that the linear relation between absorbance and concentration held for the value of absorbances considered. It was shown that the absorption-concentration relation was linear below $A \approx 1.2$ in the base-10 defined absorbance. At the intensity used for this experiment, for a concentration c expressed in moles, the penetration depth at 365nm was $\delta = c * 480\text{nm}$.
At higher concentrations, the linearity fails because of various phenomena, including aggregation of the molecules (especially with molecules like proteins or polymers), the scattering of light from big particles and stray light in the spectroscope.
We provide monochromatic illumination to stimulate the isomerisation of azobenzene using a Schott KL 1500 LCD lamp, placed at 90 degrees with respect to the incident probe light and the optical fiber that collects the light from the sample. In this way the cuvette is irradiated with UV light while the absorption spectrum is recorded along the perpendicular beam path, so the absorption can be followed in real time without interference of the illumination light. The intensity of the monochromatic light was in the order of a few tens of μW cm^{-2}.
All isomerisation reactions were followed for 90 minutes, which was a sufficient time for reaching the respective photostationary states. After this, the lamp was switched off and the absorbance was measured during thermal isomerisation in the dark. An example of spectrum measured with this technique is shown in fig. 4.

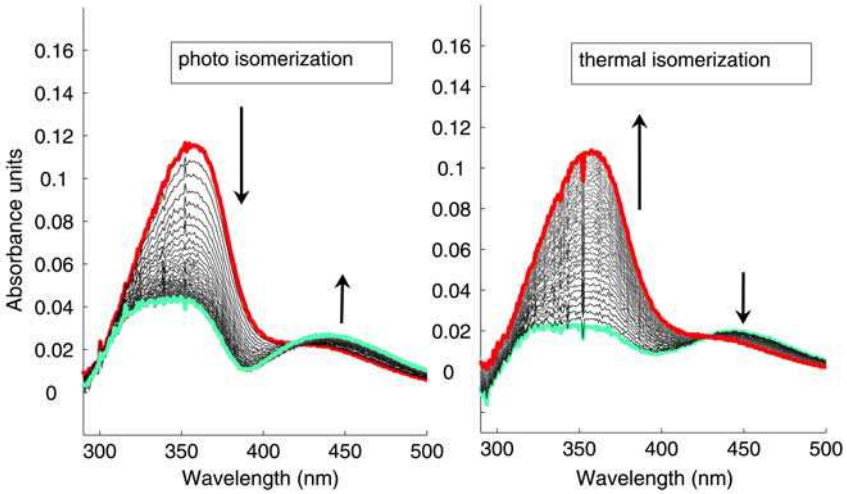


Fig. 4. Photo-induced isomerisation (a) and thermal relaxation (b) curves of AC6AzoC6 recorded as a time sequence. The arrows indicate the direction of the peak movement during the reaction.

2.2 Other chromophores

Chlorophyll was extracted from *Commelina Communis* leaves[1]. The leaves were first boiled in distilled water, in order to kill the enzymes which digest chlorophyll once the leaf is cut from the plant. The leaves were then dried and ground up with a pestle, with a few drops of

[1] Leaves were kindly provided by J. McGregor

acetone, and then left in a 50% hexane/water mixture (the hexane forms a layer on top of the water), to separate the chlorophyll from the water-soluble compounds (vitamins, etc). The extracted solution was filtered to avoid impurities, like dust particles or even intact chloroplasts which could be responsible for light scattering effects. The whole extraction process was carried out in the dark. This method of extraction does not allow the separation of chlorophyll from the carotenoids which could be present in the leaves. However, the collected spectrum shows that the stronger absorption bands are those of chlorophyll, and this means that the other compounds are present only in low concentrations. Moreover, for the purpose of our experiment, an highly purified chlorophyll is not needed, because the analysis focusses on the absorption band around 660nm, far from the absorption band of carotenoids (blue region). What is important to remark is that there are different kinds of chlorophyll, whose relative content varies from species to species. The two main chlorophyll components, called "chlorophyll a" and "chlorophyll b", differ by a carboxylic group, attached only to the porphyrine ring in chlorophyll b. The two molecules have slightly different absorption spectra, but this is not relevant for the experiments, provided that the plant species from which the chlorophyll is extracted is always the same (and has therefore the same percentage of chlorophyll a and b).

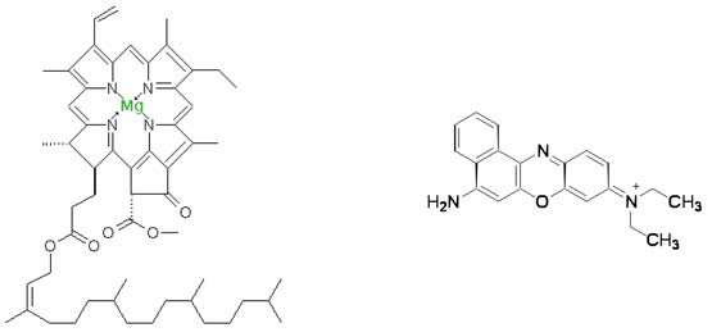
From the discussion above, it is clear that chlorophyll is a very special molecule, and has many peculiarities. In order to demonstrate that the theory is more general, a commercial dye with a strong absorption in the visible region is also investigated. Nile Blue A, a dye commonly used for staining DNA, but with spectral properties similar to chlorophyll, was selected. The chemical structure of chlorophyll and Nile Blue is shown in figure 5.

The spectroscopy experiments were conducted with an Ocean Optics USB 4000 spectrometer, equipped with optical fibers. A 25W halogen lamp with spectral range 400-880 nm, whose intensity could be tuned, was used as illumination source. The light was focussed with collimating lenses onto a 1 cm cuvette containing 3 ml of solution.

For comparison with more "conventional" spectroscopes (meaning, with a fixed sample holder and a fixed intensity of incident light), a Cary UV-Vis Spectrophotometer was used to measure spectra and absorbances of the two substances at various concentrations. The intensity of the incident light from the spectrometer was also measured with a power meter.

In order to measure the intensity of the incident light, key quantity in our experiments, the light from the source was shone onto the detector directly, in the absence of any sample or cuvette in between. Knowing the characteristic response of the spectrometer detector, it is possible to measure the intensity of light. Three different values, the number of counts at a single wavelength or the integral of the intensity over the range of wavelength which correspond to the absorption peak, and the integral over all the wavelengths could be used to quantify the incident light intensity. In all cases the outcome of the experimental results was the same. Using as a value of intensity the intensity at the single peak-wavelength made it possible to compare it to the conventional spectroscope (which produce monochromatic light). It was verified that the detector had a linear response in counts versus intensity over the range of intensities we used.

For the absorption spectra, measured as $A = \log_{10}(I_0/I)$ reference spectra for the pure solvent were taken before each measurement at every light intensity. The absorption and fluorescence spectra of these materials are shown in figure 6. We chose to refer all the absorption values to the wavelengths of the peaks in the yellow-red region.



(a) (b)

Fig. 5. Chemical structure of a) chlorophyll a and b) nile blue dye.

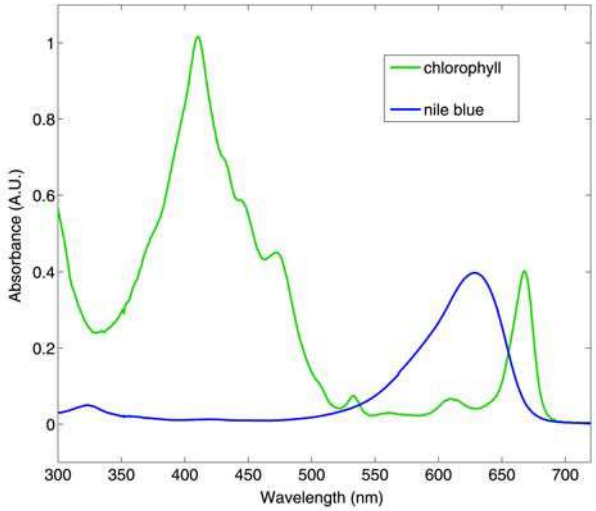


Fig. 6. Absorption spectra of chlorophyll (green curve) and nile blue (blue). The absorption peaks which were considered in this work are those at 668nm and 628nm respectively.

For each solution, the linearity of the absorption/concentration curve was verified, in order to avoid falling into a trivial nonlinear regime. The experiments were conducted in random order of light intensity, and the reproducibility was verified. The absorption of each chlorophyll solution at 660 nm was stable over a range of hours at constant incident light intensity, indicating the absence of chemical irreversible bleaching. Fluorescence from the dye was also ruled out as a possible source of disturbance, because at the light intensity we used it is not detectable with our equipment.

3. Theory

Here we present a description of the dynamical photobleaching effect in the case of azobenzene isomerisation, which was previously discussed. We will then generalise the

discussion to all the molecules with a long-lived excited state, and show how this affects the measurements of light absorption.

The non-Lambertian propagation of light through a medium has important consequences for the analysis of photo-isomerization kinetics: when the photo-bleaching becomes important, the measured absorbance no longer follows a simple (traditionally used) exponential law. In photosensitive molecules like azobenzene, irradiation with light of a certain wavelength induces a conformational change (isomerization) from an equilibrium *trans* state where the benzene rings are far apart to a bent *cis* state where they are closer. The isomerization process follows first-order kinetics. Calling the fraction of molecules in the two states *trans* and *cis* n_t and n_c we have

$$\frac{dn_t}{dt} = -Ikn_t + Ik_b n_c + \gamma n_c$$

where I is the intensity of light, k is the *trans-cis* isomerisation rate, k_b the stimulated back transition rate (*cis* to *trans*), and γ the thermal relaxation rate. In the experiments on azobenzene described below we use an illuminating light monochromated at the *trans-cis* transition wavelength. In this case the stimulated *cis-trans* isomerization is negligible (that is, $Ik_b \to 0$) and, remembering that $n_c = 1 - n_t$ the kinetic equation reduces to

$$\frac{dn_t}{dt} = -\gamma\Big([1 + Ik/\gamma]\, n_t - 1\Big). \tag{2}$$

In this equation the intensity $I = I(x)$ is the light intensity at a certain depth into the sample. It is convenient to define a non-dimensional parameter $\alpha = I_0 k/\gamma$, which represent the balance of photo- and thermal isomerization at a given incident intensity I_0. In this notation, the amount of molecules in the *trans* conformation in the photostationary state, when the balance between n_t and n_c is stable, and therefore $dn_t/dt = 0$ the equation reduces to simply

$$\gamma n_t + \frac{I}{I_0}\alpha n_t - \gamma = 0$$

therefore

$$n_t = \frac{1}{1 + \alpha I/I_0} \tag{3}$$

To express mathematically the reversible photobleaching phenomenon, it can be assumed that the change in light intensity across a thin layer of sample (thickness dx) is proportional to the number of molecules which are excited, i.e. the number of chromophores which absorbed light in a small volume of sample of thickness dx; neglecting the stimulated *cis-trans* isomerization (which is appropriate in our study), the model can be much simplified to give, per unit time:

$$\Delta I \propto Ikn_t(x,t) \cdot Area \cdot dx$$

Then, combining all the parameters, such as the photon cross section and the transition rates, we recover the penetration depth $D = \delta/c$ as the relevant parameter of the relation, and the final expression is:

$$\frac{dI}{dx} = -I\frac{n_t}{D} \tag{4}$$

with D the penetration depth, inversely proportional to concentration. In order to study this problem at the photostationary state, one needs to combine the equations (4) and (3).

$$\frac{dI}{dx} = -\frac{I}{D}\frac{1}{1+\alpha I/I_0} \tag{5}$$

Solving the differential equation, the stationary-state light intensity at a depth x is given by the relationship [Corbett & Warner 2007]

$$\ln\left(\frac{I(x)}{I_0}\right) + \alpha\left(\frac{I(x)}{I_0} - 1\right) = -\frac{x}{D}. \tag{6}$$

Looking at the equation above some important insights can be gained. The most important is that in the limit α = 0 the equation reduces to the Lambert-Beer law, i.e. an exponential decay in the transmittance through the medium. Therefore all the nonlinearity is included in α. The opposite limit, when α is very big, leads to a linear relation between transmittance and sample thickness. Figure 7 is a representation of equation 6 and it shows the intensity variation $I(x)$ for several values of the parameter α: from the plot of transmittance as a function of x, it is clear that, if the incident intensity, and therefore α, is low enough, the Lambert-Beer law is valid and the decrease is exponential, but if the incident intensity is high the bleaching of the first layers becomes progressively more relevant such that they become partially transparent to the radiation. The decay thus tends to become linear in the bulk of the sample, $I(x) \approx I_0(1 - x/\alpha D)$.

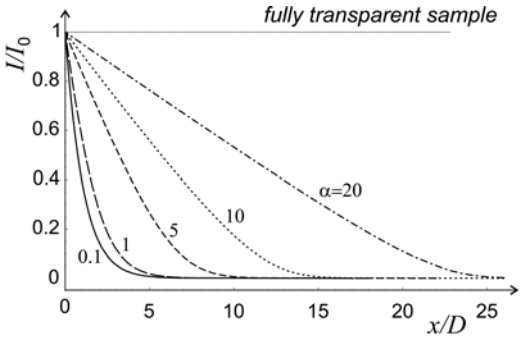


Fig. 7. Transmitted intensity ratio I/I_0 in the photostationary state as a function of the parameter x/D (proportional to sample thickness or inversely proportional to chromophore concentration) for several values of α. At small α the decay is exponential; the light penetrates deeper into the sample as α increases, as the decay tends to be linear. [Serra & Terentjev 2008b]

In order to model the dynamics of photoisomerisation, which is evidently inhomogeneous across the sample, it is not enough to model photobleaching with equation 6, but instead the equations (2) and (4) should be coupled. Calculating a time derivative of equation 4 leads to

$$\frac{d}{dt}\left(\frac{1}{I}\frac{dI}{dx}\right) = -\frac{1}{D}\frac{dn_t}{dt} \tag{7}$$

In the right hand side expression, equation 2 can be substituted, giving

$$\frac{d}{dt}\left(\frac{1}{I}\frac{dI}{dx}\right) = -\frac{\gamma}{D}\left(1 - \left[\alpha I/I_0 + 1\right] n_t\right) \tag{8}$$

This equation can be solved with the following method [Corbett et al. 2008]. Introducing the variable $y = \ln(I/I_0)$, which is a very sensible variable, being also the inverse of the absorbance, the left-hand side is greatly simplified and one obtains

$$\frac{d}{dt}\left(\frac{dy}{dx}\right) = -\frac{\gamma}{D}\left(1 - (\alpha \exp(y) + 1)\right) n_t \tag{9}$$

Also, from equation 4

$$\frac{1}{D}\frac{dn}{dt} = -\frac{d}{dt}\left(\frac{dy}{dx}\right) \Rightarrow n_t = -D\frac{dy}{dx} \tag{10}$$

Substituting n_t in equation 9 and rearranging, one finds

$$\frac{d}{dt}\left(\frac{dy}{dx}\right) = -\frac{\gamma}{D} + \gamma(\alpha \exp(y) + 1)\frac{dy}{dx} \tag{11}$$

In the next step, one has to keep in mind that

$$\frac{d}{dx}(\gamma\alpha e^y + \gamma y) = \gamma\alpha \exp(y)\frac{dy}{dx} + \gamma\frac{dy}{dx}$$

Rearranging equation 11 and exchanging the order of derivatives on the left-hand side, the equation reduces to

$$\frac{d}{dx}\left(\frac{dy}{dt} + \gamma(\alpha \exp(y) + y)\right) = -\frac{\gamma}{D} \tag{12}$$

It is now possible to integrate this expression. Integrating between 0 and x, the integral and the derivative on the left-hand side cancel out and one finds:

$$\frac{dy}{dt} + \gamma(\alpha \exp(y) + y) - \gamma\alpha = -\frac{\gamma x}{D} \tag{13}$$

The factor $\gamma\alpha$ comes from the solution of the definite integral for x=0 (the lower integration limit). In fact, if x=0, $I = I_0$ and therefore y=0 by definition.
The last step is a time integration. At time zero, the absorbance $A = -y$ must be equal to the Lambert-Beer law value x/D. Including these considerations, the integral expression for the intensity $I(x, t)$ becomes:

$$\gamma t = -\int_{x/D}^{A} \frac{dy}{\left(x/D - y - \alpha + \alpha e^{-y}\right)}. \tag{14}$$

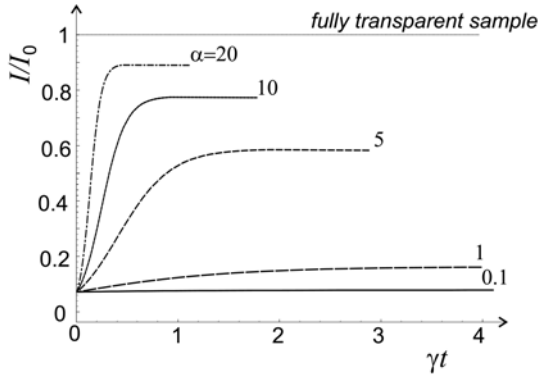


Fig. 8. Transmitted intensity ratio I/I_0 as function of time through a fixed value of x/D = 2.7 and different incident light intensities. There are several things to observe in this figure. First, as α changes, the photostationary state reaches different levels, as expected: if α is bigger, the sample becomes more transparent. The second thing is that when α increases (therefore intensity of light) the sample reaches the stationary state more quickly. The last observation, most important for our study, is that with increasing α the deviation of the kinetics from a simple exponential becomes more and more evident. [Serra & Terentjev 2008b]

The upper limit of this integral is the measurable absorbance $A = \ln[I_0/I(x, t)]$ from a sample of thickness x. Figure 8 shows a simulation predicting the time-evolution of intensity transmitted along the path x/D. Note that at $t = 0$ all curves converge to the Lambert-Beer $I/I_0 = \exp(-x/D)$, while at long times a significant portion of chromophore is bleached and the transmitted intensity increases.

We should note that the problem of non-linear photo-absorption dynamics is not only restricted to azobenzene isomerisation. Even ordinary dye molecules that do not undergo conformational changes stimulated by photon absorption, still follow the same dynamic principles, but with electronic transitions in place of *trans-cis* isomerization. Therefore, the results of this paper should be looked upon as widely applicable to other systems. In particular, the two key conclusions, that the crossover intensity into the non-linear photo-absorption regime is independent of dye concentration and that the rate of the transition is independent on solvent viscosity, are probably completely general.

The model we propose only assumes a two-configuration system, and it does not imply anything about the nature of the two states. Therefore, it is important to verify that this model has a wider and broader validity, and, in detail, that it helps to understand the behaviour of a large class of chromophores, like fluorescent molecules. Azobenzene molecule exists in two physical states, *trans* or *cis*; for chlorophyll, one could make an analogy and, considering the electronic transition, call the two states "ground" and "excited", we still find the same formula at the photostationary state

$$\ln\left(I_0/I(x)\right) + I_0(k/\gamma)\left(1 - I(x)/I_0\right) = x/D \tag{15}$$

where x is the path length of light through the sample. It is important to see here that the absorbance has a nonlinear dependence on the incident light intensity. The limits where the

LB law is recovered are either very low incident intensity (practically, it can never be achieved) or a very fast recovery to the ground state compared to the excitation.
Equation 15 can has important implications for the interpretation of absorption data. Solving the equation for the absorbance $A = \log(I_0/I)$ leads to the expression

$$A\ln(10) + I_0\frac{k}{\gamma}(1 - 10^{-A}) = \frac{xc}{\delta} \tag{16}$$

from which it is clear that the value of the absorption does not only explicitly depend on the concentration and optical path length, but also on the intensity of the incident light I_0.

4. Non-linear kinetics of photobleaching.

Azobenzene, as previously discussed, is a small molecule with two double-bonded nitrogen atoms linked to two benzyl rings. It is photosensitive, because exposure to light induces an isomerisation *trans-cis* (indicating two possible spatial arrangements of the benzyl rings) around the double bond between the nitrogens, and this results in a molecule shape change. The process is fully reversible either by stimulated backward photoconversion (with a light at a different wavelength), or by spontaneous relaxation to the equilibrium *trans* configuration.
The isomerization of azobenzene and its derivatives has been extensively studied for the last fifty years [Sudesh Kumar & Neckers 1989, Renner & Moroder 2006, Rau 1990], because this molecule has many interesting features and its applications range from electronics to biomedicine. It has been used as a model molecule for all the biological processes that involve similar reactions, like the isomerization of retinal in rhodopsin, or as a probe for measuring the free volume in polymers [Victor & Torkelson 1987]. More recently, its characteristic response to the polarization state of light made it a suitable molecule for surface patterning [Nathanson et al. 1992, El Halabieh et al. 2004]. Finally, azobenzene-containing elastomers can give rise to inhomogeneous photo-mechanical effects and their applications as photoactuators and artificial muscles are under study [Hogan et al. 2002, Finkelmann et al. 2001, Yu et al. 2004].
However, in spite of the large literature on the subject, many fundamental mechanisms and effects have not been clarified yet. It is assumed that the isomerization reaction is very sensitive to both electrical and mechanical characteristics of the environment which surrounds the molecules, but identifying and separating these effects is a difficult and often ambiguous task.
Of the two possible isomers of azobenzene, the *trans* form is the lowest energy form, since the benzyl ring electron clouds are far apart (see fig. 3), but under UV light an isomerisation occurs; once in the *cis* state, the molecules can return back to their equilibrium trans state both by stimulated isomerisation with visible light or by thermal relaxation [Rau 1990]. The rate constants of these two processes are usually different, thermal isomerisation being slower. The microscopic mechanism that leads to the isomerisation is still not clear, but there are suggestions for both rotational and inversional [Asano & Okada 1984] mechanisms may be competing.
The isomerisation of azobenzene can be monitored by UV-Vis (ultraviolet and visible light) spectroscopy, because the two *trans* and *cis* compounds have different absorption spectra in this range: the *trans* isomer absorbs around 365 nm, while the *cis* isomer at around 440 nm

[Rau 1990]. Irradiation with light at the wavelength of the *trans* peak progressively depletes the molecules in this conformation. This reaction can be "followed" by measuring the intensity of the absorbance of the spectrum peak at 365 nm, which decreases as the *trans-cis* photo-isomerisation reaction proceeds. An example of spectral evolution as isomerisation proceeds can be found in figure 4.

The models that were proposed for reaction kinetic are basically first order models, with the important exception of azobenzene in polymer matrices. The fraction of isomers in the *cis* state, n_c, varies as [Zimmerman et al. 1958, Mechau et al. 2005]:

$$\frac{dn_c}{dt} = Ikn_t - Ik_b n_c - \gamma n_c \tag{17}$$

where I is the irradiation intensity, k_b and k are the *cis-trans* and *trans-cis* constant of photoisomerisation respectively, γ is the thermal *cis-trans* isomerisation and n_t represents the fraction of molecules in the *trans* state, and it is equal to $(1 - n_c)$. In the derivation of the formula the sensible assumption was made that the *trans-cis* thermal isomerisation constant is negligible.

A basic characteristic of the photoisomerisation problem is the rate of spontaneous thermal *cis-trans* isomerisation γ. For a given azobenzene derivative, at fixed (room) temperature and sufficiently low dye concentrations to avoid self-interaction, this rate is approximately the same for all our solutions. We measured this rate after monitoring the relaxation of the spectrum after the UV illumination is switched off (see [Serra & Terentjev 2008a] for detail) and obtained $\gamma \approx 1.25 \cdot 10^{-4} s^{-1}$ (or the corresponding relaxation time of ~ 8000s).

In order to test the predictions of the theory, dynamic absorption measurements were performed for different dye concentrations and different light intensities. Considering equation (14) this is equivalent to changing x/D (where D is inversely proportional to the dye concentration) and α, which is proportional to the incident intensity I_0. With our experimental setup it was possible to follow all the isomerisation kinetics and thus the time dependence of I/I_0 [Serra & Terentjev 2008b].

We prepared three different dye solutions with (non-dimensional) weight fractions $c = 2.5 \cdot 10^{-3}$, 0.01 and 0.025, resulting in values of penetration depth ranging from D = 36 mm, to D = 3.6 mm. We recall here the physical meaning of the penetration length, which is the distance through the sample over which the light falls across a sample to 1/10 of its original intensity. The cuvette containing the sample is 1 cm long; therefore a sample with D equal to a few millimeters is almost completely opaque.

The measurements were performed using the Thermo-Oriel spectroscope described in the materials and methods section. Illumination was provided by a Nichia chip-type UV-LED, emitting at 365nm (bandwith about 10nm wide) whose output power was accurately regulated by a power supply. The LED light was attenuated by passing through a black tube of controlled length, placed in front of a quartz cuvette with 1 cm optical path. Several values of intensity were used in reported measurements, ranging between I_0 = 4 and 60 $\mu W/cm^2$. It is important to point out that these intensity values are very low and that most experiments on azobenzene isomerization are performed with intensities which are orders of magnitude higher, making the photobleaching much more of an issue. The low values of intensity allowed us to have a kinetics slow enough to detect the features which the theory predicts at short times. Every point of the spectrum was collected as an average of 100

measurements. All isomerization reactions were followed for several hours, until a photostationary state was reached. The measurements were repeated at different illumination intensities (regulated with the power supply) and at different dye concentrations.

In all cases it was important to verify that the dye concentration remained in a range where, at time t = 0, the linear proportionality between absorbance and concentration (Lambert-Beer law) held. This is important because the concentration of molecules in the *trans* state at every instant was determined from the absorbance at 365 nm. Absorbance was measured at several concentrations. The deviation from linearity started at $A \approx 1.2$, which corresponds to the dye weight fraction of c = 0.03 (3 wt%) in our 1 cm cuvette. After this point, aggregation effects start playing a role and the basic Lambert-Beer law is no longer valid, undermining the theoretical relationship given by the equation (14). We always kept the concentrations below this value, so that the linearity at t = 0 was maintained, with $A = x/D$ where the penetration depth is inversely proportional to chromophore concentration, expressed as weight fraction, $D \approx \delta / c$ with δ = 91 μm.

For our detailed dynamic experiments, a very important issue was the viscosity of the solution. In fact, at high illumination intensity we have encountered an unexpected problem. Figure 9 shows that the transmission of light through a low-viscosity dye solution (in pure toluene) displays a characteristic oscillatory behaviour. Detailed analysis of this phenomenon is under further investigation. Whether the oscillations are linked to the local convection due to the heating of the sample spot [Nitzan & Ross 1973] or to the diffusion of the less dense *cis* molecules – or whether they are intrinsic to the non-linear photochemical process [Borderie et al. 1992] – is not clear at this stage.

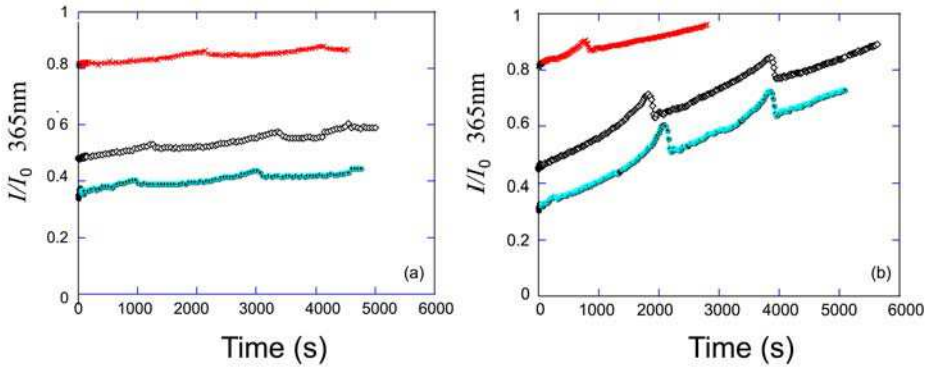


Fig. 9. Kinetics of isomerisation monitored through the observation of I/I_0 over time for 3 different values of x/D (× - 0.2, ◊ - 0.7, • - 1.1) and 2 different values of α, corresponding to: (a) I_0 = 4μW cm^{-2}, and (b) I_0 = 20μW cm^{-2}. The periodic instability was reproducible in all low-viscosity experiments. [Serra & Terentjev 2008b]

In order to avoid this difficulty, the dye solutions were prepared in a mixture of toluene and polystyrene of high molecular weight. Adding polystyrene increases the viscosity of the solution by over 2 orders of magnitude, and in this way prevents fluid motion in the cuvette on the time scales of our measurements. Polystyrenetoluene solutions were prepared at a

fixed weight ratio. Adding polystyrene to toluene increases the Rayleigh scattering of the solution, but we felt that we could safely do that because on one hand the absorption dynamics is not affected (we used the same concentration for all the measurements and for the reference spectrum), and on the other we measured the transmittance of the toluene-polystyrene solution, which is almost equal to the pure toluene solution at 365nm.

In figure 10 the representative experimental results are shown for the solution with the highest chromophore concentration (D = 4.6 mm, leading to x/D = 2.2) and three values of incident intensity. One finds that all curves converge to the same initial value corresponding to the $I/I_0 = \exp[-x/D]$, which for this concentration means quite a low transmission ($I/I_0 \approx$ 0.11). If the isomerisation didn't take place, the sample absorption would be constant in time according to the classical Lambert-Beer. A traditional description of the kinetics of isomerisation would predict an exponential decay of the absorption over time, but from figure 10 we see a strong deviation. We fit the data with the theoretical model given by equation (14) where we input the values of γ(thermal relaxation) and x/D, leaving only $\alpha = I_0 k/\gamma$ free. Two data sets at higher intensity show the transmitted $I(x, t)$ reach saturated values. In this case we are confident of the fit because we have to match both the slope and the amplitude of the curve. We obtain $\alpha \approx 60$ for $I_0 = 60\ \mu$W/cm², $\alpha \approx 20$ for $I_0 = 20\ \mu$W/cm², and $\alpha \approx 4$ for $I_0 = 4\ \mu$W/cm² (the matching of values is pure coincidence). We found that one particular output of experimental recording, the absorbance plateau value (photo-bleached) at long times, was extremely sensitive to the reading of reference intensity I_0. The latter measurement could be affected by various stray factors and in a few cases we had to rescale the raw absorbance readings with a proper reference value. This issue did not have any effect on absorbance at t = 0, or the kinetics.

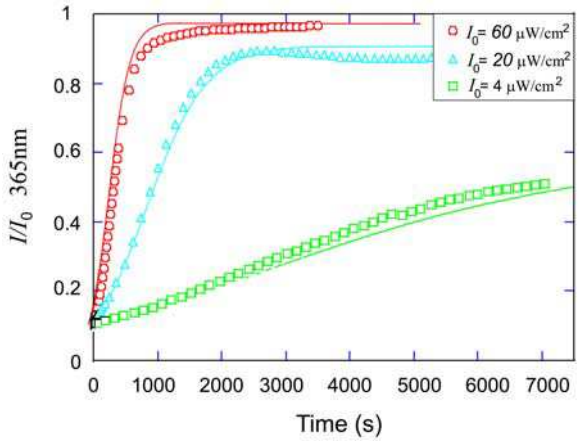


Fig. 10. The effect of photo-bleaching for samples with high dye concentration (x/D = 2.2). Three values of irradiation intensity are labelled on the plot. Solid lines are fits to the data with only one free parameter α, giving α = 60 for the highest intensity, α = 20 for the middle intensity, and α = 4 for the lowest intensity. [Serra & Terentjev 2008b]