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A polarity dependent fluorescence "switch" in live cells

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Abstract

The spectroscopic properties, ultrafast kinetics and utilization of a photochromic molecule as a bi-stable fluorescing sensor of polarity in live cells are described. This molecule is a photochromic fulgimide, 2,3-dialkylidenesuccinimide, which emits fluorescence that can be switched optically on and off. The fluorescence intensity is a function of the polarity of the molecular environment, namely it fluoresces strongly when the molecule is in its polar isomeric structure form. We demonstrate that this molecule enters live cells without inducing damage, it binds primarily to internal membranous organelles (mitochondria) and its fluorescence can be switched optically "on" and "off" repeatedly while inside the living cell. A possible use as a bi-stable, on/off sensor is discussed.

Keywords: Photochromism; Polarity; Fluorescence; Live cells; Bi-stable switch

1. Introduction

Photochromic materials have found potential applications in high capacity optical storage, optical molecular switches, optical limiters and as non-linear media. In particular, photochromic fulgimides and fulgides are being studied widely because they exhibit excellent photochromic behavior, and their two isomeric forms are thermally stable and photoreversible, which make them suitable for many electronic applications [1,2]. Even though a number of these molecules have been studied previously, additional materials have been developed because of the continuously expanding need for new photochromic molecules with optimal physical and chemical properties. This has been necessary in order to satisfy high density computer storage requirements as well as the needs of other applications. The unique property of photochromism is the reversible photoinduced conversion of the molecule to two isomeric forms. In some cases, only one of the forms emits fluorescence and that is utilized for 3D optical storage. The fulgimide utilized in the present studies fluoresces strongly only in its polar, closed ring form, see Fig. 1, while the non-polar open ring form does not fluoresce. Therefore, the inter-conversion from one form to the other is the operational mechanism for switching the fluorescence "on" and "off". This bi-stability and the fact that the fluorescence intensity is a function of polarity, suggest that this molecule could function as a sensor for changes in the polarity of species within the environment of biological and chemical systems. In this paper, we report the spectroscopic properties of this photochromic molecule as well as its use as an "on–off" fluorescence switch in live cells.

The fulgimide described here is the photochromic component of a recently described composite molecule [3], which under specific excitation conditions exhibits both photochromism and fluorescence. In addition it is also bi-stable, namely it can reside in either the "on" fluorescent state or the "off" non-fluorescent state. This "on–off" switching can be optically induced by changing the structure of the molecule from its polar to non-polar

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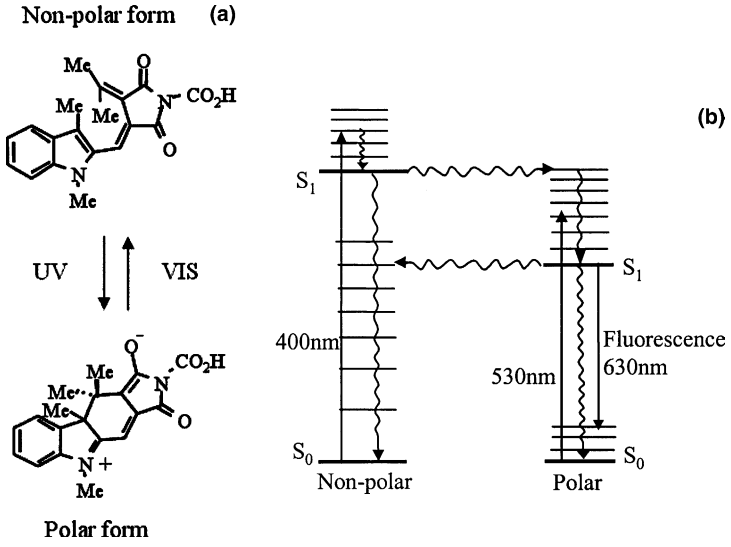

Fig. 1. (a) Chemical structure and photochromic reaction of the fulgimide molecule; (b) energy level diagram of the fluorescence and non-polar to polar conversion.

structure (Fig. 1). This switching can be performed repeatedly over time in liquid solvents, solid polymer matrices, and individual living cells by excitation at the absorption wavelength of the two forms.

2. Materials and methods

2.1. Imaging of cells

All cellular imaging experiments were performed with Zeiss a LSM 410 (Zeiss Inc., Thornwood, NJ, USA) confocal laser scanning microscope. The 488 nm light of the Ar ion laser induced the fluorescence. The fluorescence was observed through a long pass 610 nm filter. The second channel was used to observe the cells under phase contrast. A Zeiss Neofluar 100 × Ph 3 1.3 n.a. oil immersion objective was used in all experiments.

2.2. Spectroscopic measurements

All in situ spectroscopic measurements were performed with a Zeiss LSM 410 microscope, fiber optically coupled to Spectra-Pro 150 spectrograph with a 300 grooves/mm grating blazed at 500 nm (Acton Research Corp., Acton, MA, USA), interfaced to a high dynamic range TE-CCD spectrograph and camera (Princeton Instruments, Princeton, NJ, USA). CCD temperature was maintained at −40 °C.

For in situ live cell measurements switching between the closed ring polar fluorescent form and the open ring non-polar non-fluorescent form was achieved by irradiation of the sample on the microscope stage with a 100 W halogen lamp. The polar form of the molecule was induced by 1 min irradiation through a short pass 450 nm filter. The non-polar form of the molecule was generated by 1 min irradiation through a long pass 520 nm filter. Fluorescence spectra were acquired immediately after irradiation. The excitation light from 100 W Hg lamp was filtered through a narrow band pass filter centered at 550 nm. The fluorescence emission was separated from the excitation light by a long-pass 610 nm filter. The emission acquisition time was 500 ms. The fluorescence intensity was measured for both the polar and non-polar forms at 630 nm using spectra that were corrected by background subtraction.

The absorption and emission spectra in solution were obtained by means of a Shimadzu UV-1601 Spectrophotometer and Shimadzu RF-5301 PC Spectrofluorophotometer, respectively (Shimadzu Scientific Instruments, Inc., Colombia, MD, USA). The time resolved spectra and kinetics were measured with the 130 fs, 10 MHz, laser system (Tsunami 3941-MIS, Spectra-Physics Lasers, Mountain View, CA, USA) described previously [4], The fulgimide was synthesized by the previously described procedure [2].

2.3. Cell culture

PTK_2 (*Potorous tridactylis*, American Type Tissue Culture Collection, Washington, DC, USA, #CCL 56) cells were cultured in minimum essential growth medium (GIBCO, Grand Island, NY, USA) supplemented with 2 mM L-glutamine, penicillin (100 mg/ml), streptomycin (100 mg/ml) and 10% heat-inactivated fetal bovine serum. All culture reagents were purchased from Invitrogen (Carlsbad, CA, USA). Cells were maintained at 37 °C in a 7.5% CO_2 incubator. For the experiments, cells were seeded in imaging dishes at a density of 50% confluence.

2.4. Dye preparation

Stock solution of the fulgimide molecule was prepared in DMSO (dimethylsulfoxide). For the imaging study, cells were labeled with the fulgimide at a concentration of 2.4×10^{-6} M prepared in growth medium without phenol red and pH value adjusted to 6.5.

3. Results and discussion

3.1. Spectroscopy and kinetics

The open-ring form, Fig. 1, has a light yellow color which upon irradiation with 386 nm light is transformed into the closed-ring structure, whose absorption spectrum is shifted to the 485–550 nm range. The wavelength of the absorption maximum of the closed form is affected by the polarity of the solvent. This observation may be due to the large dipole moment of the fulgimide in the excited state which is stabilized by the polar solvent. The photochemical mechanism of the conversion from polar to non-polar structures and the resulting fluorescence are shown in the energy level diagram depicted in Fig. 1.

The structure of this molecule and the absorption spectra of its two photochromic non-polar and polar forms with maxima located at 386 and 539 nm, respectively, are shown in Fig. 1 and Fig. 2(A). The fluorescence shown in Fig. 2(B) is emitted strongly when the molecule is in the polar form, while the non-polar form is practically void of any emission. The polar form is formed by excitation of the non-polar isomer with 400 nm light, while 539 nm light converts the polar form to the non-polar form. The molecule is found to be stable between −55 and +55 °C, in both isomeric forms.

The intermediate spectra and kinetics of the transformation from the polar to the non-polar forms were measured by means of ultrafast time resolved spectroscopy. The experimental system was composed of an amplified Ti:Sapphire laser emitting 100 fs pulses with up to 10 mJ/pulse, that has been described previously [4]. The fundamental pulses were converted by second harmonic generation and used at a rate of 50 pps. The excited state spectra formed at various times after excitation are shown in Fig. 3 from 1.3 to 63 ps. The transient spectrum at 1.3 ps has a shape and maximum absorption that is different than the spectra recorded at later times due to index dispersion [3]. A plot of intensity at 520 nm versus time (Fig. 4) gives the rate of formation

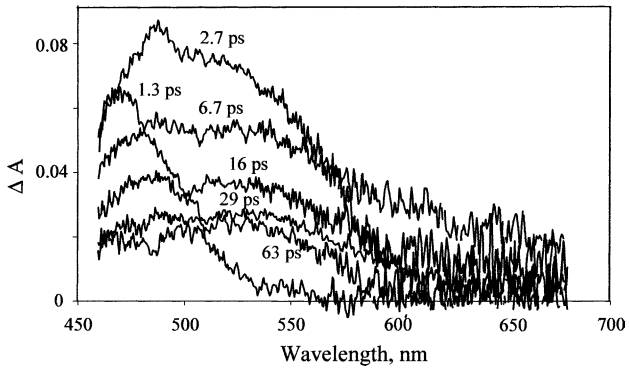

Fig. 3. Open to close form transient absorption spectra of fulgimide in acetonitrile.

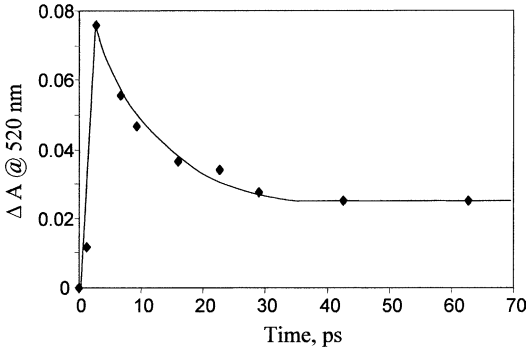

Fig. 4. Plot of ΔA vs. time measured at 520 nm for open to close form conversion.

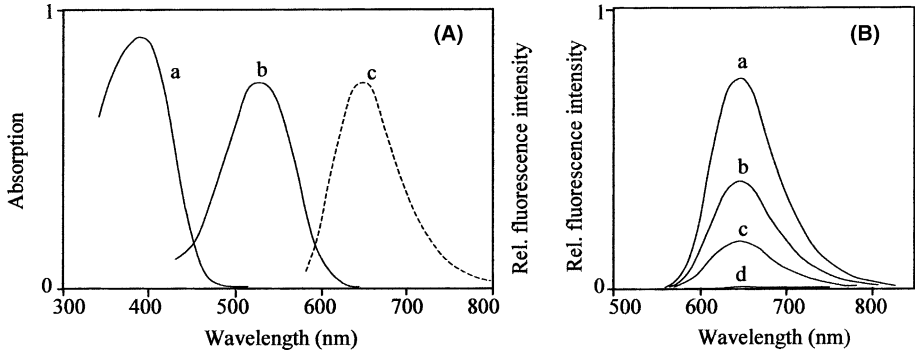

Fig. 2. (A) Absorption spectra of non-polar (a) and polar (b) forms of fulgimide and fluorescence of polar form (c); (B) change in the intensity of the fluorescence spectra of fulgimide in acetonitrile as a function of conversion from polar to non-polar by irradiation with 530 nm light. (a) Original intensity, (b), (c), (d), after 1, 2, and 5 min of illumination.

and decay of the metastable intermediate. The rise time of ~150 fs shown in Fig. 4 corresponds to vibrational decay and the growth of the $v = 0$ level of the first excited singlet electronic state. The rate of transformation from the open non-polar to the closed polar form in acetonitrile was $\sim 5 \times 10^{11}$ s^{-1} and the fluorescence lifetime was 5×10^{-8} s. These data suggest that the on–off switching is in the picosecond time scale rather than the much slower diffusion controlled rates of most chemical and biological reactions.

3.2. Photoreaction quantum yield

The quantum yield of the photochromic reaction that induces the cyclic reaction, which generates the polar closed-ring structure, Fig. 1, has been measured, as has the quantum yield of the reverse reaction. The non-polar open-ring form was converted, almost quantitatively, to the polar form by excitation with 390 nm light. However because both the polar and non-polar forms absorb in the 390 nm region, photoexcitation at 390 nm leads to the formation of a photoequilibrium mixture between the two forms. The colored form can be converted back to the open-ring form by excitation with 530 nm light. The quantum yields of these photoreactions in various solvents are listed in Table 1.

The quantum yield of the ring-opening process of the fulgimide was found to be 0.08 in acetonitrile. In non-polar hexane, the quantum yield of the ring-opening process is about two times larger than in acetonitrile. The low quantum yields in polar solvents may be due to the strong interaction between the polar excited state of the fulgimide polar form and the polar solvent, which may raise the activation energy of the ring-opening process and consequently decrease the transformation efficiency.

3.3. Fluorescence quantum yield

In contrast to the previously investigated fulgimides [5,6] which do not emit fluorescence, the closed-ring form of the fulgimide we synthesized, emits intense fluorescence. The fluorescence spectrum of the closed structure form in acetonitrile shows a broad emission band with a maximum at 650 nm, Fig. 2(B). To confirm that the closed-ring form of this fulgimide emits the observed fluorescence, as opposed to impurities or other species, the excitation spectra and fluorescence emission intensity changes as a function of open/close cycles were measured. The results show that the fluorescence intensity and excitation spectra of the closed-ring form decrease proportionally with the concentration of the fulgimide closed-ring form (see Fig. 2(B)). When the solution was completely bleached, i.e., the absorption band of the closed form completely disappeared, no fluorescence was detected. When the bleached solutions were illuminated with 390 nm light and converted to the polar form, the molecule emitted again. The fluorescence, non-polar to polar and polar to non-polar photoconversion quantum yields are shown in Table 1.

3.4. Live cell studies

The change in the polarity of the fulgimide is achieved by illumination of the polar fulgimide molecule with 530 nm light which excites it to an upper electronic state that is followed by interconversion to the ground state non-polar form. If such a compound maintains its polar-to-non-polar switching characteristics over prolonged time periods in living cells, it could be used as an intracellular chemical/molecular sensor. Localized changes such as pH, viscosity, and sub-cellular chemistry are very difficult to measure in live cells using existing methods.

The experimental observations (Fig. 5) demonstrate that this compound (1) does enter the live cell, (2) appears to associate with internal membranous organelles, especially the mitochondria, and (3) does not enter the interphase nucleus (at least in it's fluorescent polar state). The compound does not seem to bind to chromosomes in mitotic cells, and it is generally excluded from the mitotic spindle (Fig. 5(e)–(g)).

Within the live cell, the molecule was found to emit at 630 nm after excitation at 550 nm. This demonstrates that the polar "on" form of the molecule is present in live cells. Additionally, as the fluorescing molecules are converted to the non-polar "off" form by illumination with 500 nm light, the fluorescence intensity decreases proportionally (Fig. 5(a)–(d)). The molecule can then

Table 1
Fulgimide spectroscopic properties and fluorescence and reaction quantum yields

Solvent	Absorption, λ_{max} (nm)		Fluorescence		Reaction quantum yield	
	Open form	Close form	λ_{max} (nm)	Quantum yield	Coloration	Bleaching
Cyclohexane	398	504	575	0.009	0.25	0.23
Toluene	401	517	604	0.014	0.17	0.24
$C_2H_4Cl_2$	401	528	635	0.054	0.14	0.14
CH_3CN	395	527	650	0.051	0.13	0.064
CH_3OH	396	532	674	0.005	0.17	0.003
H_2O	397	565	715	0.0008	0.21	<0.003

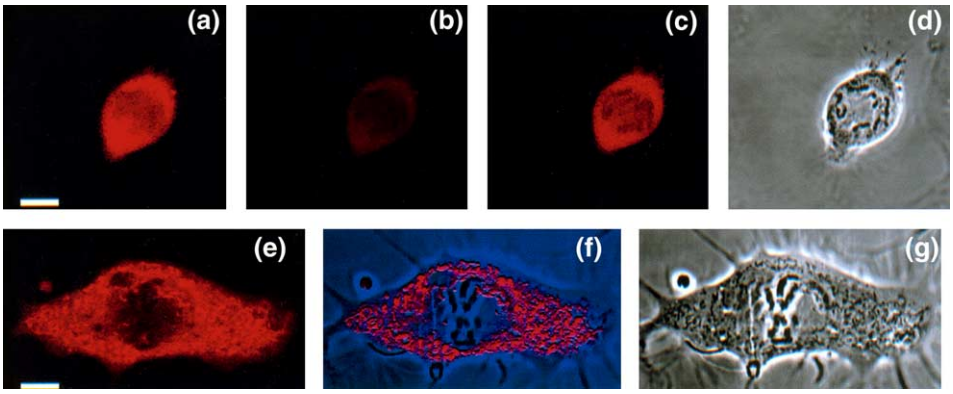

Fig. 5. (a)–(d): Fluorescence "on"–"off" cycle of fulgimide in a living cell. (a) Polar form; (b) polar form concentration decreased and non-polar form increased; (c) polar concentration increased; (d) phase contrast image of mitotic anaphase cell prior to exposure to fulgimide. Backgrounds of (a)–(c) have been calibrated to a standard. Images use false-color software. Scale bar = 12 μm. (e)–(g): Subcellular localization of fulgimide. (e) Fluorescent image of mitotic metaphase cell with emission at 630 nm; (f) same cell imaged simultaneously by phase contrast and fluorescence; note dark chromosomes in the center of the cell; (g) phase contrast image of the same cell; the discrete filamentous mitochondria are clearly visible and can be matched in the phase and fluorescence images (Figs. 5(g) and (f)). Excitation was at 550 nm and emission was at 630 nm. Images use gray-scale false-color software. Scale bar = 10 μm.

be driven back into its polar "on" state by exposure to 400 nm light and re-excited to the non-polar form with 500 nm light. The cycling, polar to non-polar form, was repeated more than seven times in the same cell over a 53 min time period. The repeated on–off fluorescence switching demonstrates that the photochromic properties of this molecule persist within the live cell. To our knowledge, fluorescence bi-stable switching in live cells has not been described previously. Such a system provides the additional capability of simultaneously using several different sensors that may fluoresce even in the same region without interference from each other because all but one sensor can be switched off at any time. Such sensors could provide a means for monitoring several cellular properties and reactions simultaneously.

Another significant property of this system is the fact that the polarity dependent fluorescence intensity and the polarity of the molecule may be switched on and off by simply illuminating the molecule with either 530 or 400 nm light. This bi-stable switching should permit the detection of polar and non-polar species within specific regions of the live cell.

The system described in this paper differs in concept and operation from the two-photon systems used for conventional multiphoton imaging [7], and ablation/manipulation of subcellular structures [8,9]. The bipolar system described here is not dependent on a high photon flux generated by short-pulse lasers because the fluorescence may be induced by one photon processes or stepwise two photon processes which have much higher absorption cross-sections than the two photon virtual transition processes. This method is non-destructive to either the cell or its organelles because orders of magnitude lower photon pulse intensities are utilized.

It should be possible to develop "on–off" bi-polar biosensor molecules that are specific to particular environmental, physical and chemical properties, and may be composed of specific groups that are targeted to particular structures and reactive centers in cells and tissues. In addition, the polar structure of these molecules renders them suitable for attachment to polar groups within the live cell, thus affording the opportunity to monitor local changes that reflect the changing chemical and physiological states of the cell. Using these specific types of molecular bi-polar probes it should be possible to detect and monitor local chemical changes that effect charge transfer and polarity within the live cell. Detection may be achieved within the timeframe of the polar-to-non-polar conversion ($\sim 5 \times 10^{11}$ s^{-1}) and the fluorescence lifetime (5×10^{-8} s). Owing to the fact that fluorescence is emitted only when the structure of the molecule and its environment are polar it becomes a means for the identification of polar moieties in the cell. Conversely, the absence of fluorescence is indicative of a non-polar environment. For example, because the fluorescence intensity increases with the polarity of the environment, it should be possible to measure intracellular pH non-invasively. Preliminary experiments suggest that a similar effect occurs as a function of viscosity. The use of optically driven bi-polar molecules to measure the chemical and physical state of cells in real time should prove very useful in biology and applied biotechnology.

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