

Revisiting the photophysics and photochemistry of methylene violet (MV)



F. Ronzani^a, A. Trivella^b, P. Bordat^a, S. Blanc^a, S. Lacombe^{a,*}

^a IPREM UMR CNRS 5254, Université de Pau et Pays de l'Adour, Hélioparc, 2 avenue du Président Angot, 64053 Pau cedex 9, France

^b ICCF, UMR CNRS 6296, Université Blaise Pascal, Campus des Cézeaux, 24 avenue des Landais BP 80026, 63171 Aubière Cedex, France

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ABSTRACT

Methylene violet (**MV**) is known for its photosensitizing properties for singlet oxygen ($^1\text{O}_2$) generation upon visible-light irradiation and various examples of its potential use in photodynamic inactivation of microorganisms and for photomedicinal purposes were reported. Notwithstanding its good photosensitization properties, there is a loss of clinical tests in the recent literature, probably related to the incomplete information concerning the photophysics of this dye and the effects of the medium on its properties. We thus studied both experimentally and numerically the solvatochromic effects and the presence of acids on the absorption and fluorescence spectra of **MV**. In chloroform, the highly favoured and reversible formation of a mono-protonated derivative of **MV**, **MVH**⁺, was clearly demonstrated experimentally and supported by ab-initio calculations. In acetonitrile, the more complex experimental absorption bands can be explained by several assumptions including a thermodynamic equilibrium between **MVH**⁺ and **MVH**₂²⁺ or the formation of a possibly oblique dimer (**MVH**⁺)₂ induced by the formation of intermolecular hydrogen bonds. All these data point out to the high sensitivity of **MV** to intermolecular hydrogen bonding and to protonation, inducing a significant influence of the chemical environment on the photosensitization mechanisms of **MV**: from transient spectroscopy and photochemical experiments, an electron transfer side-mechanism was shown to occur only in aprotic solvents, together with a very general and efficient singlet oxygen production whatever the solvent. **MV** thus represents a very good singlet oxygen sensitizer even though the influence of the chemical environment should be carefully considered for any application. Moreover, **MV** might be a sensitive probe for the detection of acids in organic non-protic solvents.

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1. Introduction

Phenothiazines (PTZ) represent a very interesting class of molecules, with suitable properties for widespread applications. Methylene blue (**MB**⁺) is probably the most well-known commercially available PTZ photosensitizer (PS) [1,2]. **MB**⁺ is used as medicinal and textile stain, antioxidant and antiseptic, diagnostic agent, antidote for cyanide and nitrate, drug for malaria, Alzheimer and other neurodegenerative diseases [2]. **MB**⁺ efficiently produces singlet oxygen [3], it is non-toxic and it absorbs light at 650 nm, which makes it suitable for photodynamic inactivation (PDI) of viruses and bacteria in blood fractions, notably for plasma sterilization [4–12], and for photodynamic therapy (PDT). Among the several **MB**⁺ derivatives, which can be prepared for tuning its

photochemical/photophysical properties, the neutral dye methylene violet (**MV**, Chart 1), which has been studied for potential applications in biology, showed interesting preliminary results. **MV** presents a higher activity than **MB**⁺ for killing intracellular viruses thanks to a much easier penetration in cells due to the absence of the positive charge. Nonetheless, **MV** is more easily trapped by lipoproteins than **MB**⁺, which can limit its activity in biological media [8,13]. **MV** can form singlet oxygen by energy transfer with relatively high efficiency and has a particular affinity towards DNA which makes it a good PS for photodynamic therapy [14,15]. Some graftable **MV** derivatives have been prepared, and enhanced singlet oxygen production was achieved by adding iodine atoms on its structure (heavy atom effect) [8,16]. Previous spectroscopic and photophysical data [17] on **MV** showed a strong dependence on the solvent. For a better understanding of **MV** properties, the solvatochromic behaviour of **MV**, its singlet oxygen quantum yield, transient absorption spectra and photochemical properties were investigated in some selected solvents.

* Corresponding author. Tel.: +335 59407579.

E-mail address: sylvie.lacombe@univ-pau.fr (S. Lacombe).

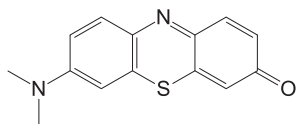


Chart 1. Chemical structure of methylene violet (MV).

On the basis of our experimental results and calculations on this compound, we could highlight an impressive sensitivity to the presence of H^+ , which makes of **MV** a very good photosensitizer and optical probe for the detection of H^+ ions.

2. Experimental part and calculation methods

Methylene violet (Bernthsen, **MV**, dye content > 72%), methylene blue (certified by Biological Stain Commission, **MB**⁺, dye content > 80%), rubrene (5,6,11,12-tetraphenyl-naphthalene, 99%), dibutylsulfide (Bu_2S , 97%) and methanesulfonic acid (CH_3SO_3H , anhydrous) were purchased from Sigma–Aldrich. All spectrograde solvents, except for triethylamine (Et_3N , Acros Organics), were purchased from Sigma–Aldrich: *n*-heptane, carbon tetrachloride (CCl_4), diethyl ether (Et_2O), toluene, dioxane, ethyl acetate (AcEt), tetrahydrofuran (THF), acetone, acetonitrile (ACN), dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), pyridine, dimethylformamide (DMF), dimethylsulfoxide (DMSO), ethanol (EtOH) and methanol (MeOH). All chemicals and solvents were used as received. The purity of **MV** was declared by the suppliers without further specifications. It was not purified to be consistent with previous published works [2,18].

UV–visible absorption spectra were recorded with a double beam Cary 5000 spectrophotometer in steps of 0.5 nm using a 1 cm quartz optical cell (Hellma). Corrected steady-state emission spectra were measured using a photon counting Edinburgh FLS920 fluorescence spectrometer equipped with a Xe lamp. Time-resolved fluorescence experiments were carried out by using a nano-second flash-lamp (nF900) and time-correlated single photon counting (TCSPC). The lifetime data were analyzed with the reconvolution fit (including instrument response) of the Edinburgh software. The concentrations of all compounds were adjusted to give an absorbance around 0.1 at the absorption maximum in a 1 cm fluorescence quartz optical cell (Hellma). For the detection of transient species a Nd:YAG laser (GCR 130-1, pulse width 9 ns, 266 nm or 355 nm) was used for sample irradiation. The monitoring system consisted of a 150 W pulsed xenon arc lamp, a R928 photomultiplier and a 05–109 Spectra Kinetics Applied Photophysics monochromator. Signals were digitized by a HP54522A oscilloscope. The samples were irradiated in fluorescence quartz cells (1 cm, Hellma); no influence of the excitation wavelength on the transient signals and spectra was noticed. The laser pulse energy (P) was measured using a joulemeter Ophir Optronics Ltd. Each data was the average of 5 measurements. The absorbance at pulse end was measured for various P values to check the linearity of the dependence of A on P and the monophotonic formation of the transient.

The spectrophotometric data recorded at various $[H^+]$ values in ACN or $CHCl_3$ were processed with Specfit program [19], which adjusts the stability constants and the corresponding absorptivities of the species formed at equilibrium. Specfit uses factor analysis to reduce the absorbance matrix and to extract the eigenvalues prior to the multiwavelength least squares fitting of non-linear parameters (equilibrium constants) of the reduced data set according to a modified Marquardt–Levenberg algorithm. Linear parameters (molar absorptivities) are computed internally using Advanced Newton–Raphson algorithm for equilibrium speciation calculations. **MV** was considered to form a protonated species, **MVH**⁺ and

the protonation constant (Eq. (1)), β_1 , may then be expressed as follows:

$$MV + H^+ \rightleftharpoons MVH^+ \quad \beta_1 = \frac{[MVH^+]}{[MV][H^+]} \quad (1)$$

As each species is characterized by its own spectrum for UV/vis experiments, the Beer–Lambert law relates the absorbance A_λ for one cm path length as:

$$A_\lambda = [MV] \times \varepsilon_{\lambda, MV} + [MVH^+] \times \varepsilon_{\lambda, MVH^+} \quad (2)$$

where ε_λ is the molar absorptivity of the species ($M^{-1} cm^{-1}$) measured at the wavelength λ .

The procedures for the detection of the transient species and the determination of the quantum yield of singlet oxygen production (Φ_Δ), via both direct and indirect detection, have been described elsewhere [18].

Preparative batch reactions were carried out to identify the products of Bu_2S photooxidation. Air-equilibrated ACN solutions (50 mL) containing **MV** ($1.0 \times 10^{-4} M$) and Bu_2S ($5.0 \times 10^{-3} M$) were irradiated in a Rayonet® reactor equipped with $16 \times 8 W$ lamps with $\lambda_{max} = 575 nm$ (RPR-5750 Å). The reaction was checked by measuring the solution absorbance with a quartz cell with optical path of 0.01 cm. The reactions were performed until no more variations in the UV–vis spectrum of **MV** were noticed (approx. 20 min). The reaction mixture was analyzed by 1H NMR on a Bruker Advance 400 NMR spectrometer in $CDCl_3$ at 25 °C using a 5 mm inverse broadband probe, at 400.13 Hz, and calibrated with respect to the solvent signal.

The photobleaching and reactivity of **MV** in solution were studied with the following set-up. 3 mL solutions (0.8 to $1.2 \times 10^{-5} M$, fluorescence quartz cells) containing the PS, magnetically stirred at 25 °C, were irradiated with a 200 W Xe–Hg Lamp; a Cornerston 260 motorized monochromator, inserted between the light source and the analytical equipment, was set to $\lambda = 0$ (white light). A Perkin Elmer double beam, double monochromator Lambda850 UV–vis spectrophotometer was used for the analysis. The quartz cells were positioned and irradiated directly on the support of the spectrophotometer (light source perpendicular to the analytical beam). The amount of bleached PS was evaluated from the decrease of the absorbance maximum, in terms of $M_{MV} s^{-1}$. From the irradiance spectrum of the light source and the absorption spectra of **MV**, the photon flux absorbed by the PS (P_a , Einsteins $L^{-1} s^{-1}$) could be obtained by means of Eq. (3) [20]:

$$\sum_{\lambda} P_{a,\lambda} = \sum_{\lambda} P_{0,\lambda} (1 - 10^{-A_\lambda}) \quad (3)$$

P_0 (emitted photon flux) was measured with an International Light ILT900 spectroradiometer. By dividing the decrease of PS concentration by P_a , the quantum yield of photobleaching ($\phi_{bleaching}$) was obtained ($mol_{MV} Einsteins^{-1}$). In the presence of Bu_2S ($15 \mu L$ of sulfide were added to the solution ($[Bu_2S] = 0.028 M$, large excess)), the photobleaching could not be estimated owing to the spectral changes induced by the formation of acids (see Section 5).

Quantum chemical calculations have been carried out using Gaussian 09 [21]. Standard calculations for structural optimization have been performed at the B3LYP/cc-pVTZ DFT level and electronic transitions have been calculated by TDDFT (time-dependent density functional theory) at the same level for consistency. The solvent is taken into account with the PCM model implemented in Gaussian 09 [22]. Vibrational frequencies have been checked to ensure that optimized conformations correspond to minima. Moreover, extra-calculations have been performed with CAM-B3LYP and WB97XD DFT functionals and extended bases as 6–31++G(3df,3dp) and AUG-cc-pVTZ. These extra-calculations confirm the results found with the previous modest strategy (B3LYP/cc-pVTZ). So, in the following discussion, interpretation is based on the results determined at the

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