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Characteristics of the excited states of Nitrofurantoin, an anti-inflammatory and photoactive nitrofuran derivative

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ABSTRACT

Spectral characteristics of bio and photoactive nitrofuran derivative drug [*N*-(5-nitro-2-furfurylidene)−1-aminohydantoin], (Nitrofurantoin, NFT) were investigated using optical absorption and fluorescence spectroscopies, both in "steady-state" and time resolved forms. The irradiation of the NFT in the UV region in aqueous solution induces the fluorescence with quantum yield $\Phi_{fl} \approx 0.03\%$ and lifetime $\tau_{fl} \approx 1$ ns with these values being practically independent of the NFT protonation state. The NFT transient absorption spectrum and lifetime (τ_T) depend on the NFT protonation state with τ_T being 0.04 μs for mono-protonated NFT state, 0.22 μs for its non-protonated one, and with quantum yield, $\Phi_T \approx 0.0001\%$ which is practically independent of its protonation state. The quenching of this transient by molecular oxygen accompanied by simultaneous singlet oxygen formation ($^1O_2$) allows associate this specie with the NFT molecules in the triplet state being the efficiency of this process dependent of NFT protonation state, $\eta_{pH10} \approx 0.53$ and $\eta_{pH2.5} \approx 0.07$. The extremely low quantum yields, Φ_{fl} and Φ_T, and short τ_{fl} and τ_T are, probably, associated with alternative ways of the dissipation of the NFT excited state energy: the NFT photoisomerization and $NO^{\bullet}$ photorelease. The synergic action of two highly reactive species such as $^1O_2$ and $NO^{\bullet}$ in biological medium is extremely valuable for applications in photochemotherapy, thus making NFT a potential candidate for further studies *in vitro* and *in vivo*.

1. Introduction

Nitrofuran derivatives (ND) are bioactive compounds widely applied in medicine and used as anti-parasitic additive in animal alimentation. Among them, Nitrofurantoin [*N*-(5-nitro-2-furfurylidene)−1-aminohydantoin] (NFT, Fig. 1) is a drug applied in clinics as an anti-inflammatory, antiseptic and antibiotic agent, especially to treat and prevent urinary tract infections [1–5]. The toxicity of NFT has been documented against several types of Gram-positive and Gram-negative bacteria [6]. Because of its broad-spectrum antibiotic activity, NFT has been used as an additive in animal feed to prevent or treat gastrointestinal infections [7–9]. NFT cytotoxicity was related with the generation of reactive oxygen species (ROS) and $NO^{\bullet}$ via its nitro-group enzymatic reduction [10–16].

In terms of photophysical properties, NFT possesses intensive absorption between 320 and 420 nm [17,18]. Moreover, it was shown that exciting the NFT molecule with a short pulse laser (~150 fs) at 780 nm results in an effective absorption of two photons (2PA) by NFT ground state [19], presenting an alternative way for NFT photoexcitation. The 2PA in the near-infrared range has the advantages of deeper penetration in tissue and lower light scattering losses [20]. This feature combined with the nitric oxide photorelease, which is a common characteristic of many nitrofuran derivatives, is important in the context of photochemotherapy and two-photon photodynamic therapy [21,22], where the combination of light in the region between 600 nm and 800 nm (known as "phototherapeutic window") associated with photoactive drugs has been used to treat several diseases, including cancer.

It was formerly demonstrated that some ND possess photoactivity toward malignant cells [23,24]. This effect was associated with nitric oxide ($NO^{\bullet}$) release at ND photoexcitation, as well as with generation of reactive oxygen species (ROS), such as singlet oxygen and superoxide anion which are generated via energy and/or electron transfer from the ND triplet state to molecular oxygen

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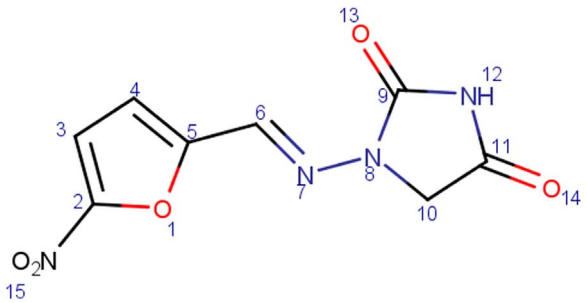

Fig. 1. [*N*-(5-nitro-2-furfurylidene) – 1-aminohydantoin] (Nitrofurantoin, NFT) molecular structure.

[25,26]. Other effect of the association of ND and UV irradiation on living organisms includes photoallergic reactions, as previously observed for NFT, a fact that confirms the potential of this drug in being photoactivated *in vivo* [27,28].

A study on the photochemistry of some ND, including NFT, has shown that both direct and indirect photolysis (via reaction with singlet oxygen) induces the NFT degradation [18]. Nitrofuraldehyde (NFA), a photolabile molecule, was reported to be the major photodegradation product of NFT and its photolysis induces the release of nitric oxide ($NO^{\bullet}$) [18,27]. Another possible photoprocess for NFT is the occurrence of photochemical *trans-cis* photoisomerization due to the rotation of its imino group (C=N) at position C6-N7 (Fig. 1). As shown previously, this process can lead to the energy loss of the molecule at excited states S_1^* or T_1^* [29–32], adding a new pathway for its energy dissipation. Moreover, changes on the environment characteristics, such as pH, polarity, and ionic strength, can affect the molecule energetic characteristics and as a consequence, influence its applications [33,34]. Nonetheless, as of now, there are no available data on the NFT excited state characteristics, such as fluorescence and triplet state spectra, lifetimes and quantum yield.

In this work we report for the first time on the spectral, energetic, and time characteristics of the both NFT singlet and triplet states. Both in its mono and non-protonated forms, using optical absorption and fluorescence spectroscopies in "steady-state" and time resolved forms. Furthermore, a new approach on the participation of the NFT triplet state in the generation of reactive species of oxygen and $NO^{\bullet}$ photorelease is presented.

2. Material and methods

NFT was obtained from Sigma-Aldrich Co. All the samples were prepared in phosphate buffer (pH 7.3; 7.5 mM) using Milli-Q® quality water. Due to the presence of nitrogen atoms in its structure, NFT can exist in a mono-protonated or non-protonated state with the pK_a point being close to neutral pH [17]. Therefore, the NFT study was performed at pH 2.5 (mono-protonated NFT form), pH 7.3 (close to the pK_a point), and pH 10.0 (non-protonated NFT form). The pH values were adjusted by adding aliquots of NaOH or HCl stock solutions to the initial NFT solutions.

The NFT concentration was controlled spectrophotometrically at pH 2.5, using molar absorption coefficient $\varepsilon_{366nm} = 1.6 \times 10^4\ M^{-1}cm^{-1}$ [17]. Linear combinations of the absorption and fluorescence spectra at pHs 2.5 and 10.0 were performed to verify the contribution of the protonated and non-protonated forms on the spectral characteristics of NFT at the physiological pH. This was performed using the software package Origin 8.5, and the result was compared with the experimental data. Optical absorption measurements were performed using Beckman Coulter DU640 and Amersham-Pharmacia 2100 Ultraspec spectrophotometers. All the experiments were run at room temperature (297 K).

2.1. Fluorescence measurements

Fluorescence measurements were made by dissolving the stock solution in phosphate buffer to final 20 μM NFT concentration, the absorbance value at the maximum being ≈ 0.3. The NFT fluorescence spectra were monitored by a Hitachi F7000 spectrofluorometer using a quartz cell with 1 cm path length with the fluorescence being excited at 377 nm. The NFT fluorescence quantum yield (Φ_{fl}) was determined via the relative method [35], using the anthracene solution in ethanol ($\Phi_{fl0} = 0.27$) as a standard [36].

The fluorescence decay curves were obtained using the time-correlated single-photon counting method. The excitation source was a Tsunami 3950 Spectra Physics titanium-sapphire laser, pumped by a Millenia X Spectra Physics solid-state laser. The pulse repetition frequency was 8 MHz, obtained using a 3980 Spectra Physics pulse picker. The laser was tuned so that a third harmonic generator BBO crystal (GWN-23PL Spectra Physics) gave the 280 nm excitation pulses, which were directed to an Edinburgh FL900 spectrometer. The spectrometer was set in L-format configuration. The emission was collected at 490 nm for NFT in ethanol, 515 and 580 nm for NFT at pH 2.5 and 10.0 respectively. The emitted photons were detected by a cooled Hamamatsu R3809U microchannel plate photomultiplier. The FWHM of the instrument response function was typically 100 ps and the time resolution was 12 ps per channel. Software provided by Edinburgh Instruments was used to analyze the individual decays.

2.2. Flash-photolysis experiments

The NFT transient was produced via irradiation of its solutions in a standard quartz cuvette (1 cm) with the 5 ns and 20 mJ pulses of the third harmonic (355 nm) of a Nd:YAG Quantel Brilliant B laser system. The transient absorption spectra were monitored in the spectral region 400 nm ≤ λ ≤ 700 nm. To study the effect of the molecular oxygen on the transient lifetimes, the samples were deaerated by purging with nitrogen or oxygenated by bubbling oxygen through the solution for 20 min.

The NFT triplet quantum yield (Φ_T) and triplet excited state extinction coefficient (ε_T) in the mono-protonated and non-protonated forms were calculated using the Singlet Depletion (SD) method [37]. For details, see Supplementary Material - SM1.

2.3. Singlet oxygen detection

The singlet oxygen formation was monitored via its phosphorescence emission, which appeared after the excitation of oxygenated NFT solution with the 30 mJ and 8 ns pulses of the third harmonic (355 nm) of a Nd:YAG Surelite I-10 laser. In view to exclude any residual fluorescence signal from the sample at λ < 800 nm the emitted light was passed through a silicon cut-off filter associated with converging lenses and a monochromator (Bentham Instruments, M300) before detection by NIR-PMT (Hamamatsu Co. R928P). Data acquisition was performed by a digital oscilloscope (Tektronix TDS340A). The phosphorescence lifetime of $^1O_2$ was monitored at 1270 nm using a germanium detector (North Coast Scientific Co, 823 A) cooled by liquid nitrogen. The experiments were performed at room temperature (297 K), in an air-saturated aqueous solution.

3. Results and discussion

3.1. Absorption spectroscopy

The NFT absorption spectra in PBS are presented in the Fig. 2.