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Spectroscopic characterization and aggregation of azine compounds in different media

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ABSTRACT

In this study, we have reported for the first time a complete experimental investigation on the compound Neutral Red and the new monobrominated derivative in different media as a function of the concentration. These compounds belong to the quinone-imine class of dyes and have good potential to be applied as photosensitizers in Photodynamic Therapy.

Although the aggregation of Neutral Red has been reported by several authors, this has not been thoroughly evaluated due to spectral changes occurring depending on the solvent and concentration of dye.

We investigated and compared the effects of aggregation properties in water, ethanol, water–ethanol mixtures, water-polyethylene glycol 400 (75:25 v/v) and *N,N*-dimethylformamide.

An analysis of the changes in the absorption spectra with respect to the solvent as a function of dye concentration together with theoretical calculations confirmed the monomeric species and the formation of different types of aggregates in this class of compounds.

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1. Introduction

The use of dyes in the biomedical area has seen a remarkable growth in research interest in recent years and is probably the most rapidly expanding area of dye chemistry at the present time. The potential biomedical applications of dyes include the general areas of bioanalysis, medical diagnostics and the therapeutic treatment of disease. There are several other applications that might be considered within biomedicine, such as histology, fluorescent biolabelling and fluorescent bioprobes [1].

Photodynamic therapy (PDT) is a therapeutic modality not only applicable for the treatment of superficially localized tumors, but is also associated with the antiviral and bactericidal properties of dyes [2–4]. PDT can be defined as the systemical, local or topical administration of a nontoxic drug or dye known as photosensitizer (PS), which acquires the desired activity only when it is excited by light of an appropriate wavelength. The excitation of PS results in the production of sufficient reactive oxygen species (ROS) to produce a cytotoxic effect [5,6].

Neutral Red (NR) is a phenazine based dye very useful as a biological probe and has been widely utilized for various purposes in many biological systems, among which is the staining of cellular particles and the intracellular pH indicator. In addition, NR has been used as a PS in PDT and good results have been obtained [7,8].

It is well known that the ionic dyes tend to aggregate in diluted solutions, leading to dimer formation, and sometimes to even higher order aggregates. In such cases the molecular nature of the dyes is strongly affected by ionic strength, temperature, presence of organic solvents, dye concentration and structure. Aggregation may rise with an increase of dye concentration or ionic strength, but will decrease with rising temperature or the addition of organic solvents [9,10].

It has been reported that the formation of aggregate in solution produces significant changes in the absorption and emission spectra [11], thus impairing the photochemical response [12] and reducing the lifetimes of the excited state, most probably as a result of enhanced radiationless excited state dissipation and lowering of the quantum yields of the excited states and of the $^1O_2$ generation [13].

On the other hand, one strategy to increase the efficiency of a spin-forbidden electronic transition from a singlet to a triplet state (intersystem crossing) and regulate the quantity of $^1O_2$ generation is the introduction of heavy atoms into a molecule [14].

Based on the above-mentioned research, we addressed the synthesis of new phenazine cationic dyes. As the chemical structure of NR permits the addition of substituents by aromatic electrophilic substitution using halogens [15], we synthesized Neutral Red monobrominated (NRBr) and Neutral Red monoiodinated (NRI), with their molecular structures being shown in Scheme 1.

In addition, we have examined the influence of dye concentration and different solvents on the aggregate formation, since the

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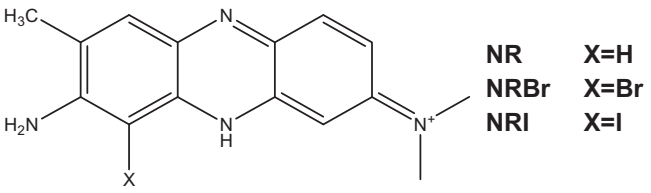

Scheme 1. Molecular structure of azine dyes used in this study.

formation of aggregates modifies the photophysical and photochemical properties of a dye, and therefore affects its ability to act as a PS [16,17]. We have chosen for this study different media such as ethanol and polyethylene glycol (PEG) 400 because they are FDA approved cosolvents for the parenteral dosage form [18,19]. Also, we investigated phenazine dyes in *N,N*-dimethylformamide (DMF) as it is known to be a good monomerizing solvent [20]. Absorption UV–vis spectroscopy was used because it is one of the most suitable methods for quantitatively studying the aggregation properties of dyes as a function of concentration [9,21].

Finally, the experimental data were compared with theoretical calculations and we confirmed the monomeric species of the dyes.

2. Experimental methods

2.1. Materials/chemicals

Neutral red hydrochloride (3-amino-7-dimethylamino-2-methyl phenazine hydrocholoride) was purchased from Sigma Chemical Co. (St. Louis, MO) (Fluka Chemicals) and used as received. We confirmed by reversed-phase high-performance liquid chromatography (RP-HPLC) analysis that its purity was >98%. All the solvents used were of pro-analysis and HPLC grade (Cicarelli, Sintorgan, Anedra). All chemicals were obtained commercially and were of the highest purity form. Solutions of bromo were prepared with molecular bromo (Carlo Erba) with a purity >99.9%.

Aqueous solutions were prepared using ultrapure water from the Milli-Q® water purification system (Millipore Corporation, USA). Deuterodimethylsulfoxide (DMSO-d_6) was purchased from Sigma Chemical Co. (St. Louis, MO) and had a purity >99.8%.

TLC were carried out using on 250 μm silica gel plates (E. Merck) with chloroform: methanol (92.5: 7.5 v:v) as the solvent system and were visualized with UV light at λ = 254 nm.

2.2. Instrumentation

Absorption spectra were carried out at room temperature with an Evolution 300 spectrophotometer using 1 cm path length quartz cells. Spectral curves were recorded in aqueous solution, ethanol, water:ethanol mixtures, DMF and water:PEG 400 mixture, from 10^{-7} to 10^{-3} M between 200 and 700 nm. All experiments were carried out at least twice with consistent results. Stock solutions of dyes were prepared in duplicate and then diluted appropriately with the same solvent.

NMR spectra were recorded on an RMN Bruker AVANCE II 400 Nuclear Instrument using DMSO-d_6 where the solvent and δ values are given in ppm.

Mass spectra were determined in solid state on a Cuadrupolar Finnigan 3300 F-100 mass spectrophotometer by electron ionization (EI-MS) at 70 eV. Electrospray ionization mass spectra (ESI-MS) were recorded on a Varian 1200L triple-quadrupole LC–MS spectrometer. ESI high-resolution mass spectra (HSMS) were recorded on a Bruker Micro QTOF II spectrometer equipped with an ESI source. Samples were introduced dissolved in methanol HPLC grade at a final concentration of 10 μg/ml.

FT-IR spectra were recorded in the range of 4000–600 cm^{-1} on a Nicolet 5SXC FT-IR spectrometer as KBr pellets.

RP-HPLC was carried out using an Agilent 1100 Series HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler, a column thermostat and a UV–vis detector set at 280 nm and 540 nm. A reversed-phase C18 (Supelco®) column (4.6 × 250 mm, 5 μm) with guard column and flow rate of 1.0 mL/min were used. The mobile-phase was prepared with methanol HPLC grade and an aqueous solution of trimethylammonium phosphate 83 mM (70:30 v/v) using Milli-Q® water, and the pH of the mobile-phase was 4.76.

2.3. Computational details

Theoretical calculations were performed with the GAUSSIAN 03 suite of programs. All geometry optimizations were computed using the hybrid density functional B3LYP and the standard 6–31 + G(d) basis set. The stationary points were located with the Berny algorithm using redundant internal coordinates. Analytical Hessians were computed to determine the nature of stationary points (one and zero imaginary frequencies for transition states and minima, respectively) and to calculate the unscaled zero-point energies (ZPEs) as well as the thermal corrections and entropy effects using the standard statistical-mechanics relationships for an ideal gas. Nonspecific solvent effects were described by using the self-consistent reaction field (SCRF) approach in Tomasi's formalism. Single point PCM [B3LYP/6-31 + G(d)] calculations were performed to estimate the UV-spectrum profile of the dyes.

2.4. Synthesis: general aspects

All the solutions were freshly prepared before being used. The reaction progress and purity of the compounds were monitored by RP-HPLC and TLC. The reaction solvent was removed under reduced pressure and the solid residue was analyzed by RP-HPLC. During all the experiments, the reaction mixture was stirred and the round-bottom flask was protected from the light.

2.5. Characterization

2.5.1. Neutral Red (NR)

IR (KBr, cm^{-1}): υ 1622, 1525, 1495 (C=C) 1194, 1009 (C–N) 880, 804 (oop C–H). t_R RP-HPLC (n = 7, min.): 4.72 ± 0.07. R_f TLC (n = 7): 0.20 ± 0.05. ^{1}H-RMN (DMSO-d_6, 400 MHz): δ (ppm) = 2.320 (s,3H), 3.219 (s, 6H), 6.764 (d,1H, J_{meta} = 2.4807), 6.894 (s,1H), 7.465 (dd,1H, J_{orto} = 9.689, J_{meta} = 2.481), 7.735 (s,1H), 7.867 (d,1H, J_{orto} = 9.689). ^{13}C-RMN (DMSO-d_6, 400 MHz): δ (ppm) = 18.181, 40.373, 92.338, 93.047, 118.979, 130.961, 131.183, 131.750, 133.941, 134.192, 135.283, 137.005, 154.127, 156.633. EI-MS (70 eV, m/z): 252 $[M]^+$, 209, 181. ESI-MS (m/z): 253.1 $[MH]^+$, MS-MS (m/z): 237.0, 210.0. HRMS (ESI): Calcd for $C_{15}H_{17}N_4$ $[MH]^+$: 253.3224, found: 253.1466.

2.5.2. Neutral Red monobrominated (NRBr)

In order to prepare NRBr, 10.0 mg (3.46 × 10^{-2} mmol) of commercial dye NR were dissolved in 10 mL of glacial acetic acid and then a solution of bromine in glacial acetic acid (3.46 × 10^{-2} mmol, 5 mL) was added dropwise with constant stirring. The reaction solvent was removed under reduced pressure at 55–60 °C, and 10 mL of ethanol was added to dissolve the reaction product which was then filtered. The ethanolic media was removed at 40 °C and the crude product did not need any further purification. The best reaction conditions were radio dye:bromine 1:1 at room temperature for 30 min (purity >98% by RP-HPLC).

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