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Photophysical properties of pyrene in interaction with the surface of melanin particles

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Abstract

Melanins perform their biological activity (photoprotection and light enhanced chemical reactivity) under the form of porous aggregates on which ions and neutral molecules can be adsorbed. For this reason, the photochemistry of natural and synthetic melanins must be investigated in the framework of the physico-chemical theory of the heterogeneous reactions and a detailed knowledge of the surface properties, is therefore, necessary. In this work, some surface characteristics of melanin particles have been investigated taking advantage of the photophysical behaviour of pyrene, a dye widely used in studies of the interface properties of micelles and colloidal semiconductors. Our fluorescence study has allowed to obtain valuable informations regarding the micro-environmental polarity of the melanin surface (that influences the vibronic structure of the emission spectra), the excimer formation, the lifetimes of the emissions and the kinetics of quenching by Cu^{2+}.

Keywords: Melanin biophysics; Pyrene fluorescence; Surface photophysics; Fluorescence quenching

1. Introduction

Surface photochemistry in such systems as micelles, micro-emulsions and colloidal semiconductors is an important field of research that has demonstrated the unique properties of interfaces to drive selected chemical reactions. In particular, charge-transfer reactions on the surface of colloidal semiconductors have been widely investigated due to their technological interest [1]. The observation in some living systems of surface reactions and photoreactions suggests that biophysics can fruitfully adopt ideas and models developed for solid state photophysics.

Melanins are widely diffuse natural macromolecules showing structural properties unusual in biology. In fact they perform their physiological role under the form of particles [2], in which photoinduced reactions with molecules interacting with their surfaces are observed [3,4]. Such heterogeneous reactions take advantage from the porous nature of the particles, that are aggregates of smaller particles [2,5] and show a fractal structure [6–8]. These features request a re-examination of the kinetics of surface adsorption processes, that can involve metal ions and neutral molecules (in particular, drugs), and a more detailed knowledge of the electronic properties of the natural aggregates and of the mobility of the adsorbed species. Many experiments with drugs indicate that the adsorption is physical in nature and that the chemical properties of the molecules are not affected by adsorption on the melanin particle [9].

Photophysical methods developed for micellar systems which enable to monitor the environments of molecules adsorbed on surfaces or at active interfaces can in principle be useful also for melanins. For example, ease of excimer formation and quenching of excited states of fluorescent probes can give informations on the state(s) of the adsorbates, measurements of fluorescence polarisation put in evidence the local degree of rigidity of bound molecules, while the fluorescence spectra can yield informations about the polarity of molecules at the sites of adsorption.

These techniques have been successfully used to study solid surfaces in inorganic materials such as aluminium oxide, silica gel, titanium dioxide and iron oxide [10–12]. This paper describes the use of the well known fluorescent probe,

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pyrene, to study the biophysical problem of the adsorption of neutral molecules on the surface of Sepia melanin particles suspended in water. Thanks to the excited state properties of pyrene, valuable informations can be obtained on the environment in which this molecule lies.

2. Materials and methods

Seventy-five milligrams of Sepia melanin (SIGMA) were suspended in 100 ml HEPES buffer (10 mM, pH 7.4). This rather concentrated turbid suspension was used in binding experiments, while for optical spectra, a 1/10 dilution was necessary. Pyrene was dissolved in EtOH as a 5 mM stock solution.

Samples were prepared in the following way: to a fixed volume of melanin suspension, pyrene was added in a quantity corresponding to a concentration 50 μM, namely higher than pyrene solubility in water. After 30 min sonication, the sample was centrifuged and, from the absorption spectrum of the supernatant, the amount of bound pyrene was evaluated. The sonication was performed at low power (45 W at 55 kHz) in order to avoid chemical modifications of the melanin or formation of free radicals, and concomitantly to obtain a good dispersion of pyrene. This procedure gave mean values of 27 μM in the supernatant from which a value of 23 μM was estimated as dye bound to melanin. Controls performed washing the precipitate with buffer revealed only traces of released dye, confirming the occurrence of a strong interaction with the pigment particles. So, within experimental errors, these values were assumed as valid, also because mainly qualitative results are expected from this kind of study. Samples with different concentrations of the components were prepared in similar way. In quenching experiments with Cu^{2+} ions, $CuSO_4$ dissolved in buffer was added directly to the melanin–pyrene suspension, in the requested amount.

Absorption spectra were performed with a Hewlett–Packard HP 8452A spectrophotometer; for steady stated fluorescence and anisotropy measurements a Yobin-Yvon Spex Fluorolog 3 spectrofluorimeter and a SLM Aminco 8000 instrument have been employed. Time-resolved experiments were performed using an apparatus based on the time-correlated single photon counting method. The excitation source was a Tsunami 3950 Spectra Physics titanium–sapphire laser, pumped by a solid state Millenia Spectra Physics laser. The repetition rate of the 5 ps pulses was set to 800 kHz using the pulse picker 3980 Spectra Physics. The laser was tuned to give output at 990 nm and a third harmonic generator BBO crystal (GWN-23PL Spectra Physics) gave the 330 nm excitation pulses that were directed to an Edinburgh FL900 spectrometer. The L-configuration of the spectrometer allowed the detection of the emission at right angle with respect to the excitation. For anisotropy measurements a Glan–Thompson polarizer in the emission beam and a Soleil–Babinet compensator in the excitation beam have been employed. The emission wavelength was selected by a monochromator, and emitted photons were detected by a refrigerated Hamamatsu R3809U micro-channel plate photomultiplier. The FWHM of the instrument response function was typically 45 ps, determined with a time resolution of 6.0 ps/channel. Measurements were made using time resolution of 48 ps/channel. A software provided by Edinburgh Instruments was used to analyse the decay curves, and the adequacy of the multi-exponential decay fitting was judged by inspection of the plots of weighted residuals and by statistical parameters such as reduced χ^2.

3. Results and discussion

3.1. Emission spectra of melanin particles doped with pyrene

Many studies have demonstrated a relationship between the vibronic fine structure of the monomeric pyrene fluorescence and the polarity of its micro-environment [13,14]. Pyrene is a strongly hydrophobic fluorescent probe, with a low solubility in water. In the presence of macromolecular systems, pyrene is preferentially solubilised in the hydrophobic regions of these aggregates. In polar solvents, the (0–0) band (band 1, $\lambda \approx 373$ nm) is enhanced while the band 3 ($\lambda = 383$ nm) is relatively insensible. The emission ratio I_1/I_3 has been used to monitor the micro-polarity experienced by pyrene in colloidal aggregates. The value increases with increasing polarity. In the present work, this ratio has been used to determine the polarity of the surface of melanin particles suspended in water.

The ratio I_E/I_M between the emission intensity of pyrene excimers at $\lambda \approx 470$ nm and the emission intensity of monomers at $\lambda \approx 373$ nm, allows to evaluate, through the ease of excimer formation, the degree of mobility, dependent on the local viscosity, of the probe molecules when adsorbed on the surface of particles [15].

Fig. 1 shows the comparison between the emission spectra of pyrene in buffer at a concentration 10 μM (a) and in melanin (about 23 μM) (b), with excitation at 328 nm. An approximative evaluation indicates that 23 μM of bound pyrene correspond to about 0.3% by weight in melanin, i.e. about 0.9 M, assuming an uniform distribution in all the porous volume (the density of melanin is about 1.57 g/cm^3 [16]). As the BET surface area is 15.7 m^2/g, as calculated from N_2 adsorption isotherms [8], the concentration of the label calculated for the surface is still higher. However, little excimer formation is observed, indicating that even in this very high concentration the mobility of pyrene is very low in melanin. Moreover, the measured fluorescence anisotropy is 0.3, a value that confirms that pyrene is strongly immobilised in this system.

After corrections for the light absorption due to melanin, the I_1/I_3 ratio results 1.35 (1.58 in buffer). In an experimental

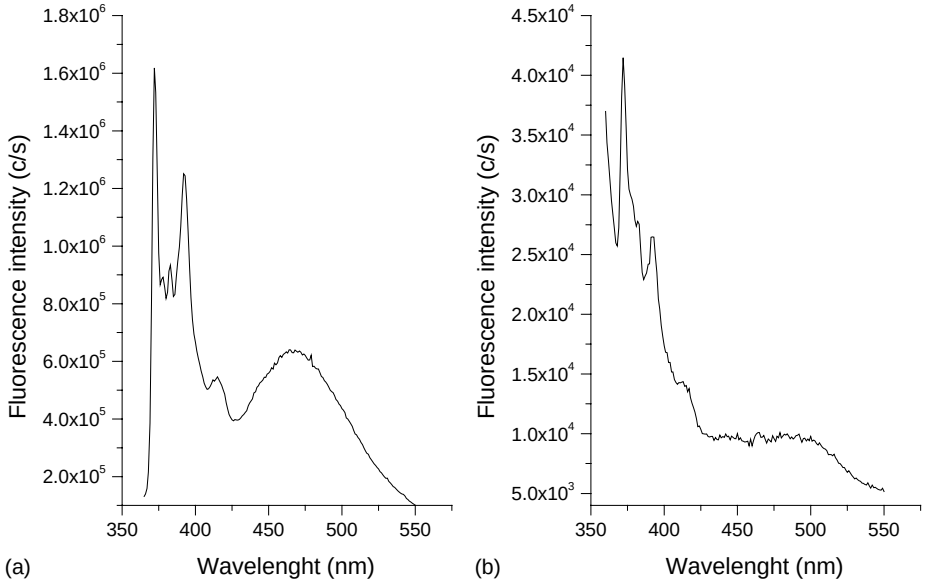

Fig. 1. Emission spectra of pyrene: (a) in buffer at a concentration 10 μM and (b) in melanin (about 23 μM). Excitation wavelenght: 328 nm.

scale, this value corresponds to a surface polarity comparable to methanol or methylene chloride [14].

The emission spectrum of pyrene adsorbed on melanin in the monomer region reproduces all the features observed in buffer solution with only the reported differences in the intensities of the various monomer bands. This observation suggests that no chemical reactions take place between the dye and the pigment. In any case it must be reminded that the optical absorption of the melanins extends into the UV range, where is particularly efficient, influencing the intensity of the observed emission.

3.2. Fluorescence lifetime studies

Different time decay curves were obtained for pyrene emission in absence and in the presence of melanin (Fig. 2). Intensity decay profiles were fitted to multi-exponential decay function given by $I(t) = \sum a_i \, e^{-t/\tau_i}$ where a_i is the pre-exponential factor and τ_i is the corresponding lifetime. Fluorescence decay of pyrene in buffer at $\lambda_{em} = 370$ nm, shown as curve a in Fig. 2, was almost mono-exponential with lifetime equal to 123 ns (Table 1), value comparable to that reported by Vanderkooi and Callis [17] for monomeric pyrene. A small contribution, 0.5% of total emission, comes from a short component of 14 ns (see Table 1), relatively unimportant in low viscosity liquids like water.

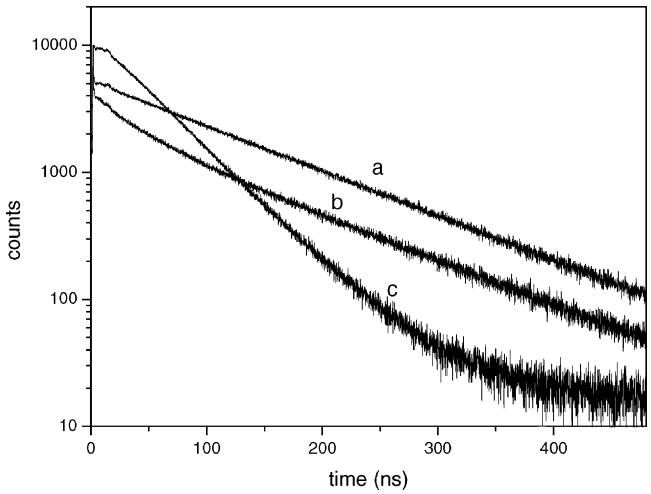

Fig. 2. Pyrene emission decay profiles in absence and in the presence of melanin, in HEPES buffer 10 mM, pH 7.4, excitation at 330 nm: (a) emission at 370 nm, pyrene concentration 1 μM; (b) emission at 370 nm, estimated concentration 23 μM, in the presence of melanin (7.5 mg/100 ml) and (c) emission in 470 nm, estimated concentration 23 μM, in the presence of melanin (7.5 mg/100 ml).

When bound to melanin surface, the decay kinetics observed at the same wavelength (Fig. 2, curve b) was best fitted to a bi-exponential function, whose main component has $\tau_1 = 116$ ns and normalised pre-exponential factor 0.61,

Table 1
Time resolved fluorescence parameters (lifetime τ_i and pre-exponential factor a_i) for pyrene (pyr) 1.0 μM in HEPES buffer 0.01 M, pH 7.4, and in interaction with melanin (pyr mel: emission at 373 and 470 nm), obtained from fit to multi-exponential decay

	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	a_1	a_2	a_3
Pyrene 373 nm	123 ± 2	14 ± 2	–	0.96 ± 0.01	0.040 ± 0.005	
Pyr mel 373 nm	116 ± 3	29 ± 1	–	0.610 ± 0.005	0.390 ± 0.004	
Pyr mel 470 nm	192 ± 30	47.0 ± 0.5	13 ± 1	0.003 ± 0.001	0.928 ± 0.003	−0.070 ± 0.003

and is responsible for 86% of total emission (Table 1). In the presence of melanin the contribution from a short component of 29 ns becomes important (Table 1), accounting for 14% of the emission. These data can be rationalised as follows: the lifetime of the main component is comparable with that measured in solutions of pure pyrene, indicating that many dye molecules are weakly interacting with the pigment particles and have emission characteristics of the dye in the monomeric form. On the other hand, a significant population of dye molecules, strongly immobilised on the surface, is subjected to a quenching effect by melanin, possibly through an enhancement of the internal conversion efficiency.

When observed at $\lambda_{em} = 470$ nm, i.e. in the excimer band, pyrene emission in the presence of melanin (Fig. 2, curve c) shows a complex decay and the best fit to experimental data was obtained with three exponentials (Table 1). The negative pre-exponential factor for the short lifetime of 13 ns is consistent with models for the excited state reaction leading to the excimer formation [17,18]. A slow decay gives only a small contribution in pyrene adsorbed in melanin granules, and a long lifetime component with same magnitude was reported by Chandrasekaran and Thomas [10] for pyrene in the presence of TiO_2, both in crystalline or amorphous form. In our measurements, the excimer decay is dominated by a lifetime component of 47.8 ns, indicating that also in this case, a quenching effect by the melanin can be effective. The above authors reported similar quenching effects promoted by crystals of TiO_2 in pyrene excimers.

3.3. Emission quenching studies: effect of Cu^{2+} ions

Addition of Cu^{2+} ions to the melanin–pyrene system causes a quenching of the pyrene monomer fluorescence, decreasing both the intensity of emission at 370 nm and the lifetime of the excited state (Fig. 3). The decay profiles were fitted to two exponential functions and average lifetimes $\langle\tau\rangle$ were calculated from the pre-exponential factor α_i and the lifetime τ_i according to $\langle\tau\rangle = \sum \alpha_i \tau_i^2 / \sum \alpha_i \tau_i$. Stern–Volmer plots for both the emission intensity and mean lifetime are linear (Fig. 4). However, the quenching observed through intensity measurements is more pronounced than that derived from the lifetime values, indicating that static and dynamic quenching may be occurring simultaneously.

From a linear fitting to the lifetime data, a Stern–Volmer constant of $0.20 \pm 0.03\ mM^{-1}$ was obtained. Using the average lifetime measured in the absence of quencher (104.4 ns), a bimolecular constant equal to $1.92 \times 10^9\ Ms^{-1}$ was calculated. The value, comparable to that reported in [10] for the pyrene quenching by iodide in amorphous TiO_2, is about one order of magnitude lower than usually obtained by relatively small species as pyrene and Cu^{2+} free in solution, in diffusion controlled quenching process. The result is an indication that pyrene loosely bound to melanin has restricted access to that ion, considering also the fact that melanins are able to bind with great efficiency metal ions, in particular Cu^{2+} and Zn^{2+}.

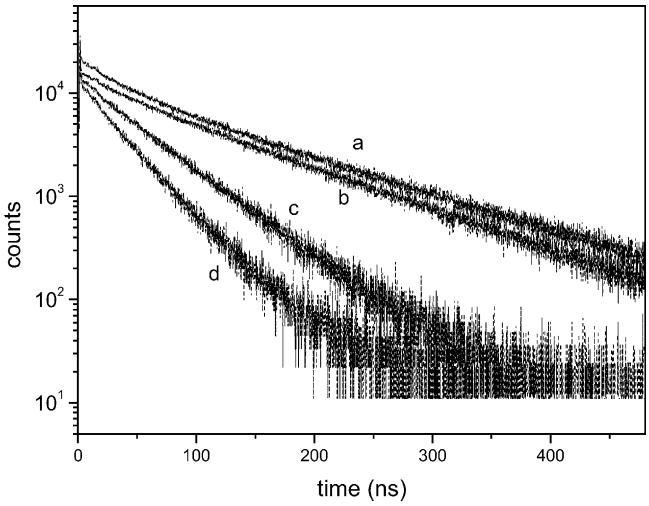

Fig. 3. Quenching of pyrene bound to melanin by Cu^{2+}. Emission decay profiles for pyrene (estimated concentration 23 μM) in the presence of melanin (7.5 mg/100 ml), in HEPES buffer 10 mM, pH 7.4, excitation at 330 nm and emission at 370 nm. Concentrations of Cu^{2+} are: (a) 0 mM, (b) 2 mM, (c) 5 mM and (d) 8 mM.

Fig. 5 shows the Stern–Volmer plot for the quenching of the excimer emission by Cu^{2+}. The mean lifetime is little affected by the presence of ions, indicating that the quenching originates from complexes where the excimer emission is halted. When pyrene is loosely bound to the melanin surface, it can be dynamically quenched by Cu^{2+}, decreasing the monomer emission; when the dye is immobilised in the form of interacting "clusters" in the cavities of the pigment particle, it produces excimers under light, and the emission can be statically quenched by the local presence of Cu^{2+} ions. Under this hypothesis, metal ions can reach and bind only a limited percentage of the dye interacting with it in

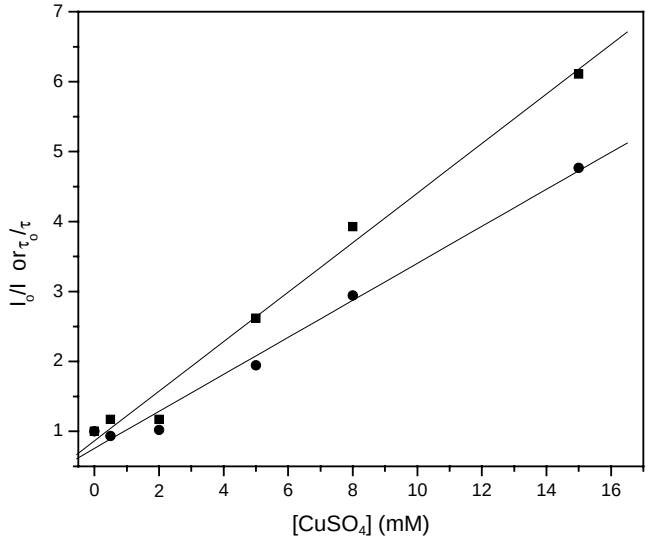

Fig. 4. Stern–Volmer plot for quenching by Cu^{2+} ions of pyrene–melanin monomer emission: (■) intensity data, (●) mean lifetime data. Solid lines resulting from linear fitting of data.

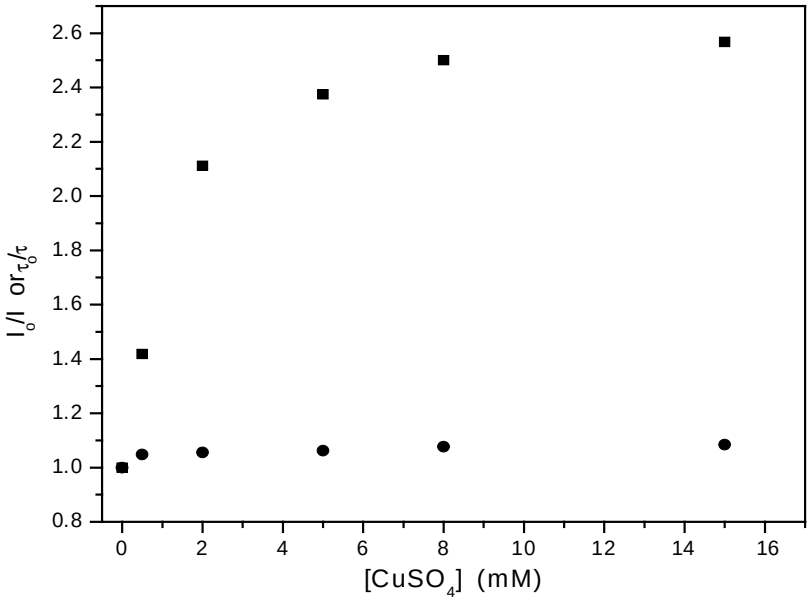

Fig. 5. Stern–Volmer plot for quenching by Cu^{2+} ions of pyrene–melanin excimer emission: (■) intensity data, (●) mean lifetime data.

form of ground state complex, leading to the observed saturation in the Stern–Volmer plot. Under this respect, the ion binding efficiency and the consequent interaction with the immobilised probe can be further kept into account.

From our experimental observations, some conclusions can be drawn:

- photophysical properties of pyrene adsorbed on colloidal melanin particles are quite different from those observed in homogeneous solutions of pyrene though no chemical reaction between pigment and dye occurs;
- the shape of the pyrene fluorescence spectrum indicates that it resides in a polar environment;
- both fluorescence lifetime and the appearance of a dynamic and a static quenching by Cu^{2+} ions indicates that pyrene interacts with melanin surface in two ways: part of the dye population (that giving origin to the little excimer band observed) is loosely bound to the surface of the pigment particle. Moreover, a percentage of the dye population is immobilised forming clusters in the cavities of the particle.

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