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Original Contribution

Generation and suppression of singlet oxygen in hair by photosensitization of melanin

Orlando Chiarelli-Neto, Christiane Pavani, Alan S. Ferreira, Adjaci F. Uchoa [1], Divinomar Severino *, Maurício S. Baptista *

Department of Biochemistry, Instituto de Química, Universidade de São Paulo, São Paulo, SP 05509–900, Brazil

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ABSTRACT

We have studied the spectroscopic properties of hair (white, blond, red, brown, and black) under illumination with visible light, giving special emphasis to the photoinduced generation of singlet oxygen ($^1O_2$). Irradiation of hair shafts (λ_{ex}>400 nm) changed their properties by degrading the melanin. Formation of C3 hydroperoxides in the melanin indol groups was proven by ^{1}H NMR. After 532-nm excitation, all hair shafts presented the characteristic $^1O_2$ emission (λ_{em} = 1270 nm), whose intensity varied inversely with the melanin content. $^1O_2$ lifetime was also shown to vary with hair type, being five times shorter in black hair than in blond hair, indicating the role of melanin as a $^1O_2$ suppressor. Lifetime ranged from tenths of a nanosecond to a few microseconds, which is much shorter than the lifetime expected for $^1O_2$ in the solvents in which the hair shafts were suspended, indicating that $^1O_2$ is generated and suppressed inside the hair structure. Both eumelanin and pheomelanin were shown to produce and to suppress $^1O_2$, with similar efficiencies. The higher amount of $^1O_2$ generated in blond hair and its longer lifetime is compatible with the stronger damage that light exposure causes in blond hair. We propose a model to explain the formation and suppression of $^1O_2$ in hair by photosensitization of melanin with visible light and the deleterious effects that an excess of visible light may cause in hair and skin.

Light absorption in biological tissues is extremely important for the health and care of skin and hair [1,2]. The effects of light exposure have been extensively studied in skin. However, few studies have been published considering the effects of light overexposure in the structure and properties of hair [2–5]. Also, the effects of photosensitization reactions, which can occur during exposure of these tissues to both visible and UV light, are not completely understood [1,2].

Although the composition of skin is much more complex than that of hair, the outer layers of skin and of hair fibers are composed mainly of keratin, melanin, and lipids. Keratin is the major structural protein present in these tissues. It is transparent in the UVA and visible regions of the light spectrum and does not play any role in the initiation of the photoinduced processes that will be treated herein. Melanin, which can be separated into two main types, i.e., eumelanin and pheomelanin, determines the color of hair and skin [6,7]. Melanin pigments, which are granular structures mainly located in the cortex region of the hair fiber [8,9], absorb light in the UV/visible/near-infrared (NIR) regions of the electromagnetic spectrum and have been reported to protect hair and skin from extensive solar exposure by absorbing/scattering light as well as by playing several antioxidant roles [6–11]. However, it is likely that under certain circumstances the excited species of melanin may generate reactive oxygen species. Therefore, under these conditions, melanin could trigger damaging instead of protecting mechanisms. Similar effects have been observed in the reaction centers of photosynthetic organisms. Under high-intensity illumination, reaction centers generate a considerable amount of singlet oxygen ($^1O_2$) by photosensitization, which can damage the photosynthetic apparatus [12].

Singlet oxygen is a highly electrophilic molecule and is capable of efficiently reacting with biomolecules by several mechanisms, including adding to double bonds of lipids, proteins, and nucleic acids and oxidizing –SH groups. In terms of amino acids, $^1O_2$ is especially reactive against cysteine, tyrosine, tryptophan, methionine, and histidine and derivatives of these molecules [13]. Therefore, $^1O_2$ can surely cause damage in skin and hair. Photooxidation caused by singlet oxygen has been classified as a type II mechanism. After light absorption it is also possible that a type I mechanism proceeds, in which excited triplet molecules engage in one-electron redox processes (electron or electron coupled to H^+ or direct hydrogen atom transfers) with biomolecules forming a variety of radical species [12–14].

The mechanism of hair damage caused by UV exposure has been studied before [3–6,10,14]. There is strong evidence that free radicals, including hydroxyl radical ($^{\bullet}$OH), are the main damaging species, although the mechanism of their generation is not clear [14]. The involvement of $^1O_2$, and consequently of photosensitization reactions,

* Corresponding authors. Fax: +55 11 3815 5579.
E-mail addresses: divinomar@gmail.com (D. Severino), baptista@iq.usp.br (M.S. Baptista).
[1] Present address: Unicastelo, São José dos Campos, SP, Brazil.

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has been hypothesized, but it was never proven experimentally [3–5,15]. The possible generation of these reactive oxygen species by melanin and/or its precursors or derivatives has motivated meticulous photophysical studies [16–19].

Because of the difficulty in studying melanin itself, several experimental models have been proposed to understand its dual protecting/damaging roles [18]. Recently the properties of singlet and triplet states of derivatives of dihydroxyindol (DHI), a fragment of melanin, were shown to have very short singlet (10 ps) and triplet (200 ps) lifetimes [19]. Efficient deactivation channels of DHI excited states have been proposed as one of the mechanisms by which melanin protects skin and hair. However, it is important to note that reactive species can be generated even in the presence of short-lived excited states, because these excited states can be generated in the vicinities of reactants and diffusion does not always necessarily occur.

Other experiments have demonstrated the damaging roles of melanin and its components [16]. Superoxide anion radical was detected by electronic paramagnetic resonance after photoexcitation of melanin [17]. The authors also suggested that $^1O_2$ could be generated [17]. However, $^1O_2$ has never been detected directly from hair or from melanin. Efficient $^1O_2$ suppression ($k_2 \sim 10^8\ M^{-1}\ s^{-1}$) by L-dopa [20] suggests the possible quenching of $^1O_2$ by melanin, although this has never been precisely characterized.

We have used a sensitive instrument to detect $^1O_2$, which is based on the classical measurement of its NIR phosphorescence emission with a maximum at around 1270 nm. In our instrument, an NIR photomultiplier tube (PMT) with extremely low dark current (<500 cps in the dark) provides highly sensitive detection of $^1O_2$ [12,21–23]. Using this equipment we were able to detect the characteristic $^1O_2$ phosphorescence coming directly from hair shafts and from solutions prepared with melanin, showing unequivocally that $^1O_2$ is generated in hairs by photosensitization of melanin.

Experimental procedures

Materials

All solvents were of spectroscopic grade. Water was double-distilled from an all-glass apparatus and was further purified via a Millipore Milli-Q system. Urea was recrystallized from methanol. D_2O (99%; Aldrich, Milwaukee, WI, USA) was used as received. Melanin (synthetic; Sigma, St. Louis, MO, USA) was used as received. All other materials were of the best analytical grade available. NaOD was prepared by three cycles of dissolution and evaporation of the initial solid of NaOH (1 g) in D_2O (10 g). Hair samples were obtained from human subjects that were working in the laboratory. They were washed with water and dried at room temperature. Eumelanin and pheomelanin were synthesized as described by Haywood et al. with modifications [24]. Eumelanin was prepared from L-tyrosine (2.5 mg/ml) in pH 7.4 phosphate buffer (50 mM) and mushroom tyrosinase (150 U/ml) in bovine serum albumin solution (5 mg/ml). Pheomelanin was synthesized from L-dopa (0.5 mg/ml) and L-cysteine (1.5 mg/ml) in pH 7.4 phosphate buffer (50 mM) and mushroom tyrosinase (100 U/ml). The reactions were performed for 24 h at room temperature under stirring. We also utilized eumelanin and pheomelanin samples kindly provided by Dr. S. Ito [25,26].

Optical microscopy

Microscopic images were obtained in an Axiovert 200 (Carl Zeiss, Göttingen, Germany). The same filter set ($\lambda_{ex} = 450–490$ nm; $\lambda_{em} \geq 515$ nm) was used to obtain all emission images shown. Samples were introduced into a glass slide and observations were made typically at 400× magnification and photographed on a Canon Power Shot G10. ImageJ Launcher was used for data analysis.

Absorption and fluorescence UV/Vis spectra

Fluorescence spectra were obtained using a Spex Fluorolog 1681 0.22 m spectrofluorimeter in the front face acquisition mode with excitation/emission slits of 1.5 mm/2.0 mm, respectively. Excitation/emission wavelengths used in the experiments are shown in the figure legends. Reflectance spectra of hair shafts were obtained in a fiber-guided spectrophotometer (USB-2000; Ocean Optics, Dunedin, FL, USA). Origin 7.0 software was used for data analysis.

Artificial irradiation

Irradiation (hairs and melanin solutions) was performed in a home-made photoreactor, which consisted of a 4-W halogen lamp providing light in the visible region (400–800 nm). Absorption/reflectance/emission spectra were obtained before and after irradiation. Melanin solutions (0.04 g/L) were prepared in 0.1 M NaOH/D_2O and were either purged with argon (20 min) or oxygen (20 min) or allowed to equilibrate in air. Indol emission was accessed by exciting samples at 290 nm and quantifying emission from 300 to 540 nm. One should be aware that melanin is a complex structure and it is likely that there are other chemical groups present in melanin that generate emission spectra similar to that of indol. Because hydroxyindol groups are one of the major structures present in eumelanin, we can assume that we are monitoring either indol chromophores or related structures that probably react by similar mechanisms. For the NMR measurements, 10 mg of melanin (Aldrich) was dissolved in 0.7 ml of D_2O/NH_4OH (pH 10). ^1H NMR spectra were obtained at 25 °C on a Bruker DRX500 apparatus operating at 500 MHz.

Singlet oxygen detection

Singlet oxygen measurements were performed in a specially designed Edinburgh F900 instrument (Edinburgh, UK) that consisted of a Continuum Surelite III laser (5-ns duration, 10 pulses/s, 1 mJ/pulse), cuvette holder, silicon filter, monochoromator, and liquid-nitrogen-cooled NIR PMT (R5509) from Hamamatsu (Hamamatsu Co., Bridgewater, NJ, USA) and a fast multiscaler analyzer card with 5 ns/channel (MSA-300; Becker & Hickl, Berlin, Germany) [12,23]. $^1O_2$ emission spectra were obtained by measuring emission intensities from 1180 to 1360 nm with 1- to 5-nm steps. Lifetime measurements were performed by accumulation of ~2000 decays. Emission spectra were automatically constructed by the instrument software by acquiring decays at various wavelengths, accessing the maximum emission intensity in each wavelength, and plotting the maximum intensity as a function of the wavelength. Hairs were suspended in a specific medium (CCl_4, H_2O, D_2O, ethyl alcohol, and air) and were directly excited at 532 nm. An equivalent mass of all hair types (120 ± 10 mg) was used to prepare shafts that were fixed inside a fluorescence quartz cuvette. The mass of hair that was actually irradiated by the Nd:YAG laser was quantified to be 30 ± 1 mg. Melanin samples were prepared in D_2O (pure D_2O, NaOD 0.1 M, H_2O_2 1.0 mM, and urea 6.0 M). NIR spectral and lifetime measurements were performed 1 h after sample preparation and also after a specific period of incubation (25 °C) that varied from a few hours to 14 days. For measuring the efficiency of singlet oxygen generation (η_Δ), the absorption of samples and standard was matched at the excitation wavelength. Methylene blue, which was dissolved in D_2O: ethanol (1:1) to avoid aggregation and keep its quantum yield constant ($\Phi_\Delta = 0.52$), was used as standard [12,23]. Emission transients were obtained and the maximum values of emission intensities, which were observed typically 100 ns after the laser pulse, were used in the η_Δ calculations. We utilized the term "efficiency of singlet oxygen" instead of "quantum yield" because melanin has a complex molecular structure and may be highly aggregated.

Statistical analysis

In most of the experiments reported herein, measurements were performed as three independent repetitions and average values are expressed as means ± standard deviation. The level of significance was assessed using Student's *t* test employing Origin data analysis software (version 7.0). Statistical significance was assigned at a *P* value of ≤0.05.

Results and discussion

Under UV light excitation, hair is known to fluoresce, mainly because of the absorption and emission of specific amino acid residues (tryptophan, tyrosine, and phenylalanine). Absorption of UV radiation has also been shown to affect hair color and structure [3–7,10,11]. To our knowledge there is no report about the fluorescence of hair induced by irradiation with visible light, which can originate only from the absorption and emission of molecules absorbing in the visible, i.e., melanin and its precursors and derivatives.

We start this report by describing the optical properties of various types of hair under illumination with visible light. For the sake of simplicity only two extreme hair colors are shown, i.e., blond and black. Intermediately colored hairs, i.e., light and dark brown, etc., have intermediate spectroscopic features. Note that both blond and black hairs present similar structural features at the microscopic level (Fig. 1A); however, their emission profiles are different. Fluorescence from blond hairs (Fig. 1A, top right) is more intense than that detected from black hairs (Fig. 1A, bottom right). The weaker emission from black hair fibers is compatible with the weaker emission spectra measured in the spectrofluorimeter (Fig. 1C). Another interesting difference from the emission profile of black and blond hairs is the punctuation profile. Note that the emission coming from black hairs is not continuous, but instead, it has a punctuated profile that is due to the presence of remaining nuclear and organelle residuals, including melanin granules [8,9]. Note also that the amount of light absorption in black hairs is much larger, because of the larger melanin content (Fig. 1B), suggesting that the weaker fluorescence of black hairs is due to an inner filter effect (less light reaches the deeper layers of the hair) and light reabsorption (emitted light is reabsorbed).

The only species that absorbs visible light in hairs is melanin, indicating that melanin is the probable species that is emitting light. Because melanin is not a simple molecule, it is not expected to have a well-defined absorption/emission spectrum. Because of its structural diversity, we expect that the melanin pool (including the various types of melanin in different stages of maturation) has several absorbing and emitting species, similar to other complex substances found in nature such as petroleum [27]. By varying the excitation wavelength we observed changes in the wavelength of maximum emission, indicating that different fluorophores were present (Fig. 1D), which is a behavior that one could expect from a complex substance such as melanin. The fact that the fluorescence spectra and the microscopic images of hair under visible light excitation are sensitive to the melanin content allows us to use these techniques to evaluate specific damages to melanin and their respective effects in hairs.

UV light is known to cause damage in melanin and in other hair structures [2–4,6,7,10,11]. However, the effect of visible irradiation has not been carefully investigated. By irradiating hairs with visible light we observed changes in the absorption/emission properties of hair shafts (Fig. 2). The increase in light emission of both blond and black hair (Fig. 2A) was accompanied by a decrease in melanin absorption in both types of hair (Figs. 2B and C), indicating that melanin was being degraded. Note also that the effect was more pronounced in blond hairs (Fig. 2B). Therefore, visible light, in addition to interacting physically (scattering, diffraction, absorption, and emission) with the structures present in hairs, also causes chemical modifications, which is similar to the effect observed by exposure of hairs to UV light [2–5,10,11].

To characterize the possible chemical modifications taking place in hairs (especially in melanin), we studied changes in the fluorescence and ^{1}H NMR spectra of melanin (Aldrich) samples before and after irradiation with visible light. The properties of the indol groups, which are one of the most reactive sites in melanin, were analyzed. The

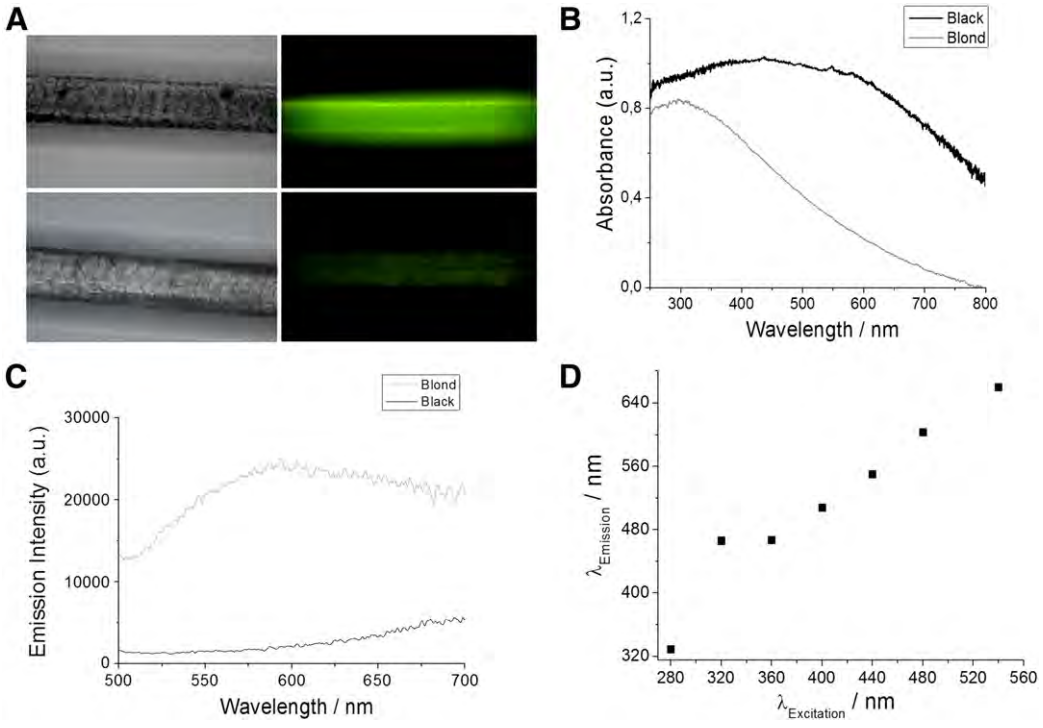


Fig. 1. Microscopic and spectroscopic properties of blond and black hairs. (A) Left: typical transmission microscopic images of blond (top) and black hair (bottom). Right: typical fluorescence microscopic images of blond (top) and black hair (bottom). Original magnification 400×. (B) Absorption spectra of black and blond hairs. (C) Fluorescence spectra of blond and black hairs (λ_{ex} = 480 nm). (D) Correlation between excitation wavelength and the emission maxima of the emission spectra of blond hair.

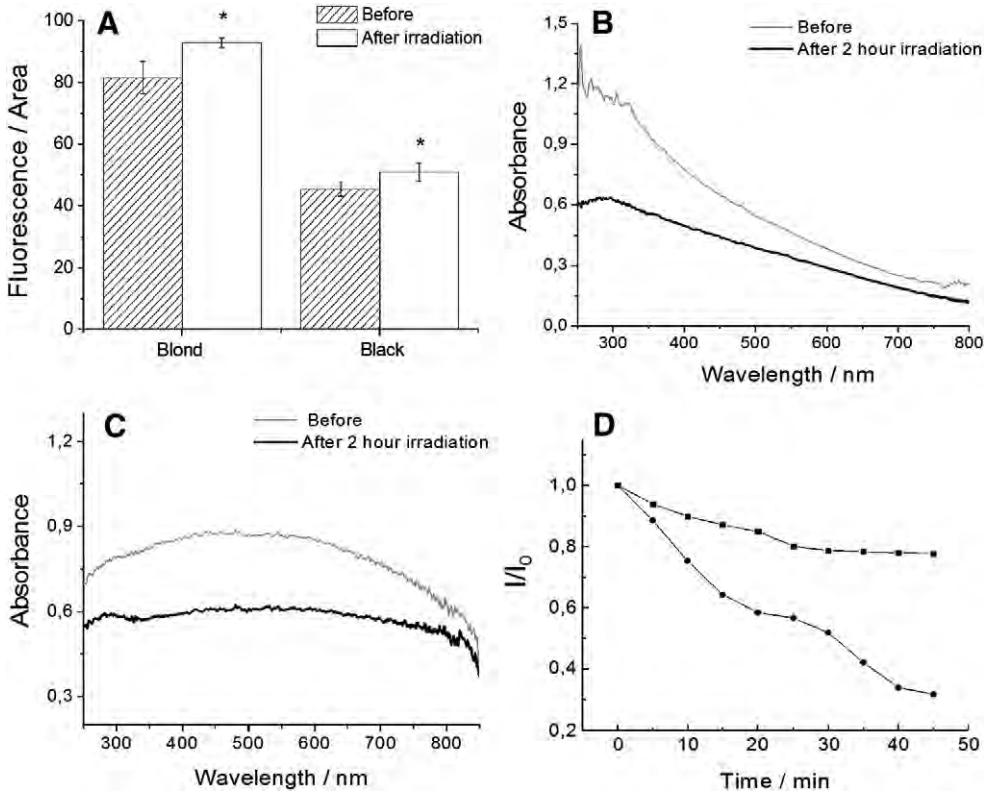


Fig. 2. (A–C) Absorptive and emissive properties of hair shafts (blond and black) before and after 2-h irradiation with halogen lamp. (A) Integrated emission intensities of blond and black hair samples before and after irradiation. Data are the averages of the fluorescence of 10 hairs obtained from microscopy images, integrated using ImageJ at a constant area. Absorption spectra of (B) blond and (C) black hairs before and after irradiation. (D) Integrated emission ($\lambda_{ex} = 290$ nm, $\lambda_{em} = 310$–500 nm) of melanin (Aldrich) solutions in D_2O ([NaOD] 10^{-4} M) irradiated with halogen lamp purged with argon for 20 min (■) and equilibrated with air (●). Measurements were performed on the same day of sample preparation. *$P<0.05$, significantly different by t test.

emission of indol chromophores ($\lambda_{ex} = 290$ nm, maximum emission at ~350 nm) present in melanin was characterized as a function of irradiation time in samples equilibrated with air (higher oxygen concentration) and purged with argon (lower oxygen concentration) (Fig. 2D). It is clear that the characteristic indol emission was being suppressed during irradiation. Similar effects have been observed after the reaction of $^1O_2$ with indol groups of tryptophan and tryptophan derivatives [28–31]. The initial products of this reaction have been characterized to be either a dioxetane or a hydroperoxide (C3 position). Using isotope labeling, Ronsein and co-authors characterized the first step as the formation of the hydroperoxide at the C3 position [31]. These initial unstable species will decompose under normal conditions. In the case of tryptophan, the prevalent reaction product is *N*-formylkynurenine [28–31].

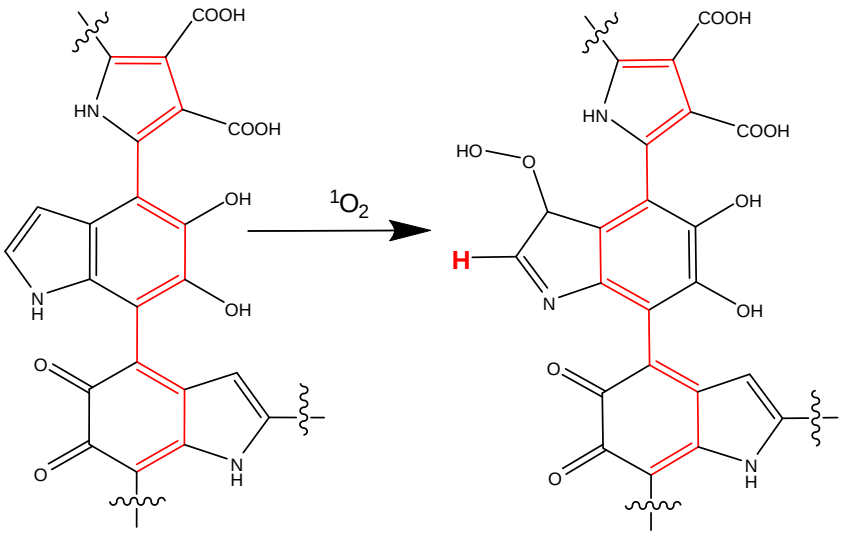
Note also that the rate of indol oxidation is inhibited by decreasing the concentration of dissolved oxygen (Fig. 2D), indicating the importance of oxygen and possibly of $^1O_2$ in the oxidation reaction. If the photooxidation was to progress through a type I mechanism (direct reaction of triplets with substrates), oxygen could inhibit the reaction, because it is an efficient suppressor of triplet species [32].

The same photooxidation procedure was repeated and melanin (Aldrich) solutions were analyzed by ^{1}H NMR. The ^{1}H NMR spectrum of melanin has already been obtained, analyzed, and published by others [33]. After irradiating melanin we observed the formation of a peak at 8.4 ppm (singlet) with a consecutive decrease in the peaks of the aromatic indol protons (7.7 to 7.8 ppm). These changes in the ^{1}H NMR spectrum of melanin were also observed by Katritzky and co-authors after the chemical oxidation (hydrogen peroxide) of melanin. Katritzky and co-authors also predicted that this peak at 8.4 ppm was due to the presence of an "−N=CH-Ar" group [33]. Knowing that the main reaction route of $^1O_2$ with indol is the formation of a hydroperoxide at the C3 carbon, we realized that the dislocation of the double bond to the vicinity of nitrogen can explain the formation of such a chemical group (Scheme 1). Unfortunately, this reaction product is not specific to $^1O_2$ because other reactive species can also generate it. However, considering that we show, without doubt, that $^1O_2$ is being generated in hairs and in melanin solutions (see below), it is very likely that the initial step of melanin degradation is the reaction shown in Scheme 1. Therefore, visible light is able to induce degradation of the indol ring of melanin and the initial step of this reaction is due to $^1O_2$ forming a hydroperoxide at the C3 position of the indol groups. However, it is necessary to learn if and how $^1O_2$ is being generated.

After light absorption, melanin forms excited-state species that live long enough to induce excited-state reactions [16–18,34]. One possibility to explain the photodamage of melanin is that triplet species are formed, allowing type II photosensitization reactions and formation of $^1O_2$ that can add to double bonds (Scheme 1) and react with –SH groups. Singlet oxygen can damage melanins and lipids, as well as react with amino acids, for example, cysteine residues present in keratin [13,35,36].

Previous work has hypothesized that UV-induced chemical damage in hair could be caused by $^1O_2$. However, there is no report in the literature proving the photoinduced generation of $^1O_2$ in hair [3,4,16,17]. It is expected that UV excitation of hairs should produce $^1O_2$, because of the direct excitation of photoactive amino acids [37]. On the other hand, we suspect that melanin itself can produce $^1O_2$. To test this hypothesis, direct measurements of NIR phosphorescence of hair shafts were performed after 532 nm excitation, a wavelength that is absorbed only by the melanin pool in hairs.

Hairs were suspended in water and in D_2O, and NIR phosphorescence spectra were measured after 532 nm excitation (Fig. 3). It can be observed that the emission band is centered at 1270 nm, which is the fingerprint of the $O_2(a^1\Delta_g) \rightarrow O_2(X^3\Sigma_g^-)$ transition [12,20–22]. The



Scheme 1. Proposed reaction mechanism for melanin photooxidation by singlet oxygen, which is compatible with the formation of the ^{1}H NMR peak at 8.4 ppm (in red) and with the known reaction mechanism of singlet oxygen with indol groups.

signal intensity is stronger in D_2O, which is another indication that the emission is coming from $^1O_2$. Because the lifetime of $^1O_2$ is around 11 times longer in D_2O than in water, there is a considerable increase in the steady-state concentration of $^1O_2$-emitting species in D_2O. To further prove the generation of $^1O_2$ by a type II mechanism, the NIR emission spectra of hairs was measured in solutions having various concentrations of dissolved oxygen, i.e., samples purged with argon or with molecular oxygen or samples equilibrated in air (Fig. 3, inset).

It is clear that the presence of a lower oxygen concentration causes a considerable decrease in the 1270 nm emission (samples purged with argon). The reduction is not complete ($^1O_2$ emission was decreased by ~50%), showing that oxygen molecules remained in the reaction medium. One could expect a stronger decrease in the amount of $^1O_2$ generated after argon purging if the photosensitization process were occurring in isotropic solution. In fact, this result suggests that the photosensitization occurs inside the hair structure, i.e., argon purging is not able to efficiently remove oxygen from the inside of hairs (see further discussion below). Emission increases proportionally with the increase in the concentration of dissolved oxygen, giving further confidence that it is coming from the $^1O_2$ decay formed in type II photosensitization reactions.

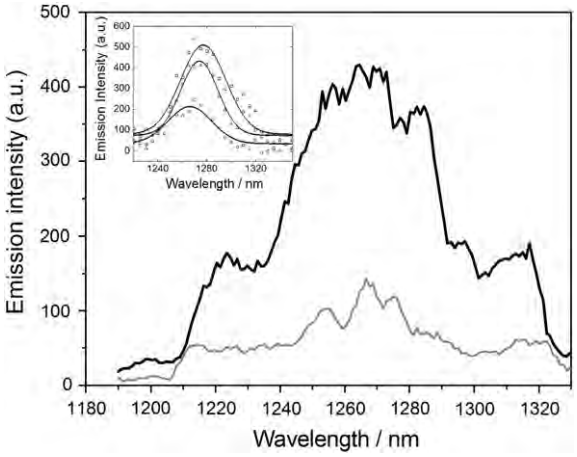


Fig. 3. NIR phosphorescence emission spectra of a black hair suspended in D_2O (thick line) and in H_2O (thin line). Inset: NIR phosphorescence spectra of blond hair suspended in CCl_4 purged with argon (○), air equilibrated (△), and purged with oxygen (□). Excitation at was 532 nm (10 pulses/s; 1 mJ/pulse; 10 ns/pulse).

The intensity of $^1O_2$ emission depends also on the hair type. Fig. 4 shows the intensity of $^1O_2$ signals coming from hairs of different colors. Black and brown hairs presented similar intensities. For the other hairs, significantly higher $^1O_2$ emission was observed ($P<0.01$, t test), being strongest in blond hairs. Therefore, $^1O_2$ signal intensity is inversely proportional to the melanin content in hair shafts (Figs. 4A and D), which is a similar trend to that observed in the fluorescence signals (Fig. 2A).

In addition to the magnitude of the NIR spectral signal, the lifetime of $^1O_2$ is another important parameter to be analyzed, because it gives an indication of the environment of $^1O_2$, as well as allowing one to study the presence of physical and chemical quenchers that cause a decrease in $^1O_2$ lifetime. Hairs were suspended in various solvents and NIR emission decays were measured at 1270 nm (Fig. 4A, inset). Note that there is a clear distinction of the signal measured at 1200 nm (signals at 1180 nm show only dark noise) and those measured at 1270 nm, in both hair types (Figs. 4B and C), proving that photons are due to the $^1O_2$ decay. Although the kinetic decays are more complex than those expected from the presence of a single emitting/suppressor pair, we fitted all data to monoexponential functions, because there was no time resolution to fit the signal to more precise kinetic schemes. Therefore, the $^1O_2$ lifetimes obtained should be considered an approximation. By fitting the emission decay at 1270 nm to monoexponential functions we obtained the following lifetimes (hair colors, lifetime in μs in CCl_4): black, 0.8; brown, 2.2; blond, 3.7; and white, 4.0 (Table 1, for black and blond hairs). The first interesting aspect to be considered is that the emission decay observed in blond hairs is longer compared with that observed in black hairs (4.7 times shorter in black hairs). It is interesting to note that black hairs have approximately five times more melanin than blond hairs [7,26], and $^1O_2$ is suppressed approximately five times more efficiently in black hairs, which is strong evidence that melanin is the main suppressor species of $^1O_2$. Hairs of other colors have increased $^1O_2$ lifetime with decrease in melanin concentration. These observations are in agreement with the known suppressor activity of $^1O_2$ by melanin. It is also in agreement with the larger magnitude of $^1O_2$ emission observed from blond hairs (Fig. 4).

Another interesting aspect is that the lifetime of $^1O_2$ in any type of hair is much (several orders of magnitude) shorter than the known $^1O_2$ lifetime in the respective isotropic solvents in which the hair shaft is suspended (Table 1). In the case of CCl_4 the $^1O_2$ lifetime is several tenths of a millisecond (~87 ms) [20], and the signals coming from hairs suspended in CCl_4 are 3.75 and 0.8 μs for blond and black hairs,

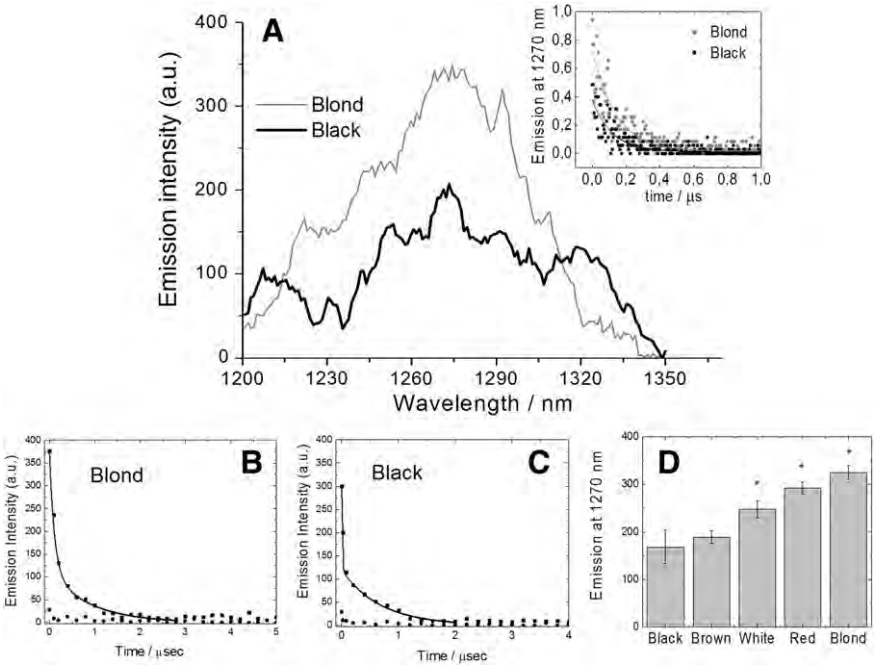


Fig. 4. (A) $^1O_2$ emission from blond and black hair samples excited at 532 nm in CCl_4. Inset: emission decays of $^1O_2$ (at 1270 nm) in black and blond hairs in D_2O. Emission decays at 1270 (■) and 1200 nm (●) of (B) blond and (C) black hairs in CCl_4. (D) $^1O_2$ emission intensity (at 1270 nm) from five types of hair. *$P<0.01$, significantly different by t test.

respectively. The same observation is valid for other solvents (Table 1). This result is different from that obtained in other complex systems such as whole cells in culture. When $^1O_2$ is generated inside cells the lifetime is long enough to allow $^1O_2$ to exit cells and experience the characteristic lifetime of the isotropic solvents in which the cells are suspended [38,39]. Therefore, our result indicates that in the case of hairs, $^1O_2$ must be generated and suppressed within the hair structure.

Although $^1O_2$ lifetime is much smaller in hairs compared to the value expected in isotropic solvents, the medium in which hairs are suspended also interferes with the $^1O_2$ lifetime. Note that $^1O_2$ lifetime increases in solvents that favor longer $^1O_2$ lifetimes (CCl_4, D_2O) and decreases in water. This effect must have a relation to the penetration of the solvent molecules in the hair structure [40].

In the experiments shown above we presented evidence that melanin generates and suppresses $^1O_2$. As mentioned in the introduction, although the building-block constituents of melanin are known to be photoactive, no one has observed emission of $^1O_2$ coming directly from melanin excitation. By dissolving synthetic melanin (Aldrich) in various types of solution (0.1 M sodium hydroxide, 0.1 M hydrogen peroxide, and 6 M urea) and exciting it at 532 nm we were able to observe the characteristic phosphorescence emission of $^1O_2$ (Fig. 5A). Different types of melanin were tested, including melanin from Aldrich (Fig. 5A), eumelanin (Fig. 5C) and pheomelanin (Fig. 5D) synthesized in our laboratory, and samples of eumelanin and pheomelanin synthesized by Dr. S. Ito (Fujita Health University) (Table 2). All of them generated $^1O_2$ in similar amounts. By performing experiments in which the amount of photons absorbed by the samples was matched and by comparing the emission of the melanin samples with the emission of a standard (methylene blue, see Experimental procedures), we were able to quantify that the efficiency of $^1O_2$ generation was around 1–2% for all melanin samples, being slightly larger (difference without statistical significance) for pheomelanins.

Table 1
Singlet oxygen lifetimes (in µs) in blond and black hairs after 532-nm excitation in various media.

Medium	Blond hair	Black hair
CCl_4	3.7	0.8
D_2O	0.2	0.09
H_2O	0.1	0.05
Ethanol	0.2	0.08
Air	0.2	0.09

Standard deviations were within 20% of the average lifetime value.

NIR emission decays from melanin (synthesized by us) solutions at 1270 and 1200 nm are clearly different, giving further confidence on the validity of the NIR signal being related to $^1O_2$ decay. The transients at 1270 nm were fitted to monoexponential functions providing lifetimes of 1.7 ± 0.2 and 1.4 ± 0.1 µs for eumelanin and pheomelanin, respectively. Both lifetimes are much smaller than the $^1O_2$ lifetime expected in D_2O (35–40 µs), indicating that $^1O_2$ is efficiently suppressed by melanin. Note that the amount of O–H present in melanin solutions was reduced by performing the experiments in D_2O:NaOD solutions. The smaller lifetime observed in pheomelanin solutions indicates a more efficient suppression by pheomelanin, but better time-resolution experiments are needed to reach a definitive conclusion in terms of comparing the suppression efficiencies of eumelanin and pheomelanin. In conclusion, the data shown here prove that both eumelanin and pheomelanin generate $^1O_2$ and suggest that pheomelanin generates and suppresses $^1O_2$ with slightly better efficiency compared with eumelanin.

We also obtained $^1O_2$ emission spectra at days 1, 4, 10, and 14 after sample preparation and there was always an increase in $^1O_2$ emission with the time of incubation. The integrals of the emission spectra obtained in days 1 and 14 are presented in Fig. 5B. There was a considerable increase in the amount of $^1O_2$ generated with incubation time when melanin was dissolved in sodium hydroxide and hydrogen peroxide (Fig. 5B). Therefore, hydrolysis and oxidation of melanin induced by sodium hydroxide and hydrogen peroxide, respectively, allow the excited species of melanin to generate more $^1O_2$. This finding supports the hypothesis that fragments of melanin were responsible for the generation of $^1O_2$ as predicted by Toffoletti [17]. More intriguing, however, are the data obtained from urea solutions of melanin. In this case, melanin is integral (dialysis experiments proved the absence of melanin fragments). The only change induced

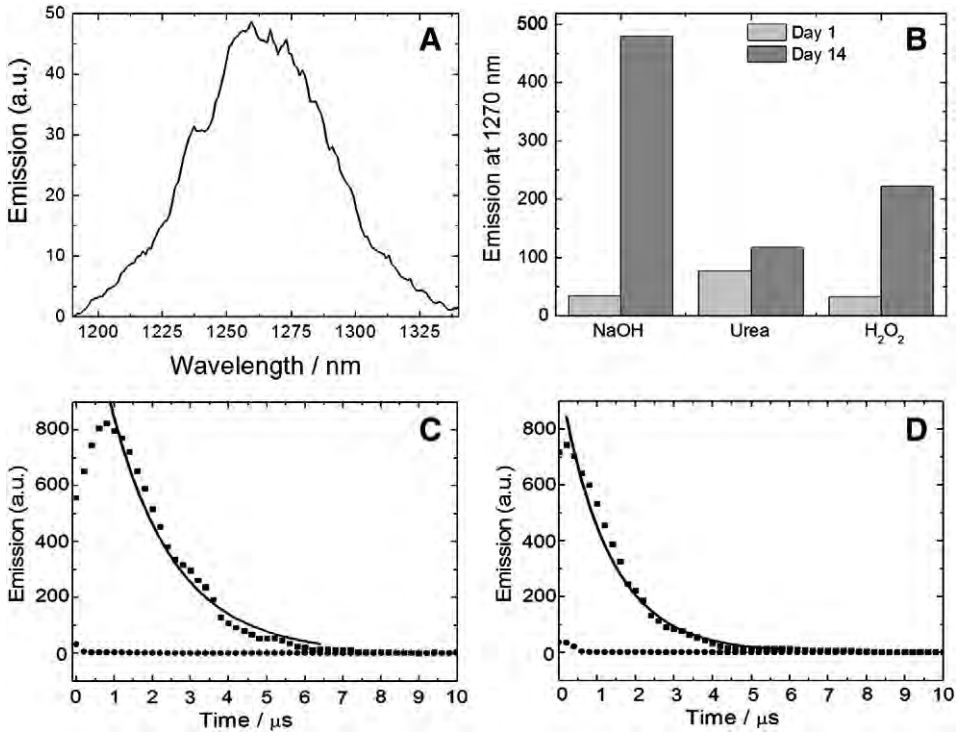


Fig. 5. (A) $^1O_2$ emission spectra of melanin in 0.1 M NaOH solution obtained on the day of sample preparation. (B) $^1O_2$ emission intensity in days 1 and 14 in 0.1 M NaOH, 6.0 M urea, and 0.1 M H_2O_2. Transient decay at 1270 (■) and at 1200 nm (●) of (C) eumelanin and (D) pheomelanin dissolved in D_2O in the presence of [NaOD] 10^{-4} M and measured on the same day of sample preparation. Melanins were synthesized following Haywood et al. [24]. Data fitting was performed using a monoexponential function providing $\tau_{eumelanin} = 1.7 \pm 0.2$ μs ($R^2 = 0.97$) and $\tau_{pheomelanin} = 1.4 \pm 0.1$ μs ($R^2 = 0.98$).

by urea was the denaturation of the biopolymer, i.e., opening of the melanin structure and exposure of its photoactive residues. Therefore, when melanin suffers chemical or physical treatments that cause a change in its structure and exposure of its photoactive residues, it generates $^1O_2$ with better efficiency.

Nofsinger and co-workers have observed ROS generation in eumelanin irradiated by UVB. They quantified that aggregated eumelanin pigments produce 10 times less superoxide anion radical ($O_2^{\bullet -}$) than unaggregated oligomers [41]. Both in our work and in the Nofsinger work the photoefficiency of melanin increased by opening its polymeric structure and by exposure of its photoactive residues. This effect seems to be a general trend in melanin's photochemistry. Therefore, the general state of hydration and other properties that are important for the maintenance of the melanin structure in skin and in hair may be critical for avoiding damage in these tissues by sun exposure.

Fig. 6 summarizes the photophysical processes taking place within a hair shaft. Our results provide strong evidence that under visible light irradiation hair suffers damage to its structure and color because of the generation of $^1O_2$ by photosensitization of melanin.

Table 2
Efficiency of singlet oxygen generation (η_Δ) from various types of melanin samples in D_2O.

Sample	η_Δ
Eumelanin[a]	0.015 ± 0.003
Eumelanin[b]	0.015 ± 0.004
Pheomelanin[a]	0.017 ± 0.004
Pheomelanin[b]	0.016 ± 0.003

$\lambda_{ex} = 532$ nm; 1 mJ/pulse. Three independent measurements using a methylene blue solution in D_2O:ethanol (1:1) were taken as standard ($\phi_\Delta = 0.52$).
[a] Samples prepared in our laboratory following Haywood et al. [24].
[b] Samples prepared in the laboratory of Dr. Ito [25,26].

Conclusions

$^1O_2$ is photochemically generated in hairs by visible light excitation. Inside hair, $^1O_2$ has a short lifetime depending on the melanin content. $^1O_2$ generation correlates with hair photodamage under visible light illumination. Chemical modifications of melanin induced by irradiation were shown to be related to the formation of a hydroperoxide at the C3 position of the indol ring. Therefore, our

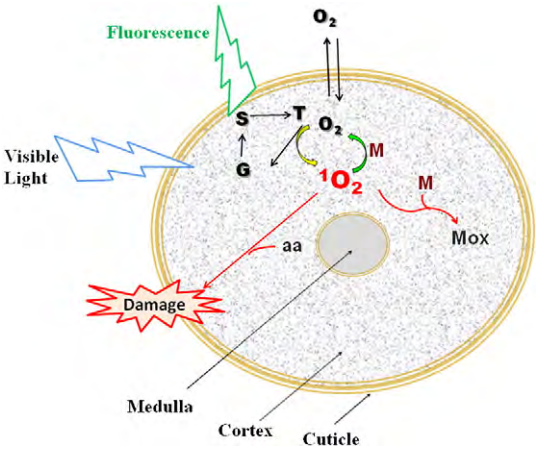


Fig. 6. Photophysical and photochemical processes in a hair shaft under irradiation showing production of singlet oxygen ($^1O_2$) by photosensitization after irradiation with visible light. The process starts mainly in the region where melanin is present (cortex). G is the ground state of melanin; S is its singlet state; T is its triplet state. G absorbs light, forming S that converts to T through intersystem crossing, and T reacts with oxygen, forming $^1O_2$. $^1O_2$ can be suppressed by three main processes: physical quenching (mainly by melanin-M) returning heat and molecular oxygen, chemical quenching by melanin forming oxidized melanin (Mox), or reacting with other amino acid residues (aa) such as cysteine of keratin, causing damage in the hair structure. Cross section of the hair shows cuticle, cortex, and medulla, which are out of scale.

results prove the role of $^1O_2$ in the mechanism of hair damage induced by photosensitization of melanin. We also have opened a new window of investigation concerning the effect of overexposure of biological surfaces to visible light. This understanding is important to develop new strategies to protect skin and hair from the effects of overexposure to the sun.

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References

[1] He, G. L.; Kutala, V. K.; Kuppusamy, P.; Zweier, J. L. In vivo measurement and mapping of skin redox stress induced by ultraviolet light exposure. *Free Radic. Biol. Med.* **36:**665–672; 2004.
[2] Liebmann, J.; Born, M.; Kolb-Bachofen, V. Blue-light irradiation regulates proliferation and differentiation in human skin cells. *J. Invest. Dermatol.* **130:** 259–269; 2010.
[3] Nogueira, A. C.; Richena, M.; Dicelio, L. E.; Joekes, I. Photo yellowing of human hair. *J. Photochem. Photobiol. B* **88:**119–125; 2007.
[4] Nogueira, A. C.; Dicelio, L. E.; Joekes, I. About photo-damage of human hair. *Photochem. Photobiol. Sci.* **5:**165–169; 2006.
[5] Santos Nogueira, A. C.; Joekes, I. Hair color changes and protein damage caused by ultraviolet radiation. *J. Photochem. Photobiol. B* **74:**109–117; 2004.
[6] Hennessy, A.; Oh, C.; Diffey, B.; Wakamatsu, K.; Ito, S.; Rees, J. Eumelanin and pheomelanin concentrations in human epidermis before and after UVB irradiation. *Pigment Cell Res.* **18:**220–223; 2005.
[7] Ito, S.; Wakamatsu, K. Quantitative analysis of eumelanin and pheomelanin in humans, mice, and other animals: a comparative review. *Pigment Cell Res.* **16:** 523–531; 2003.
[8] Liu, Y.; Hong, L.; Wakamatsu, K.; Ito, S.; Adhyaru, B.; Cheng, C. Y.; Bowers, C. R.; Simon, J. D. Comparison of structural and chemical properties of black and red human hair melanosomes. *Photochem. Photobiol.* **81:**135–144; 2005.
[9] Barnicot, N. A.; Birbeck, M. S. C. The electron microscopy of human melanocytes and melanin granules. Biology of Hair Growth. Academic Press, New York, pp. 239–252; 1958.
[10] Blarzino, C.; Mosca, L.; Foppoli, C.; Coccia, R.; De Marco, C.; Rosei, M. A. Lipoxygenase/H_2O_2-catalyzed oxidation of dihydroxyindoles: synthesis of melanin pigments and study of their antioxidant properties. *Free Radic. Biol. Med.* **26:** 446–453; 1999.
[11] Liu, Y.; Simon, J. D. Isolation and biophysical studies of natural eumelanins: applications of imaging technologies and ultrafast spectroscopy. *Pigment Cell Res.* **16:**606–618; 2003.
[12] Uchoa, A. F.; Knox, P. P.; Turchielle, R.; Seifullina, N.; Baptista, M. S. Singlet oxygen generation in the reaction centers of Rhodobacter sphaeroides. *Eur. Biophys. J.* **37:** 843–850; 2008.
[13] Michaeli, A.; Feitelson, J. Reactivity of singlet oxygen toward amino acids and peptides. *Photochem. Photobiol.* **59:**284–289; 1994.
[14] Inbaraj, J. J.; Gandhidasan, R.; Murugesan, R. Photodynamic action of some naturally occurring quinones: formation of reactive oxygen species. *J. Photochem. Photobiol. A* **124:**95–99; 1999.
[15] Millington, K. R. Photoyellowing of wool. Part 1. Factors affecting photoyellowing and experimental techniques. *Color. Technol.* **122:**169–186; 2006.
[16] Forest, S. E.; Simon, J. D. Wavelength-dependent photoacoustic calorimetry study of melanin. *Photochem. Photobiol.* **68:**296–298; 1998.
[17] Toffoletti, A.; Conti, F.; Sandron, T.; Napolitano, A.; Panzella, L.; D'Ischia, M. Time-resolved EPR observation of synthetic eumelanin–superoxide radical pairs. *Chem. Commun. (Cambridge)* **33:**4977–4979; 2009.
[18] Rozanowska, M.; Sarna, T.; Land, E. J.; Truscott, T. G. Free radical scavenging properties of melanin interaction of eu- and pheo-melanin models with reducing and oxidising radicals. *Free Radic. Biol. Med.* **26:**518–525; 1999.
[19] Gauden, M.; Pezzella, A.; Panzella, L.; Napolitano, A.; d'Ischia, M.; Sundstrom, V. Ultrafast excited state dynamics of 5,6-dihydroxyindole, a key eumelanin building block: nonradiative decay mechanism. *J. Phys. Chem. B* **113:**12575–12580; 2009.
[20] Wilkinson, F.; Helman, W. P.; Ross, A. B. Rate constants for the decay and reactions of the lowest electronically excited singlet state of molecular oxygen in solution: an expanded and revised compilation. *J. Phys. Chem. Ref. Data* **24:**663–678; 1995.
[21] Wilkinson, F.; Helman, W. P.; Ross, A. B. Quantum yields for the photosensitized formation of the lowest electronically excited singlet state of molecular oxygen in solution. *J. Phys. Chem. Ref. Data* **22:**113–263; 1993.
[22] Redmond, R. W.; Gamlin, J. N. A compilation of singlet oxygen yields from biologically relevant molecules. *Photochem. Photobiol.* **70:**391–475; 1999.
[23] Severino, D.; Junqueira, H. C.; Gugliotti, M.; Gabrielli, D. S.; Baptista, M. S. Influence of negatively charged interfaces on the ground and excited state properties of methylene blue. *Photochem. Photobiol.* **77:**459–468; 2003.
[24] Haywood, R. M.; Lee, M.; Andrady, C. Comparable photoreactivity of hair melanosomes, eu- and pheomelanins at low concentrations: low melanin a risk factor for UVA damage and melanoma? *Photochem. Photobiol.* **84:**572–581; 2008.
[25] Ito, S. Optimization of conditions for preparing synthetic pheomelanin. *Pigment Cell Res.* **2:**53–56; 1989.
[26] Ito, S. The IFPCS Presidential Lecture: a chemist's view of melanogenesis. *Pigment Cell Res.* **16:**230–236; 2003.
[27] Vieira, V. C.; Severino, D.; Oliveira Jr., O. N.; Pavinatto, F. J.; Zaniquelli, M. E.; Ramos, A. P.; Baptista, M. S. Langmuir films of petroleum at the air–water interface. *Langmuir* **25:**12585–12590; 2009.
[28] Gracanin, M.; Hawkins, C. L.; Pattison, D. I.; Davies, M. J. Singlet-oxygen-mediated amino acid and protein oxidation: formation of tryptophan peroxides and decomposition products. *Free Radic. Biol. Med.* **47:**92–102; 2009.
[29] Saito, I. M. T.; Nakagawa, M.; Hino, T. Peroxidic intermediates in photosensitized oxygenation of tryptophan derivatives. *Acc. Chem. Res.* **10:**146–352; 1977.
[30] Criado, S.; Bertolotti, S. G.; Soltermann, A. T.; Garcia, N. A. Kinetic studies of the photosensitized oxidation ($O_2(^1\Delta_g)$-mediated) of tryptophan-alkyl esters in Triton X-100 micellar solutions. *J. Photochem. Photobiol. B* **38:**107–113; 1997.
[31] Ronsein, G. E.; Oliveira, M. C.; Miyamoto, S.; Medeiros, M. H.; Di Mascio, P. Tryptophan oxidation by singlet molecular oxygen [$O_2(^1\Delta_g)$]: mechanistic studies using ^{18}O-labeled hydroperoxides, mass spectrometry, and light emission measurements. *Chem. Res. Toxicol.* **21:**1271–1283; 2008.
[32] Baptista, M. S.; Indig, G. L. Effect of BSA binding on photophysical and photochemical properties of triarylmethane dyes. *J. Phys. Chem. B* **102:** 4678–4688; 1998.
[33] Katritzky, A. R.; Akhmedov, N. G.; Denisenko, S. N.; Denisko, O. V. ^{1}H NMR spectroscopic characterization of solutions of Sepia melanin, Sepia melanin free acid and human hair melanin. *Pigment Cell Res.* **15:**93–97; 2002.
[34] Napolitano, A.; De Lucia, M.; Panzella, L.; d'Ischia, M. The "benzothiazine" chromophore of pheomelanins: a reassessment. *Photochem. Photobiol.* **84:** 593–599; 2008.
[35] Machado, A. E. D.; Deandrade, M. L.; Severino, D. Oxidation of an electron-rich olefin induced by singlet oxygen—mechanism for tetraphenylethylene. *J. Photochem. Photobiol. A* **91:**179–185; 1995.
[36] Igarashi, N.; Onoue, S.; Tsuda, Y. Photoreactivity of amino acids: tryptophan-induced photochemical events via reactive oxygen species generation. *Anal. Sci.* **23:**943–948; 2007.
[37] Chin, K. K.; Trevithick-Sutton, C. C.; McCallum, J.; Jockusch, S.; Turro, N. J.; Scaiano, J. C.; Foote, C. S.; Garcia-Garibay, M. A. Quantitative determination of singlet oxygen generated by excited state aromatic amino acids, proteins, and immunoglobulins. *J. Am. Chem. Soc.* **130:**6912–6913; 2008.
[38] Jimenez-Banzo, A.; Sagrista, M. L.; Mora, M.; Nonell, S. Kinetics of singlet oxygen photosensitization in human skin fibroblasts. *Free Radic. Biol. Med.* **44:**1926–1934; 2008.
[39] Ragas, X.; Agut, M.; Nonell, S. Singlet oxygen in *Escherichia coli:* new insights for antimicrobial photodynamic therapy. *Free Radic. Biol. Med.* **49:**770–776; 2010.
[40] King, G. Sorption of vapours by keratin and wool. *Trans. Faraday Soc.* **41:**325–332; 1945.
[41] Nofsinger, J. B.; Liu, Y.; Simon, J. D. Aggregation of eumelanin mitigates photogeneration of reactive oxygen species. *Free Radic. Biol. Med.* **32:**720–730; 2002.