

Do thermal treatments influence the ultrafast opto-thermal processes of eumelanin?

Adalberto Brunetti · Marcella Arciuli · Leonardo Triggiani · Fabio Sallustio · Anna Gallone* · Raffaele Tommasi*[†]

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Abstract After light absorption melanin converts very rapidly the energy gained into heat. The time scale of this process ranges from tens of femtoseconds to a few nanoseconds. Femtosecond transient absorption allows for exploration of such photoinduced carrier dynamics in order to observe the de-excitation pathways of the biological complex.

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[†] Corresponding author

* A.G. and R.T. shared senior authorship

Anna Gallone

Tel.: +39 080 544 85 53

Fax: +39 080 544 85 38

E-mail: anna.gallone@uniba.it

Raffaele Tommasi

Tel.: +39 080 544 85 61

Fax: +39 080 544 85 68

E-mail: raffaele.tommasi@uniba.it

A. Brunetti · M. Arciuli · F. Sallustio · A. Gallone · R. Tommasi

Department of Basic Medical Sciences, Neuroscience and Sense Organs, University of Bari Aldo Moro, Policlinico Universitario, Piazza G. Cesare 11, 70124 Bari (Italy)

L. Triggiani

Department of Chemistry, University of Bari Aldo Moro, Campus Universitario, Via E. Orabona 4, 70125 Bari (Italy)

R. Tommasi · L. Triggiani

Institute for Physico-Chemical Processes (CNR-IPCF) – Bari Division, Italian National Research Council, c/o Department of Chemistry, University of Bari Aldo Moro, Via E. Orabona 4, 70125 Bari (Italy)

Here we report on the ultrafast relaxation of suspensions of Sepia melanin in DMSO at room temperature using a femtosecond broadband pump&probe technique by photoexciting in the UV and probing in the entire visible range. In particular, we focus on the possible role that different heat treatments, performed in the temperature range 30-80 °C might have on the relaxation of charge carriers photogenerated by UV radiation in such suspensions. Experimental data indicates that in all the investigated suspensions, photoexcited carriers always follow a tri-exponential route to relaxation. Moreover, we find that the relaxation time constants are essentially the same in all cases, within the experimental error. We take this as evidence that all the investigated suspensions essentially exhibit the same relaxation dynamics, regardless of the temperature at which the heat treatment has been performed, and of the heat-induced denaturation of the proteinaceous compounds bound to the photoactive pigment. Our experiments represent a significant step towards the understanding of the stability of melanin with respect to temperature changes.

Keywords Melanin · Transient Absorption · fs pump&probe · Ultrafast Spectroscopy

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1 Introduction

Melanin is the conventional name given to a family of macromolecular pigments present in the human body, as in most animal species [1], fungi [2], bacteria [3], and plants [4]. Human melanin is found in several organs, including the inner ear, central nervous system, and most importantly skin, eyes, and hair [5], where it plays a fundamental role as a protecting shield against thermal, chemical and biochemical stresses [6,7].

Furthermore, melanin shows other technologically relevant properties, like hybrid ionic-electronic conductance and redox reversibility, which make it an interesting candidate for biosensors, batteries, capacitors, and other novel organic-electronics devices [8–13].

Biologically speaking, natural melanin behaves as a double-edged sword, as it is basically a photo-protective intracellular antioxidant agent able to scavenge reactive nitrogen oxide species formed after UV radiation in skin melanocytes and retinal epithelial cells [14–16], but under specific stress conditions it can also act as a potentially harmful pro-oxidant agent [17]. Thanks to this combination of unique properties, melanin has acquired a role in biomedicine, *e.g.* in photothermal therapy, chemotherapy, and theranostics [13]. Aside from its antioxidant activity, melanin also efficiently binds metal ions, thanks to the chelating properties of its functional groups [18,19], and acts as a UV shield, being able to absorb tissue-damaging radiation and dissipate its energy by converting it into heat with very high efficiency [20–24]. Melanin is found in nature mainly under three forms, namely eumelanin (a dark-brown pigment that is prevalent in humans and microorganisms), pheomelanin (a yellow-to-reddish pigment), and neuromelanin (a pigment mainly found in human brain) [25].

Physically speaking, melanin is a system characterized by a set of unique optical properties, such as broad UV-Vis light absorption [26], low luminescence and scattering [6], and the ability to convert ionizing radiation into chemical energy [27]. The photophysical and photochemical processes involved in melanin response to light are extremely fast, acting on a timescale ranging from tens of

femtoseconds to some nanoseconds [28]. Thanks to our spectroscopic system (thoroughly described in Materials and Methods) we are able to investigate such ultrafast dynamics. Eumelanin has been shown to convert most of the UV energy absorbed into heat in less than 1 ns [6], but the microscopic mechanism of this photo-conversion process, as well as the structural effects on this and other relaxation pathways available in melanin complexes, are unclear [23,24].

Chemically speaking, eumelanin is a copolymer constituted of 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) units, and their derivatives [18]. However, the structural organization of this pigment is still debated. This is a critical issue for understanding its optical and charge-transport properties, and further complicates a full description of structure-property relations. In 2006 Kaxiras and coworkers [29] proposed a structural model of eumelanin that has been recently supported by electrochemical experiments [10]. In this model, the building blocks of eumelanin pigments are depicted as small planar porphyrin-like oligomers containing 4 to 8 DHI and DHICA monomer units, which in turn layer in groups of 3-4, forming π -stacked nanoaggregates. Together with this scheme, other models, according to which different cyclic molecular arrangements can exist in eumelanin-like matter, have been proposed and corroborated by computational studies [30,31]. In any case, regardless of the actual structure, it is a fact that oligomer units can exhibit radically different functionalities, due to the intrinsic chemical diversity of DHI and DHICA groups. This gives a significant chemical heterogeneity to the assembled macroscopic pigments, enabling them to perform a wide range of functions.

Eumelanin isolated from the ink sac of *Sepia Officinalis* (traditionally known as *Sepia melanin*, SM) is currently accepted as a standard for natural melanin [32,33]. It has been demonstrated that SM oligomeric backbone contains approximately 20% of DHI- and 75% of DHICA-derived units [34,35]. Some of these units are chemically and sterically able to anchor functional groups of proteins forming complexes called *melanoproteins*, so that the

melanin core is always surrounded by a certain amount of proteinaceous matter [13,36]. SM has been demonstrated to share several features with neuromelanin: they form aggregates very similar in size and shape, with analogous surface-to-volume ratio and surface chemistry [37]. The last one is a crucial parameter when considering interactions with solutes and oxidative processes.

Neuromelanin is supposed to have a role in neuroprotection and in Parkinson's disease; in fact, a loss of neuromelanin has been correlated to an increase of iron levels in the brain of Parkinson's disease patients [18,38–40].

It is well known that high temperatures alter the thermodynamics of folding and assembly of proteins, primarily through reduction in configurational entropy [41]. This fine balance between stability and lability, which is found in virtually all proteins, in all taxa, is a strikingly consistent feature of protein evolution [42]. Most proteins begin to lose their functional structure at temperatures higher than about 50 °C [43]. On the other hand, melanins are reported to show a high thermal stability in a wide temperature range, depending on the origin of the pigments [44]. In any case, for most of them, the structure is preserved at temperatures at least up to 100 °C [45–48]. Furthermore recently developed polyimide/sepia eumelanin nanocomposites have shown enhanced thermal properties with degradation temperatures above 500 °C [49].

The aim of this work is to investigate the influence of thermal treatments performed at different temperatures on the photoconversion property of eumelanin by means of femtosecond Transient Absorption (TA) spectroscopy. TA is a powerful technique enabling the measurement of ultrafast photoinduced processes occurring in excited electronic states on the scale of femtoseconds. It is a pump-probe time-resolved optical spectroscopy technique with a wide range of applications including the investigation of ultrafast biophysical processes [50,51]. We prepared suspensions of SM, performed different thermal treatments on them, and then we applied TA to explore the ultrafast charge carrier dynamics of each sample. In order to monitor only the effects of thermal treat-

ment on eumelanin, minimizing all other possible sources of damage, we have taken care to operate always in conditions of low photoexcitation intensity. Indeed it is known that when exposed to light, melanins can degrade, photoionize or produce free radicals [52–55], although eumelanin was shown to have a quite high resistance to this kind of damage [53]. Here, we demonstrate that SM shows a relaxation dynamics that is independent of thermal treatments up to at least 80 °C, even though temperature is well known to affect the stability of proteinaceous compounds surrounding the melanin core.

2 Materials and methods

2.1 Sample preparation

Commercial Melanin powder from *Sepia officinalis* purchased from Sigma Aldrich was used to prepare the investigated melanin suspensions.

Dimethylsulfoxide (DMSO) melanin suspension was prepared by dissolving 4 mg of eumelanin powder into 20 ml of DMSO-CH₃OH mixture (ratio 1 : 20). The above mixture was initially sonicated for 30 min and then centrifuged (Heraeus Megafuge 1.0 R) at 3000 rpm at 4 °C for 30 min, in order to remove larger suspended aggregates. The investigated melanins were obtained starting from the supernatant containing the native pigment with a proteinaceous fraction ranging from 5% to 7.6% [56,57]. Different samples were prepared by heating the supernatant on a hot plate and keeping them for an hour at a fixed temperature, ranging from 30 to 80 °C respectively, with a 10 °C step, and then used for optical measurements.

2.2 Optical spectroscopy investigations

Femtosecond TA experiments were carried out in a typical non-collinear configuration at room temperature (Fig. 1). A commercial diode-pumped Ti:Sapphire femtosecond oscillator (Mai Tai, Spectra Physics), operating at a repetition rate of 78 MHz produced pulses of $\simeq 100$ fs width. These pulses were stretched and then amplified by a regenerative Ti:Sapphire

amplifier (Spitfire Pro, Spectra Physics), pumped by a Q-switched Nd³⁺:YLF laser (Empower, Spectra Physics) at 1 kHz repetition rate, and finally compressed to produce more than 3 mJ, $\simeq 95$ fs pulses at 799 nm. The amplified laser beam was divided by a beam splitter into two parts (90% reflected and 10% transmitted); the reflected pulses were sent to an OPA (optical parametric amplifier, TOPAS-C, Spectra Physics) that provided pump pulses tunable in a broad spectral range (290 – 1600 nm). At the selected pump wavelength, the single pulse energy was $\simeq 3\mu\text{J}$. The pump beam crossed a depolarizer, neutral density filters, then an optical chopper and eventually it was focused onto the sample. The pump pulses were focused onto a spot of $\simeq 100\ \mu\text{m}$ diameter, yielding excitation energy densities on the sample of $\simeq 2\ \text{mJ} \cdot \text{pulse}^{-1} \cdot \text{cm}^{-2}$ (corresponding to about $3.5 \cdot 10^{15}$ photon $\cdot \text{pulse}^{-1} \cdot \text{cm}^{-2}$). The average pump power on the suspension was $\simeq 1.5\ \text{mW}$. In biological tissues, usually laser pulses with duration less than 1 μs are not responsible of thermal damage unless their repetition rate is higher than 1 kHz [58,59]. However, ever ultra-short laser pulses (duration < 10 ps), each of which has negligible thermal effect, may result in a significant increase in temperature if in the focal volume the rate of heat removal is lower than the rate of heat generation [58,60]. For this reason, we employed a magnetic centrifugal stirrer to mix the suspension in the cuvette. This stirring, together with low average pump power, allows us to rule out any possible significant rise of temperature due to heat accumulation in the focal volume. The beam transmitted by the beam splitter after the output of the Spitfire Pro was time delayed by using a variable optical delay line, and then focused onto a CaF₂ crystal to generate a white light continuum (WLC) in a broad spectral range (450 – 700 nm). WLC was used as a probe and focused onto the sample within the pump spot area. After passing through the sample, the WLC was detected using a fiber-coupled CCD spectrometer. The time resolution of the experiment was obtained by the width of the temporal cross-correlation between pump and probe pulses ($\simeq 200$ fs). For each transient absorption measurement the chromatic aberrations were significantly

reduced using a chirp correction software procedure (Surface Xplorer by Ultrafast Systems).

Transient Absorption. In femtosecond-TA measurements, the measured differential absorption ΔA is a function of the probing wavelength (λ) and time delay (τ) between the pump and probe laser pulses [61]; at each time delay the transmitted intensity of the probe pulse both in absence and in presence of excitation is recorded, and the signal is given by

$$\Delta A(\lambda, \tau) = -\log_{10} \left(\frac{I_{TP\&p}(\lambda, \tau)}{I_{Tp}(\lambda)} \right) \quad (1)$$

where $I_{TP\&p}(\lambda, \tau)$ and $I_{Tp}(\lambda)$ are the transmitted intensity of the probe pulse in presence and in absence of the pump pulse, respectively [62]. A positive $\Delta A(\lambda, \tau)$ is achieved when the transmitted intensity of the probe pulse in presence of excitation is smaller than the transmitted intensity without the pump pulse, which means that a photoinduced absorption process occurs; on the contrary, a negative $\Delta A(\lambda, \tau)$ is related to photobleaching and/or photoemission processes. This kind of technique gives the possibility to investigate spectral changes due to electronic transitions, which occur in the sample after excitation [63], and compare the de-excitation dynamics among natural melanin pigments which have undergone different thermal treatments.

3 Results and discussion

The typical suspension studied is characterized by the well-known UV-Vis absorption spectrum of eumelanin, *i.e.* strong absorption in the UV spectral region and monotonic decrease with increasing wavelengths. Such a profile is well known in the literature and it has been partially attributed to scattering and electronic effects at the molecular level [26]. All samples investigated in this paper exhibit the same expected absorption features (data not reported), confirming that the thermal treatments do not affect the UV-Vis light absorption mechanisms in SM.

TA measurements were performed pumping the SM suspensions in the spectral region of maximum absorption ($\lambda_{pump} = 350\ \text{nm}$) and probing

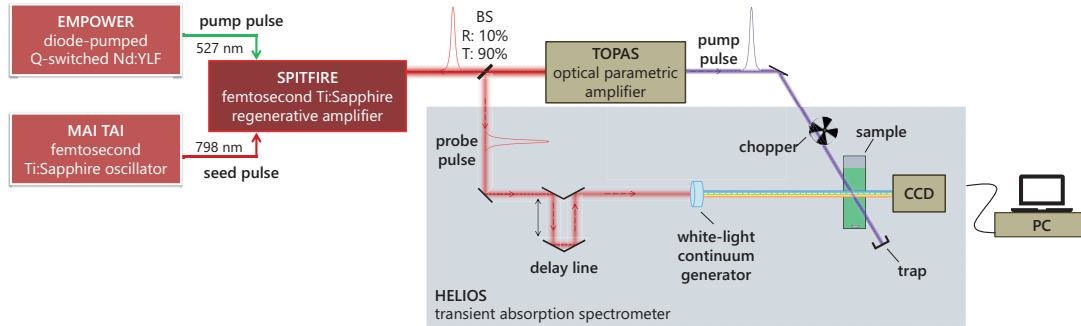


Fig. 1 Schematic representation of experimental pump-probe setup used.

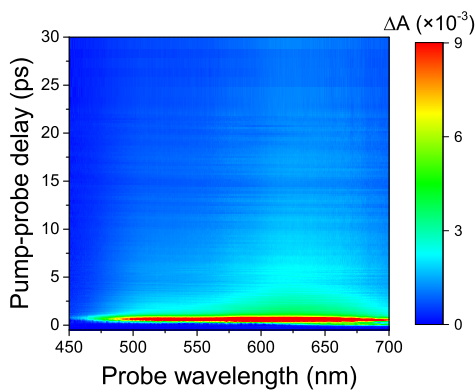


Fig. 2 Contour plot of transient absorption ΔA of pristine melanin suspension pumped at 350 nm and probed in the Vis range (450-700 nm) as a function of pump-probe delay (restricted to the first 30 ps for clarity).

over the entire Vis range (Fig. 2). In these experimental conditions we avoid any kind of nonlinear effects due to Raman activity of the solvent [64]. In Fig. 2 we show the TA of the untreated melanin sample (pristine). Contour plots obtained from the other samples are all consistent with the untreated one. The signal is always positive, demonstrating that after melanin pigments are excited in the UV region by pump pulse, the probe pulse will experience absorption from excited states throughout the investigated range [65,28]. The spectral response is extended over the whole visible range with maximum ΔA positive signal around 625 nm recovering in a few tens of picoseconds. This behavior clearly confirms that SM is a system characterized by an efficient thermal relaxation that allows UV

energy absorbed to be converted into heat via very fast internal processes. In this respect, it is worth noting that most of the signal decays in less than 2 ps. The absence of features ascribable to fluorescence arises from the small luminescence quantum yield of melanins [66]. As a result, non-radiative relaxation processes prevail in carrier recombination [67], allowing an effective conversion of UV photon energy into heat [24], and this process is the basis of melanin's physiological function of skin shielding.

In detail, we investigated the ultrafast dynamics probing at the maximum TA signal wavelength, as shown in Fig. 3 depicting normalized femtosecond-TA decays as black open circles. The decay of an untreated suspension is also reported for comparison (upper trace). All these decays are fitted (red lines in Fig. 3) with the convolution function $C(t)$ between the instrumental response $R(t)$ and a sample response $S(t)$:

$$C(t) = R(t) \otimes S(t) = \int_0^{+\infty} R(t') \times S(t-t') dt' \quad (2)$$

The instrumental response function (IRF) $R(t)$ is assumed to be Gaussian [68]:

$$R(t) = \frac{1}{\sqrt{2\pi} \cdot \sigma} e^{-\frac{t^2}{2\sigma^2}} \quad (3)$$

where σ is related to the experimental full width at half maximum (FWHM) of the cross correlation between pump and probe pulses according to the equation $\text{FWHM} = 2\sqrt{2\ln 2}\sigma$. The sample response $S(t)$ is a tri-exponential decay function with time constants τ_i and amplitudes A_i ($i=1,2,3$),

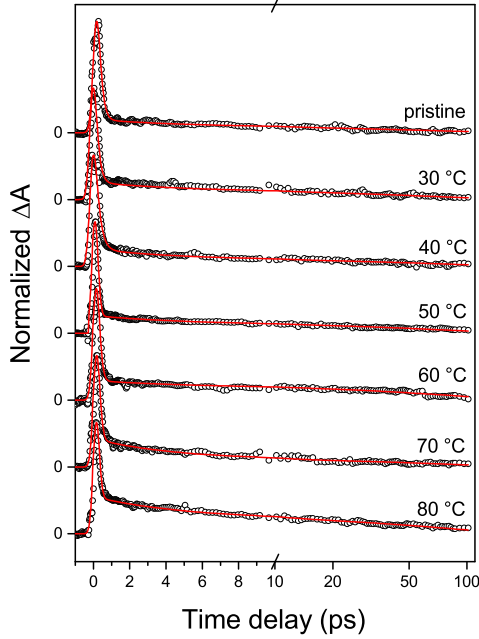


Fig. 3 Femto-TA decay at $\lambda_{probe} = 625$ nm as a function of the time delay between pump and probe pulses, upon excitation at $\lambda_{pump} = 350$ nm of melanin suspensions which have undergone to different thermal treatments. x axis is linear for the first 10 ps and logarithmic above in order to highlight the ultrafast dynamics occurring close to the zero-delay region. Each decay curve is normalized to the peak value for the sake of comparison. The curves have been vertically shifted for clarity.

Table 1 Fitting parameters of tri-exponential decay function (Eq. 4) and corresponding τ_{av} . All time constants are expressed in ps.

Temperature	τ_1	τ_2	τ_3	R^2	τ_{av}
Pristine	0.15	5.7	70	0.977	56.9
30 °C	0.15	5.2	65	0.975	57.0
40 °C	0.15	4.8	65	0.962	58.8
50 °C	0.15	4.8	65	0.945	62.0
60 °C	0.15	5.0	65	0.976	61.6
70 °C	0.15	5.5	67	0.980	54.8
80 °C	0.12	5.3	65	0.924	59.1

in agreement with Ref. [69] for similar melanin samples:

$$S(t) = \sum_{i=1}^3 A_i e^{-\frac{t}{\tau_i}} \quad (4)$$

The fitting parameters are reported in Table 1 together with the average decay time evaluated by using equation 5 according to Ref. [70]:

$$\tau_{av} = \frac{\sum_{i=1}^3 A_i \tau_i^2}{\sum_{i=1}^3 A_i \tau_i} \quad (5)$$

The relative experimental uncertainty never exceeds 20%. For all samples the decay time associated to the first component of TA dynamics (τ_1 in equation 4) is comparable with $\sqrt{2} \cdot \sigma$, *i.e.* the characteristic time constant of the Gaussian IRF profile (as defined in equation 3), experimentally evaluated in about 150 fs. This means that for τ_1 close to σ , in the first picoseconds, the fit decay approaches the cross-correlation function and it is not possible to find a clear signature of τ_1 in the convolution function $C(t)$. Furthermore, the slower component of the decay ($A_3 \cdot e^{-\frac{t}{\tau_3}}$) appears to be the dominant one over the whole recombination process, as evidenced by the magnitude of τ_{av} which is only slightly lower than τ_3 . Interestingly, Table 1 also highlights that the dynamics associated with all samples present very similar decay parameters, all lying within experimental uncertainty, and comparable to those of the pristine suspension. It is worth noting that, on random samples, we performed control measurements a week after thermal treatment and then, again, about one month after to monitor for possible variations but no significant changes were observed.

These results give a strong experimental evidence that the thermal treatment does not alter the dynamics of photo-induced carriers, allowing us to propose that the structure of the photoactive part of SM is not compromised. Since proteins are subjected to denaturation at temperatures higher than about 50 °C, the proteinaceous part of our samples will have lost its functional structure when Sepia melanin was treated with temperatures of 50-80 °C. Nonetheless, our results clearly demonstrate that the electronic recombination channels of melanin complexes are not affected by this protein denaturation process, suggesting that the capacity that these melanins have to convert UV radiation into heat is fully preserved in the investigated temperature range.

4 Conclusion

In summary, Sepia melanin suspensions in DMSO were prepared and then heat treated at different temperatures in the range 30-80 °C. Their broadband transient absorption was investigated in the Vis range by a femtosecond pump&probe technique. All experimental data, obtained by photoexciting at 350 nm, were fitted using a tri-exponential response function, with the evaluated time constants comparable to those previously reported in literature. We found that heat treatments do not in any way affect the relaxation dynamics of eumelanin, indicating that the ultrafast recombination of charge carriers excited in the UV does not change even if the proteinaceous component surrounding the photoactive core of eumelanin is drastically denaturated by the thermal treatment. This shows that the recognized melanin protection function against cell damage caused by UV radiation, in particular the melanin capability to convert UV-Vis radiation into heat, is preserved even at relatively high temperatures.

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