Metal ion influence on eumelanin fluorescence and structure

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Abstract

Melanin has long been thought to have an unworkably weak and complex fluorescence, but here we study its intrinsic fluorescence in order to demonstrate how metal ions can be used to control the rate of formation, constituents and structure of eumelanin formed from the well-known laboratory auto-oxidation of 3, 4-dihydroxy-L-phenylalanine (L-DOPA). The effect on eumelanin absorption and fluorescence of a range of solvated metal ions is reported including Cu, Zn, Ni, Na and K. Monovalent cations and Zn have little effect, but the effect of transition metal cations can be considerable. For example, at pH 10, copper ions are shown to accelerate the onset of eumelanin formation, but not the rate of formation once it commences, and simplify the usual complex structure and intrinsic fluorescence of eumelanin in a way that is consistent with an increased abundance of 5,5-dihydroxyindole-2-carboxylic acid (DHICA). The presence of a dominant 6 ns fluorescence decay time at 480 nm, when excited at 450 nm describes a distinct photophysically species, which we tentatively assign to small oligomers. Copper is well-known to normally quench fluorescence, but increasing amounts of copper surprisingly leads to an increase in the fluorescence decay time of eumelanin, while reducing the fluorescence intensity, suggesting copper modification of the excited state. Such results have bearing on diverse areas. The most accepted morphology for melanin is that of a graphite-like sheet structure, and one which readily binds metal ions, an interaction that is thought to have an important, though as yet unclear bearing on several areas of medicine including neurology. There is also increasing interest in bio-mimicry by preparing and labelling sheet structures with metal ions for new electronic and photonic materials.

Keywords: Melanin, eumelanin, 3, 4-dihydroxy-L-phenylalanine, L-DOPA, copper ions, fluorescence, polymerization

1. Introduction

Metal ions play many important roles in human metabolism, including acting as reaction centres in protein and enzymes, and facilitating catalysis, transport, signalling and aggregation. In addition to being vital for normal biological function, metal ions (and transition metal ions in particular), in the absence of regulation, are thought to be implicated in the generation of reactive species such as free radicals, that can lead to oxidative stress and the cell damage associated with several diseases. These include neurological conditions such as Alzheimer's, Parkinson's and Huntington's. In addition, transition metal ions are known to accelerate the aggregation of certain biological systems. For example, the aggregation of beta-amyloid, that is associated with the origins of Alzheimer's disease, is accelerated by copper and zinc ions, which are also found on autopsy at abnormally high concentrations (~ mM) in neuro senile plaques and fibrils [1]. Therapies capable of inhibiting and reversing this aggregation are the subject of global research [2].

Here we focus on melanin, a ubiquitous pigment perhaps best known for its photo-protection of skin, but also implicated in melanoma, the most aggressive form of skin cancer. The synthesis of the most common natural form of melanin (eumelanin) occurs in melanosomes, which are organelles within melanocytes, and proceeds from the amino acid L-tyrosine though a series of chemical and enzymatic steps facilitated by the copper-containing glycoprotein tyrosinase [3]. A very close representation of natural eumelanin is readily synthesized in the laboratory from the auto-oxidation of 3, 4-dihydroxy-L-phenylalanine (L-DOPA) [3]. Both natural and synthetic melanin are understood to be heteropolymers composed predominantly of 5,6- dihydroxyindole (DHI) and 5,5-dihydroxyindole-2-carboxylic acid (DHICA) units and their redox forms. The evidence points towards these constituents being arranged in a π–stacked graphite-like sheet structure [4-7], although the actual structural arrangement of the constituents remains unknown [8]. Figure 1 summarizes the main stages of the synthesis [3] we will consider.

There is an interesting range of disease correlations around melanin, including those in neurology and cancer. For example Parkinson's disease is associated with abnormally low levels of neuromelanin, increased risk of melanoma [9], and can be treated by L-DOPA, which has been shown to disaggregate amyloid fibrils α-synuclein and beta-amyloid that are known to be associated with Parkinson's disease and Alzheimer's disease respectively [10]. Also, analogous to beta-amyloid, organic accelerators and inhibitors of eumelanin aggregation are being investigated [11].

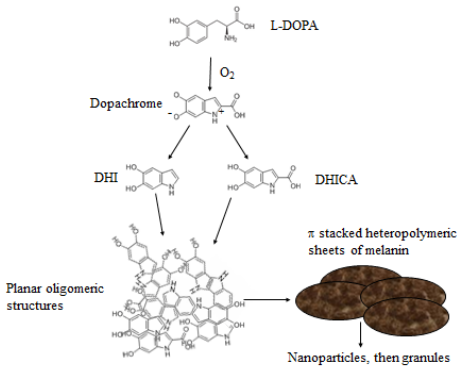

Figure 1. Simplified auto-oxidation scheme of L-DOPA to produce eumelanin

One difference between natural and synthetic melanin is the presence of copper in the former. Correlating with this is the greater relative abundance of DHICA with respect to DHI in natural melanin (50%) as compared to melanin synthesized from L-DOPA (10%), and in eumelanin synthesized at higher than physiological pH [12]. Moreover, addition of metal ions, particularly Cu^{2+}, has previously been found to accelerate the formation of eumelanin from L-DOPA. Cu^{2+} has been suggested to bind predominantly to hydroxyl groups at pH 7-11[13] and increase the concentration of DHICA in synthetic eumelanin formed from L-DOPA in the presence of tyrosinase by catalyzing the re-arrangement of the intermediate dopachrome in favour of DHICA rather than DHI [14].

Recently melanin has taken on a wider research interest beyond biology in terms of a possible technology platform for memory, switching and photonics [15, 16]. Such potential opportunities have been realized largely because of melanin's unique combination of physical properties, which include a broad spectral absorption, low fluorescence quantum yield, paramagnetism, semiconducting and hydration-dependent conduction and photoconduction [8]. Although melanin structural modification for applications in technology can in principle be achieved using pH and dopants such as metal ions, melanin synthesis is complex and the pathway to the end products poorly charted.

Indeed, while eumelanin is well-known to strongly bind metal ions the effect on the formation kinetics, structure and associated fluorescence have been little explored. In this paper we investigate such effects in respect of findings that have bearing on biology as well as the modification of eumelanin's sheet structures for potential use in bio-mimicked technology.

Although ideal for tracking changes in many biological processes, fluorescence has not been the technique of choice in melanin research and application because of melanin's low fluorescence quantum yield, overlapping spectral components and complex fluorescence decay kinetics. Nevertheless here we report the usefulness of fluorescence spectroscopy in monitoring and characterizing the effect of metal ions in modifying eumelanin's structure and report how copper ions in particular are effective in simplifying the end products and concomitant fluorescence photophysics.

2. Experimental

While eumelanin is formed from L-DOPA over a wide range of pH through auto-oxidation, the time it takes for the reaction to conclude is generally shortened at alkaline pH with respect to physiological pH. A stock solution of L-DOPA

at 1mM concentration was first mixed by ultra-sonication for 30 min and measurements were typically taken at an L-DOPA concentration of 40 μM. Metal ion salts were added from aqueous stock solution to a final concentration range of 4 μM to 4 mM. Salts used were $CuSO_4$, $CuCl_2$, $ZnCl_2$, $NiCl_2$, NaCl and KCl. DHICA was purchased from Toronto Research Chemicals Inc, Canada. All other chemicals were obtained at the highest available purity from Sigma Aldrich, UK. To measure fluorescence spectra we used a Horiba Scientific FluoroLog fluorimeter. Absorption measurements were performed using a Jasco V-660 Spectrophotometer. To obtain kinetics of fluorescence and absorption, spectral measurements were at a few minute intervals until the spectrum reached steady-state. To initiate the start of the eumelanin synthesis 50 μl ammoniac was added to adjust to pH 10. Fluorescence decay measurements were performed using time-correlated single-photon counting [17] with a Horiba Jobin Yvon IBH FluoroCube. Excitation was with either a 1 MHz NanoLED [18] laser diode emitting at 452 nm and < 200 ps fwhm or a 40 MHz DeltaDiode at 378 nm.

3. Results and discussion

3.1 Absorption spectra

Given the weakness of melanin fluorescence, absorption studies were the basis of much of the early structural information that could be gleaned. Melanin is characterized by its broad-band absorption spectrum covering the visible spectrum all through to the near UV. The temporal evolution of this absorption spectrum during the formation of eumelanin from L-DOPA reveals a highly organized process with characteristic phases. Within the first minute of adjusting the pH to 10 two absorption peaks appear at 240 nm and 295 nm that are typical of indole structures (see figure 2(a)). During the first phase these peaks rise with the peak at 295 nm displaying a slight bathochromic shift. At, for examples, 40 μM L-DOPA and pH 10 the first phase lasts for about 12 min. In a second phase isobestic points form within the absorption spectrum and the absorption evolves towards the characteristic melanin broad-band absorption spectrum (figure 2(a)) with a new peak initially appearing that centres at ~ 440 nm. The identification of these peaks is today still not totally unambiguous. For example, 440 nm is close to, but not identical to the 475 nm absorption reported for dopachrome at pH 5.5 [19, 20]. Such a long wavelength of 440 nm would not seem to be attributable to monomeric indole structures such as DHI and DHICA, but might represent their extended polymeric π conjugations. The absorption peak ~ 290 nm, which undergoes with time a bathochromic shift in figure 2(a), has also been attributed to both DHICA [19, 20] and dopachrome [21]. However, the latter is an unstable intermediate and it is generally agreed that DHI, DHICA and their redox forms are the dominant stable species [8]. The absorption peak measured for DHICA alone at ~ 310 nm in Figure 2(b) confirms the "end-point" species absorbing in figure 2(a) to be DHICA.

This highly organized process in eumelanin formation is greatly altered in the presence of metal ions. For example, with the addition of 400 μM Cu^{2+} the initial phase showing the formation of DHICA from dopachrome is either absent or too rapid to be observed and the absorption is strongly shifted towards shorter wavelengths with the peak at 440 nm already present at the outset (figure 2(c)). In addition the final absorption spectrum is more akin to the structureless and

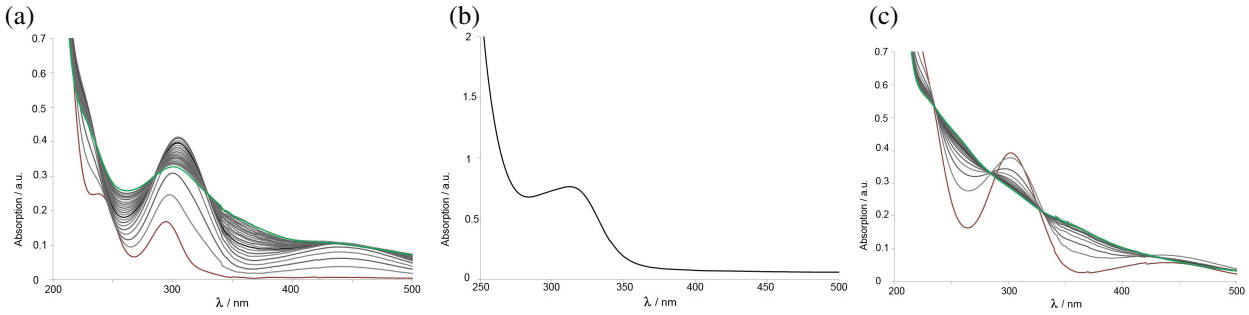

Figure 2. Evolution of absorption spectra at pH 10 for eumelanin synthesis. (a) For 40 μM L-DOPA, (b) DHICA absorption spectrum, (c) melanin formed from 40 μM L-DOPA in the presence of 400 μM $CuSO_4$. For figures (a) and (c) the first measurement marked in red is at 1 min after adjusting the pH to 10, the measurement after 30 min is marked in black, and the final spectrum recorded after 90 min is marked in green. Measurements in between these times are at 4 min interval and are in grey. For 2(a) the peak at ~ 305 nm is reached after 22 min.

exponential-like continuum of natural eumelanin with increasing absorption at shorter wavelengths. Figure 3 compares the effect of Cu^{2+}, Ni^{2+}, Zn^{2+} and K^{+} on the eumelanin synthesis in comparison to having no metal ions present. Cu^{2+} leads to an increase in absorption apart from above 375 nm suggesting arrestment of the eumelanin synthesis before extended π conjugations of polymeric forms of indoles are formed. In the case of 400 μM Cu^{2+} the absorption at 480nm is decreased by 70%. With 400 μM Ni^{2+} the reduction is even more pronounced at 80%. Other physiologically relevant ions like Zn^{2+} or K^{+} do very little to alter the absorption of eumelanin. Indeed, there is no marked decrease in eumelanin absorption at wavelengths > 375 nm, suggesting less interaction. For example 400 μM Zn^{2+} only decreases eumelanin absorption at 480 nm by 6% and 400 μM K^{+} leads to no reduction. Only Cu^{2+} causes a dramatic change in the final eumelanin absorption spectrum at wavelengths < 350 nm. Absorption at 275 nm is increased by 250% in the presence of Cu^{2+}. The presence of Ni^{2+} leads to a slight decrease in absorption over a broad range from 230 nm to 500 nm.

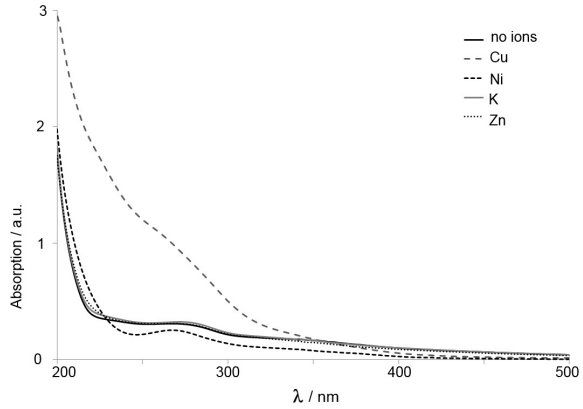

Figure 3. The absorption of melanin formed in the absence or presence of different metal ions. Metals have been added at the start of the melanin formation as solutions of $CuSO_4$, $NiCl_2$, $ZnCl_2$ and KCl each at a concentration of 400 μM. The melanin formation took place at pH 10 and the absorption spectra were measured 4 days after the onset of the reaction.

3.2Fluorescence spectra

The changes in the absorption spectrum of eumelanin induced by metal ions are reflected in the fluorescence spectrum. Exciting at 450 nm the relative fluorescence intensity in the presence of Cu^{2+} and Ni^{2+} ions mirrors the arrestment of species responsible for the absorptions above 375 nm (figure 4).

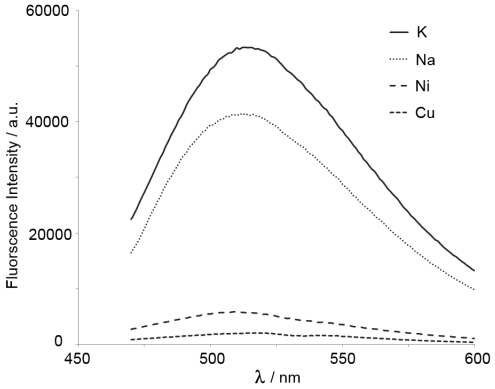

Figure 4. The fluorescence emission of eumelanin in the presence of ions excited at 450 nm. Metal ions have been added as solutions of $CuSO_4$, $NiCl_2$, NaCl and KCl, each at a concentration of 400 μM. The eumelanin formation took place at pH 10 and the spectra were measured 4 days after the onset of the reaction.

In the absence of copper the formation of melanin yields a characteristic peak around 520 nm (figure 5(a)) that has previously been loosely attributed to polymer structures [22]. Adding Cu^{2+} to the reaction changes the evolution of the spectra [23]. At a 4 µM $CuSO_4$ concentration we observe fluorescence rapidly rising at 480 nm (Fig 5(b)). This becomes more pronounced with increasing Cu^{2+} concentration. At 4 mM $CuSO_4$ the initial peak at 530 nm nearly vanishes and the emission at 480 nm becomes dominant (Fig 5(c)) demonstrating how copper ions significantly modify the synthesis.

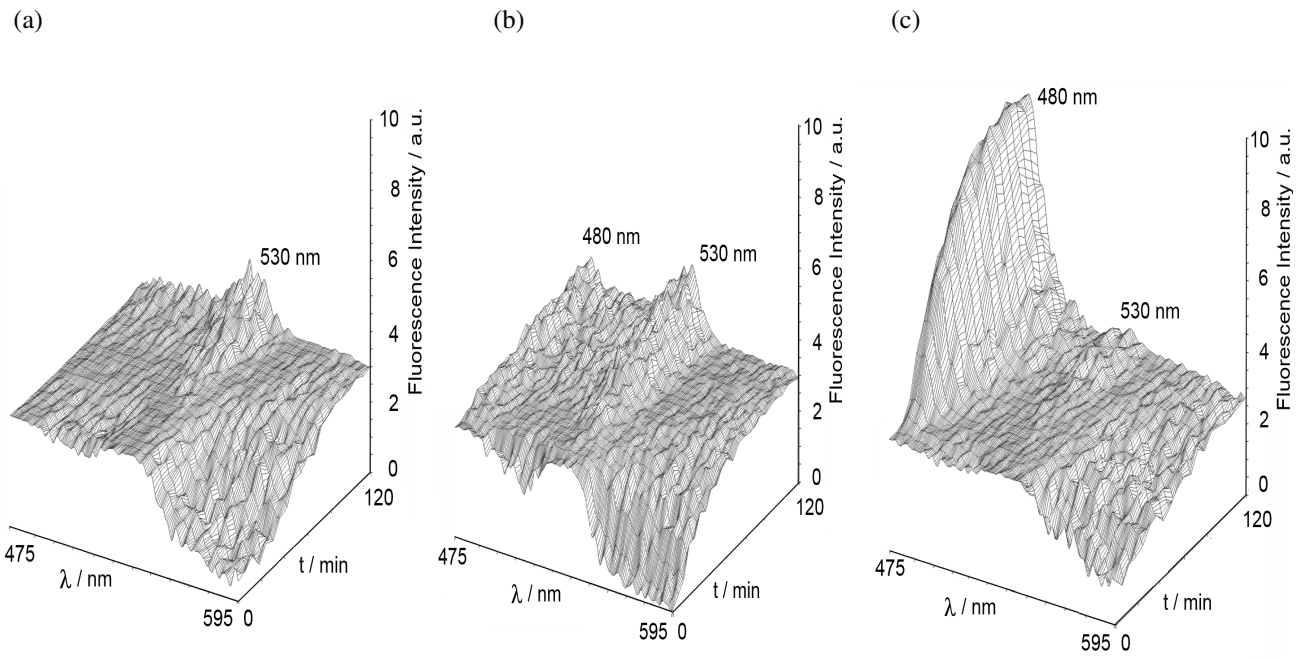

Figure 5. The evolution of eumelanin intrinsic fluorescence excited at 450 nm. (a) No copper present (b) 4 µM. $CuSO_4$ (c) 4 mM. $CuSO_4$ [23].

Exciting the eumelanin at 375 nm as it forms reveals a more complex emission spectrum than at 450 nm. Monitoring fluorescence from a solution of 40 µM L-DOPA over 1 hour after shifting to pH 10 shows an initial fluorescence peak around 530 nm and after about 10 min a distinct peak at 460 nm develops and the initial peak at 530 nm shifts towards shorter wavelength (figure 6(a)), eventually being less intense than the peak at 460 nm. The presence of 400 µM Cu^{2+} leads to the omission of the initial peak at 530nm (figure 6(b)) and this is consistent with when exciting at 450 nm (figure (5)). Over the course of 1 hour fluorescence slowly rises but remains about 85% below the fluorescence without Cu^{2+}. Such effects are less pronounced with a lower concentration of 40 µM Cu^{2+}. The presence of Cu^{2+} leads to the formation of melanin with much increased absorption and decreased fluorescence characteristics. Both K^+ as well as Zn^{2+} had little influence on melanin fluorescence.

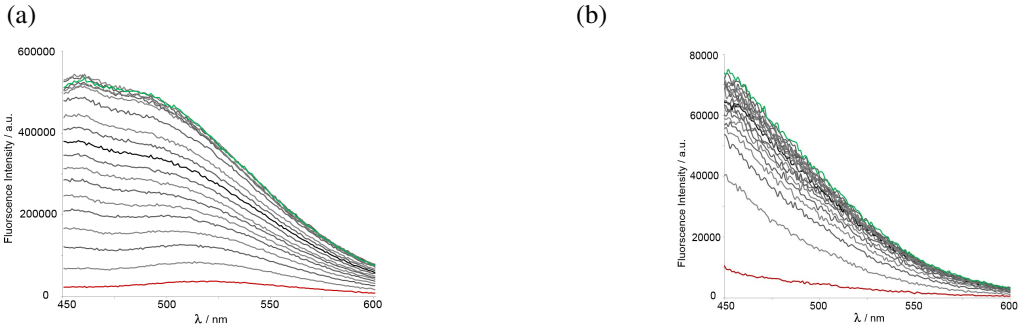

Figure 6. Fluorescence spectrum of 40 μM L-DOPA at pH 10 excited at 375 nm in the presence of (a) No ions and (b) 400 μM $CuSO_4$. The first spectrum recorded is marked in red. Subsequent spectra were taken at 3 min intervals with the spectrum at 30 min marked in black and the final spectrum taken at 60 min marked in green.

Recently we have investigated the sheet structure of eumelanin formed from L-DOPA using the sheet-sensing extrinsic probe thioflavin T (ThT) that has been widely used to investigate sheet structures in beta-amyloid aggregation [7]. The rise of extrinsic fluorescence *I(t)* due to ThT could be described well by a sigmoidal function with its inherent property to describe the time delay associated with protomolecule formation and subsequent sheet structures [7]. Namely,

$$I(t) = I(0) + \frac{\alpha}{1+\exp(-k(t-t_{1/2}))} \qquad (1)$$

where *I(t)* is the fluorescence intensity at a time *t*, α the maximum fluorescence above the background, $t_{1/2}$ the time taken for the fluorescence to reached half its maximum and k the rate of eumelanin formation.

Figure 7(b) shows a typical curve we obtained with ThT fluorescence at 480 nm in the absence of a metal ion that is described by equation 1, reflecting a time delay of $t_{1/2}$ = 91.6 min, k = 0.15 ± 0.05 min^{-1} at pH 10 as eumelanin sheets are formed [7]. Figure 7(a) compares the growth in intrinsic fluorescence, also at 480 nm, and taken from figure 5(c) without ThT, but with 4 mM of copper present. When fitted to equation 1 the rise in fluorescence caused by the copper yields k = 0.099 min^{-1}, $t_{1/2}$ = 28.2 min and correlation coefficient = 0.998. The natural conclusion is that the species giving rise to the 480 nm fluorescence is formed rapidly and does need either a protomolecule or sheets to be formed and hence represents species formed in the early stage of eumelanin polymerization. Given the known influence of copper in directing dopachrome ring closer towards DHICA rather than DHI [14] it is tempting to attribute the 480 nm fluorescence to DHICA. However, it is too long a wavelength for DHICA monomer [19, 20].

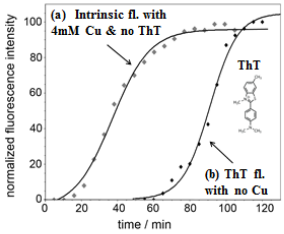

Figure 7. Sigmoidal fit to eumelanin at 480 nm (a) intrinsic fluorescence with 4 mM $CuSO_4$ added and (b) ThT (inset) fluorescence in the absence of copper.[7]

The strong increase in fluorescence at 480 nm and decrease at 530 nm (figure (5)) also correlates with the absorption increase at 450 nm in the presence of Cu^{2+} as shown in figure 2c. The addition of 1 mM Ni^{2+} ions produces a similar effect to Cu^{2+} giving k = 0.056 min-^{1}, $t_{1/2}$ = 45.21, min and correlation coefficient = 0.999.

At pH 10 the hydroxyl, carboxyl and amine groups in eumelanin monomers DHICA and DHI can be deprotonated and hence a colloid stabilized by electrostatic repulsion is produced. At somewhat lower pH the same concentrations will aggregate more rapidly to produce the brown/black pigment associated with melanin. Hence in addition to the ring closure of dopachrome favouring formation of DHICA rather than DHI [14], the effect of Cu^{2+} ions is that they can bind to such anionic groups, disturb the stability, and cause the more rapid aggregation as we observe. In the absence of Cu^{2+} ions at pH 9 dark melanin aggregates are present, but absent at pH 10 as borne out by the transparent (red/brown) solution observed. In the presence of Cu^{2+} ions at pH 10 the dark aggregated phase returns.

3.3 Fluorescence lifetime decay

Finally, we studied the fluorescence lifetime behaviour in the presence of Cu^{2+}. The fluorescence decay of eumelanin, in various forms, is well-known to be usually complex, often requiring at least three exponentials for a satisfactory description [24-26]. However, we find that in the presence of Cu^{2+} the fluorescence decay of melanin can be considerably simplified.

(a) **(b)**

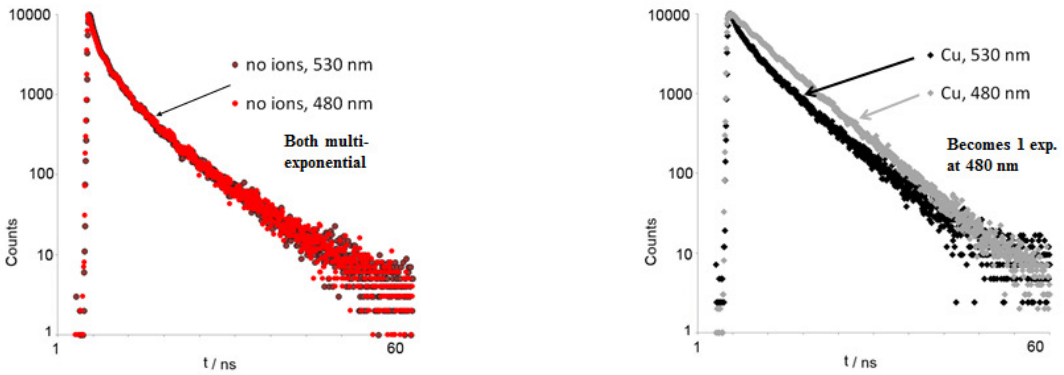

Figure 8. Change of fluorescence decay at 480 nm and 530 nm during excitation at 452nm (a) without Cu^{2+} ions and (b) with 4 µM Cu^{2+} ions [23].

The intrinsic fluorescence decay at both 480 nm and 530 nm emission requires a three exponential model (equation 2), but in the presence of only 4 µM Cu^{2+} at 530 nm the fluorescence decay requires a two-exponential function and at 480 nm a single exponential function (figure 8, table 1) [23]. A single decay component such as that ~ 6.33 ns shown in table 1 suggests only one species is dominant in emitting at this wavelength. When combined with the knowledge that DHICA increases when copper ions is present and yet DHICA monomer cannot be excited at 450 nm (see figure 2(b)) the logical conclusion is that a single species of oligomer is dominant, and given the early stage nature of the rapid polymerization, possibly a covalently bound dimer or trimer of DHICA/DHI or their oxidised forms. However, at pH 10 DHICA will be deprotonated into a dianion form and the intramolecular excited state proton transfer reaction used to describe eumelanin photophysics at neutral pH unlikely to be the same [27].

$$i(t) = B_1 exp(-t/\tau_1) + B_2 exp(-t/\tau_2) + B_3 exp(-t/\tau_3) \qquad (2)$$

Table 1. Fluorescence decay components of eumelanin formed from L-DOPA with and without copper when excited at 452 nm [23].

Sample, Fl. Wavelength/nm	τ_1/ns	B_1 / %	τ_2/ns	B_2 / %	τ_3/ns	B_3 / %
no Cu^{2+}, 480	4.88 ± 0.12	18	1.01 ± 0.04	23	0.03 ± 0.05	59
no Cu^{2+}, 530	4.87 ± 0.17	17	1.03 ± 0.17	26	0.24 ± 0.01	57
4 µM Cu^{2+}, 480	6.33 ± 0.03	100				
4 µM Cu^{2+}, 530	6.24 ± 0.03	81	1.21 ± 0.11	19		

The fluorescence decay of melanin varies strongly with the concentration of Cu^{2+} present. With the concentration increasing from 4 µM to 40 µM and 400 µM and excitation at 378 nm the decay is dominated by increasingly longer lifetime components (figure 9(a)) although the overall fluorescence intensity is found to still be reduced. In figure 9(b)

(a) (b) (c)

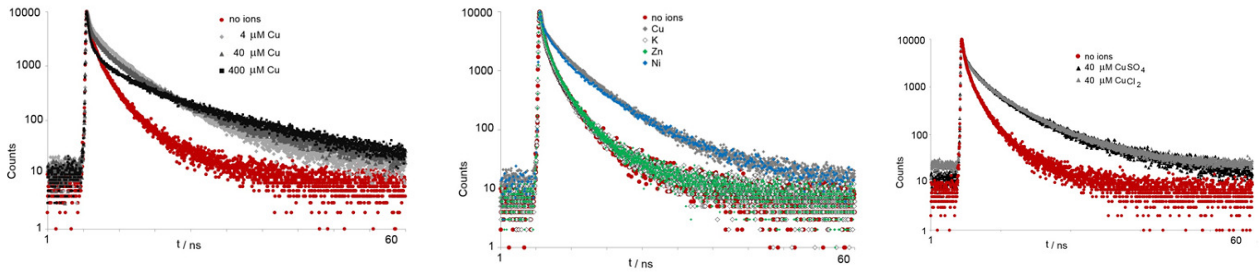

Figure 9. The fluorescence lifetime decay of eumelanin is measured at one hour after the onset of the synthesis at 480 nm using excitation at 378 nm with a Delta Diode. (a) With no copper ions present (red dots) and at 4, 40 and 400 µM of Cu^{2+}. The presence of increasing amounts of Cu^{2+} during the formation process leads to an increasingly dominant longer decay component. (b) With 4 µM K^+ or Zn^{2+} ions, which do not alter the fluorescence decay, and with 4 µM Cu^{2+} or Ni^{2+}, each having a similar decay with a longer component of increased intensity. (c) With addition of 40 µM $CuCl_2$ compared to 40 µM $CuSO_4$ and no metal cation present.

the effect of Cu^{2+} is compared with Ni^{2+}, which shows a similar decay profile at 4 µM concentration, and with K^+ or Zn^{2+} which have little if any effect. Similarly figure 9(c) shows that it is the divalent metal ion that is most effective in inducing change as using copper salt with a chloride rather than sulphate anion makes little difference. The complete fluorescence decay analysis in the presence of different copper concentrations and different metal ions is shown in table 2.

Table 2. Fluorescence decay components of eumelanin formed from L-DOPA in the presence of different metal ions at one hour after the onset of the synthesis at 480 nm using excitation at 378 nm. The chi-squared (X^2) goodness of fit criterion shows that a 3 exponential model provides quite a good description under all the conditions.

Sample, Fl. Wavelength/nm	τ_1/ns	B_1	τ_2/ns	B_2	τ_3/ns	B_3	X^2
no added ions, 480	1.87 ± 0.03	39	6.94 ± 0.15	9	0.42 ± 0.05	30	1.210
4 µM Cu^{2+}, 480	3.06 ± 0.06	48	7.24 ± 0.07	28	0.48 ± 0.01	9	1.127
40 µM Cu^{2+}, 480	3.11 ± 0.05	42	10.16 ± 0.08	28	0.48 ± 0.01	12	1.172
400 µM Cu^{2+}, 480	3.16 ± 0.06	23	12.35 ± 0.08	37	0.38 ± 0.01	14	1.010
4 µM Ni^{2+}, 480	2.45 ± 0.05	39	7.09 ± 0.05	35	0.48 ± 0.01	12	0.974
4 µM Zn^{2+}, 480	1.80 ± 0.03	41	6.22 ± 0.13	10	0.41 ± 0.01	26	1.188
4 µM K^+, 480	1.83 ± 0.03	38	6.62 ± 0.13	10	0.41 ± 0.01	29	1.100

The key questions highlighted by these results are what is the role of metal ions in the synthesis of eumelanin from L-DOPA and what is their effect on the eventual structure? Although it is generally agreed that DHICA, DHI and their redox forms according to pH, are the main constituents, the synthesis is well-known to be complicated and little understood in the presence of metal ions. Clearly the photophysics of the species formed, and certainly their fluorescence, reflect this complexity but, given the importance of copper in melanogenesis, metal ions in pathology and electronic properties metal ions might impart to potential bio-mimicked devices, progress in understanding is sorely needed.

Of course copper is a well-known quencher of fluorescence, but given that the monovalent ions Na^+ and K^+ do not increase the fluorescence it might be thought that the growth in fluorescence at 480 nm as shown in figure 5 can be explained by each divalent Cu^{2+} ion binding to two deprotonated groups and indeed perhaps playing a role in bridging between sheet structures of eumelanin. However, if the changes in intrinsic fluorescence in the presence of Cu^{2+} ions we observe correlate with sheet formation from ThT fluorescence, the primary effect of adding copper ions seems to be to reduce the time delay before the sheets start to form, as once started the rates with and without [7] copper are comparable (i.e. k = 0.099 and 0.15 ± 0.05 min^{-1}, respectively, c.f. figure 7). Given the oxidative nature of transition metal ions it would not be surprising if the fluorescence results for copper in particular reflect enhanced polymerization of DHICA/DHI, which, circumstantially lead to a single species emitting at 480 nm as shown by the mono-exponential decay of ~ 6.3 ns (Table 1) at this wavelength; this species tentatively ascribed here to a small oligomer. Previously a decay constant of ~ 6 ns has been observed as one component of a 3 exponential decay description during eumelanin synthesis without copper ions present, at both pH 7 and 10, but no decay component as long as the ~ 10 ns and 12 ns components in the presence of copper ions shown in table 2 was observed even after 14 days after starting the synthesis [26]. The increase in the long decay component (from ~ 7 to 12 ns at 4 μM to 400 μM respectively) with increasing copper concentration shows that copper does not go on facilitating more of this species as a photophysically isolated form or the decay time would stay constant at ~ 6 ns with an increasing amplitude (B_2). So for example, our observations would not fit naturally into the interpretation of the model of eumelanin as a polymer of photophysically isolated porphyrins [28] with copper at its centre, but they would be consistent with oligomeric forms of the latter linked by polymerization-induced extended π–electron conjugation. However, cross-sheet as well as in-plane sheet polymerization cannot be ruled out as the mechanism for increasing π–electron conjugation and the sources of the fluorescence emission at wavelengths as long as 480 nm when excited at 450 nm, where simple indole monomers like DHICA cannot be excited. Clearly not all the copper (or other metal ions) in the solution we have studied is interacting with the eumelanin synthesis. Indeed the unusual combination of a reduction in fluorescence and an increase in decay time with increasing copper concentration (figure 4 and table 2) that we observe suggests a dual environment. For example, if excess solvated copper ions in solution quench some of the exposed fluorescent sites while other copper ions enhance the polymerization of fluorescent sites that are shielded from further quenching.

Conclusions

Quite simply, like many of the questions surrounding melanin in general, the molecular species responsible for the fluorescence emissions observed are, as of now, largely unknown. Undoubtedly fluorescence alone cannot provide all the answers to unravelling the complexity of metal ions and eumelanin. However, we hope that we have further demonstrated here that, rather than often being neglected because of its complexity and low intensity, fluorescence is still a useful analytical tool for studying eumelanin. Moreover, despite the many undoubted complexities and contradictions in interpreting the absorption spectra of eumelanin, fluorescence studies can lead to some simplification that might help point towards a clearer identification of the fundamental building blocks of eumelanin, assist in designing melanin films for bio-inspired devices or indeed in better understanding eumelanin's biological function in the presence of metal ions.

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