Mapping the Formation of Eumelanin using Coupled Measurements

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Abstract

Melanin plays a crucial role as a pigment all through the animal kingdom. Being a macromolecule just on the divide between an ordered crystalline or a purely amorphous form melanin has proven a challenge to structure-function analysis.

Melanin assembles from small molecules much like a jigsaw and much like in a jigsaw the fine detail quickly vanishes in the overall picture. With Melanin being first and foremost a photo-active molecule we focus on spectral properties for the characterization of its structure using linked measurements of excitation and emission to identify 'areas of interest' in the Excitation-Emission Matrix (EEM). We then probe for characteristic fluorescence lifetimes in the identified areas to track melanin building blocks through the formation pathway.

1. Emulating Nature: Making Melanin in the Lab

In the human body eumelanin derives from the amino acid Tyrosine which is then converted by enzymatic action and oxidisation into the molecule L-Dopa to start the melanin formation pathway. Starting the reaction directly from L-Dopa has proven to be a realistic method to investigate melanin in laboratory conditions [1].

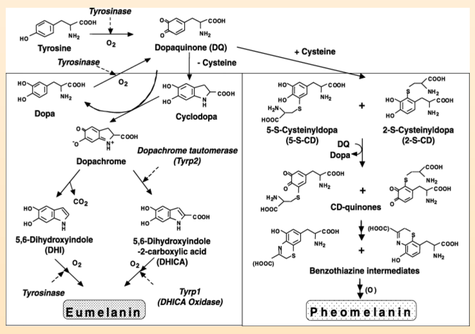


Figure 1: **The melanin formation pathways**

Melanin is generated through a complex network of reaction-pathways leading to eumelanin (black-brown pigment) and pheomelanin (reddish). By adding or omitting Cysteine and the enzyme Tyrosinase we can generate pure eumelanin or a mixture of both melanin types.

2. A finely balanced System: Eumelanin derived from L-Dopa

The formation of melanin can be easily tracked by recording the evolution of its characteristic absorption spectrum. Taking absorbance scans over time reveals several isosbestic points in the spectrum. While an isosbestic point is not in itself an indication of the conversion of one species into another or of an equilibrium between two species it does show that the stoichiometry (the relative quantity of reactants and products) remains constant.

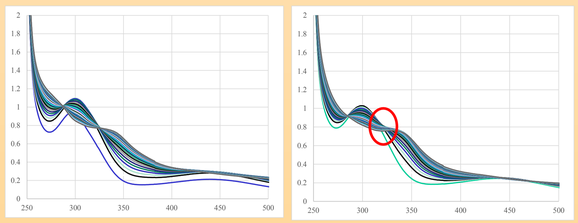


Figure 2: **Evolution of the absorption spectrum of eumelanin**

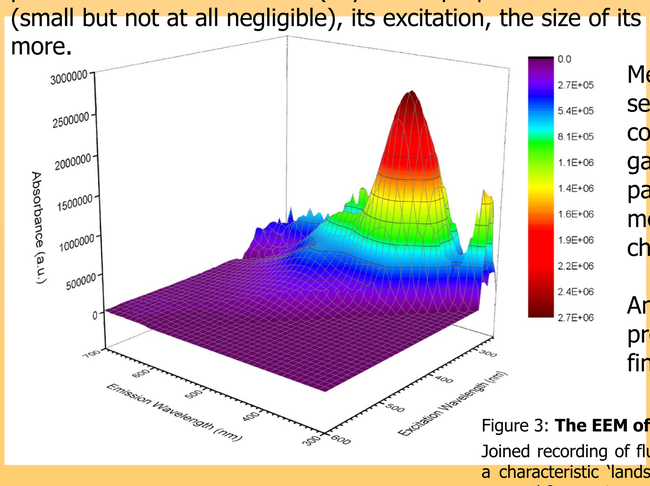
Shown are the absorption spectra of *de novo* forming melanin recorded in 5 minute intervals at pH 8 (left) and pH 8.3 (right). The pH change leads to the blurring of the isosbestic point at 320nm emission.

With the melanin absorption spectra showing several isosbestic points we have clear indication of a highly regulated, highly interlinked formation network [2].

A small change in an external parameter – here the pH – is sufficient to blur one isosbestic point (Figure) indicating a change in the chemical composition.

3. Joining Parameters: Excitation-Emission Matrices (EEM)

A photo-active molecule like melanin can be characterized through a wide range of parameters: its absorbance (key to its purpose as our 'natural sunscreen'), its fluorescence; (small but not at all negligible), its excitation, the size of its particles, its conductivity and many more.



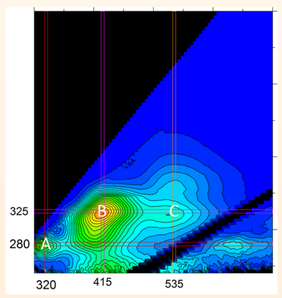
Measuring melanin parameters separately often leaves difficulties in comparing and linking findings. To gain control over this plethora of parameters we conducted joined-measurements recording melanin characteristics simultaneously.

An Excitation Emission Matrix (EEM) provides a highly detailed 'spectral fingerprint' of the melanin.

Figure 3: **The EEM of synthesised eumelanin**

Joined recording of fluorescence excitation and emission yields a characteristic 'landscape' of melanin fluorescence: a unique spectral fingerprint.

4. Areas of Interest: The Building Sites of Melanin

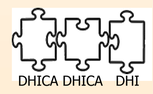


Tacking the evolution of EEMs during the formation process of melanin allows to identify specific sites where the absorption generates significant fluorescence emission. Some of these area show a transient character suggesting that fluorescent structures are later transformed or integrated into non-fluorescent parts of the molecule.

Figure 4: **Areas of interest in the excitation-emission matrix**

Recording EEMs at different times during the melanin formation process reveals regions of high excitation and emission. We observe a characteristic area 'A' where the initial L-Dopa molecule absorbs and fluoresces. Areas 'B' and 'C' show a longer joint excitation wavelength around 325nm and emission at 415nm and 535nm respectively.

5. Tracking Pieces of the Puzzle: Characteristic Lifetimes in the EEM



Excellent work has been done to characterize single building blocks of melanin [3,4]. We can use these characterisations to identify components of melanin present during the formation process. Tracking the fluorescence lifetimes in Region 'B' reveals three characteristic decay times, two showing a transient nature and a third component rising over the course of melanin formation. The component with the lifetime of 1.79 ns is likely to be 5,6-Dihydroxy-Indole (DHI) [3].

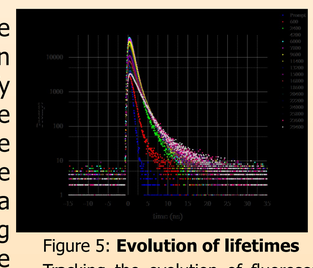
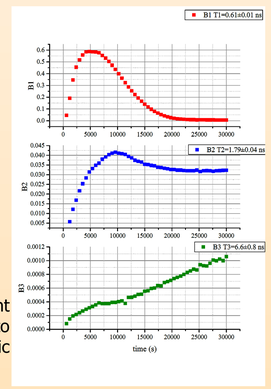


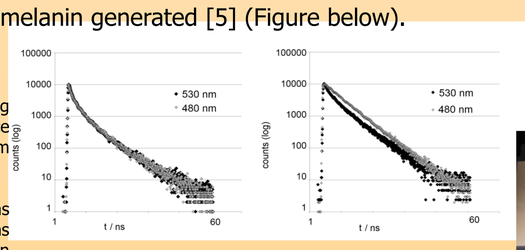
Figure 5: **Evolution of lifetimes**

Tracking the evolution of fluorescent lifetimes in areas of interest allows to pinpoint the presence of characteristic melanin components.



6. Closer to Nature: Influence of Effectors on Melanin Structure

The production of eumelanin in the lab is a well established technique yielding highly reproducible results. Alas melanin extracted from natural sources does show small but intriguing differences to lab generated melanin. The addition of micro-nutrients like transition metal ions to the reaction can alter the characteristics of the melanin generated [5] (Figure below).



Sample, F1 wavelength/nm	τ_1/ns	B_1 / %	τ_2/ns	B_2 / %	τ_3/ns	B_3 / %
+ Cu^{2+}, 480	4.88 ± 0.12	18	1.01 ± 0.04	23	0.03 ± 0.05	59
+ Cu^{2+}, 530	4.87 ± 0.17	17	1.03 ± 0.17	26	0.24 ± 0.01	57
10 µM Cu^{2+}, 480	6.33 ± 0.03	100				
10 µM Cu^{2+}, 530	6.24 ± 0.03	81	1.21 ± 0.11	19		

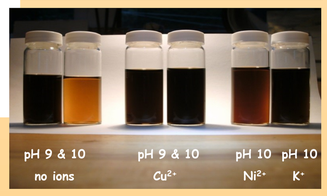
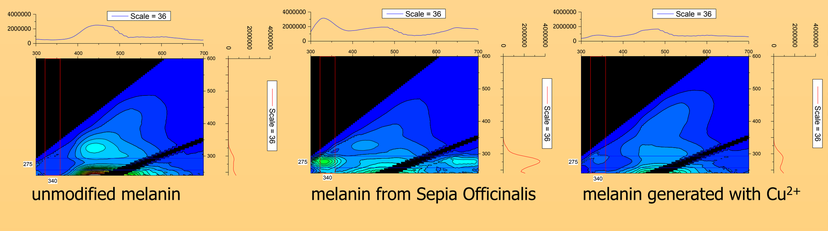


Figure 6a: **Micro-nutrients influence eumelanin structure**

Adding copper to *de novo* forming melanin simplifies the fluorescence decay of melanin emitting at 480nm and 530nm (1) [5].

Variations in the pH as well as addition of transition metal ions leads to striking differences in melanin colour (2).

Using EEMs we identify differences between lab generated and naturally occurring melanin and show how the addition of the micro nutrients can lead to a melanin closer in characteristics to the natural form (Figure below).



unmodified melanin — melanin from Sepia Officinalis — melanin generated with Cu^{2+}

Figure 6b: **The effect of micro-nutrients on EEMs**

Comparing the EEM of laboratory melanin (1) with that of natural sepia melanin (2) we find characteristic differences like a short wavelength area present in natural melanin but not in lab melanin (ex: 275nm, em:340nm). Addition of 10 µM copper ions partially restores this area (3).

Conclusions & Outlook

Melanin offers great opportunities as a bio-engineering tool to build bio circuitry or as a immuno-neutral cover for implants or nano-medical compounds. To fully exploit these opportunities we need greater understanding of melanin formation. Tracking joined characteristic parameters during the formation process of complex macro-molecules opens opportunities to identify and trace separate building blocks of the molecule.

References:

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