Towards Probing Skin Cancer using Endogenous Melanin Fluorescence

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INTRODUCTION

The main pigment of human skin, whether, it is a blemish such as café au lait spots, lentigos, or dark areas under the eye, is a heterogeneous polymer called *melanin* that is entangled with proteins. In the human skin there are two types of melanin, namely, *eumelanin* and *pheomelanin* (Teuchner et al 1999). Eumelanin and pheomelanin are produced as a result of the enzyme tyrosine, which induces the biosynthesis of melanin. Of the two types of melanin, eumelanin is the predominant type in human skin. The main biological function of native melanin is to protect the skin from photodamage, especially in the uv-wavelength range. In other words, melanin (especially eumelanin) acts as a sun screen. However, the photoinduced by-product of pheomelanin is free radicals that may activate carcinogenic processes (Teuchner et al, 1999). The underlying mechanism, that triggers either the beneficial action of melanin in the skin or its malignant transformation, is not fully understood.

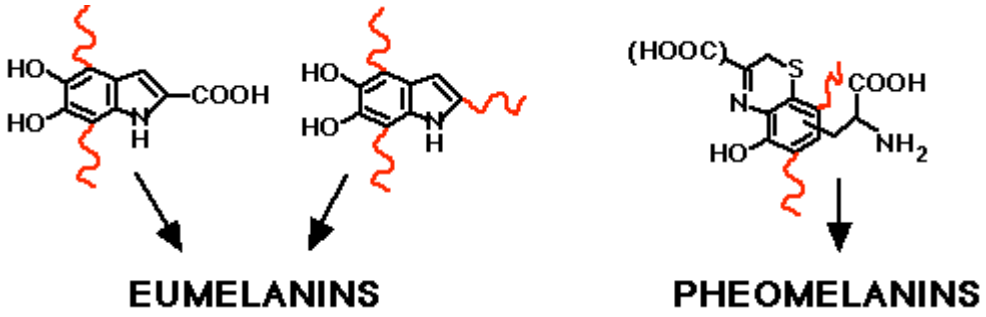

Figure 1: The building blocks of eumelanin and pheomelanin. Curly red lines indicate sites of attachment to the extended polymer and possibly to proteins. The details of these attachments in the total complex structure are not known.

To understand the photophysical properties of melanin and therefore the associated biological functions, synthetic chemists have created synthetic derivatives of eumelanin, which mimics the physiology of natural eumelanin (Teuchner et al. 1999). The synthetic eumelanin allows for comparative studies of the natural eumelanin in a control environment, and then ultimately there will be a correlation between these model molecular systems and the native biomolecules in biological tissues (Hoffman et al, 2001). These studies on synthetic melanin represent the preliminary steps toward *in vivo*

studies for in-depth understanding of their biological functions. Melanin fluorescence has been extensively studied, in solution, as a potential probe for skin diseases such as cancer. For that reason, there are major interests in differentiating between cancer and healthy tissues using mainly native melanin fluorescence as a reporter. So far, however, the experimental approaches have suffered from the lack of systematic and comprehensive investigation of the molecular dynamics of melanin in its native environment with high spatial and temporal resolution.

The long term of this objective is to elucidate the excited-state molecular dynamics of melanin under different and controlled environments. Furthermore, our goal is in-depth understanding of the correlation between such ultrafast dynamics and the structural conformations of melanin in their native biological environments for optical diagnostics of skin cancer. Here, we report our preliminary studies on oxidation effects on melanin fluorescence in solution. Hydrogen peroxide is a molecule that is well known in industry and in the household. In the body, H_2O_2 plays a dual role, as both a dangerous toxin and as a valuable signaling molecule. In this report, we have used H_2O_2 for oxidizing melanin. We also used a combination of steady-state and ultrafast time-resolved fluorescence microscopy and spectroscopy techniques. The key advantage of those techniques is providing quantitative biomolecular dynamics, with both spatial and temporal resolution of biomolecules (melanin in this case) in their native biological environments. In this report, we provide preliminary results on synthetic melanin, *in vitro*, using ultrafast laser spectroscopy. These studies were carried out under different oxidation conditions to test the potential of melanin fluorescence as a reporter for different physiological conditions in living skin tissues. This characterization is critical as an early optical probe of skin cancer compared to harmless skin lesions. Our long term goal is to extend this study of melanin fluorescence to *in vivo* using the endogenous fluorophore for early skin cancer detection.

II. MATERIALS AND METHODS

II.a Chemicals*:* Synthetic melanin and the oxidation agent (hydrogen peroxide, H_2O_2) were purchased from Sigma-Aldrich. The non-oxidized melanin was maintained in different solvents such as dimethylsulfoxide (DMSO), phosphate buffer saline (PBS), and water. The procedures of oxidation for melanin were followed by Kayatz et al. (2001). The complete oxidation of synthetic melanin takes approximately 3 ½ - 4 hours when isolated in 30 % hydrogen peroxide.

II.b Absorption and Fluorescence Measurements*:* The absorption spectra of *in vitro* melanin was measured and recorded on a spectrophotometer (Model DU800 from Beckmann and Coulter) over a 300-800nm range as a function of oxidation and solvent type. Fluorescence spectra were measured with a fluorimeter (Spex Fluorolog F212) using an excitation wavelength of 470 nm with a 10-nm bandpass, and 1.5 nm resolution. Steady state anisotropy measurements were also recorded using the emission wavelength at 540 nm.

II.c Time-trace of Melanin Oxidation using Steady State Spectroscopy*:* In order to investigate the oxidation effect on melanin fluorescence, we have measured time traces of the absorption and emission spectra as a function of time after melanin oxidation by incubation in 30% aqueous solution of hydrogen peroxide. Oxidation time-traces were followed approximately 200 minutes for both fluorescence and absorption spectroscopy. The first time point for both spectra was measured approximately 15 minutes after melanin had been in the hydrogen peroxide solution (~15 min to dissolve).

II.d Time resolved Anisotropy and Lifetime Decay Measurements: Time Correlated Single Photon Counting module (SPCM830; Becker and Hickl) was used for time resolved fluorescence and anisotropy measurements. The sample was staged on an inverted microscope (Olympus IX81) for recording the fluorescence decays at parallel, perpendicular, and magic-angle polarizations with respect to the excitation laser.

III. RESULTS AND DISCUSSION

III.a Steady State Time-Traces of In Vitro Melanin Oxidation: Synthetic melanin exhibits low solubility in organic solvents (e.g., DMSO and PBS) with an absorption spectrum similar to that shown in Figure 2.a. In contrast, the oxidized melanin was dissolved completely within ~15 minutes in hydrogen peroxide with similar absorption spectra. While oxidization does not affect the absorption spectrum of melanin, it does reduce the optical density compared with non-oxidized melanin.

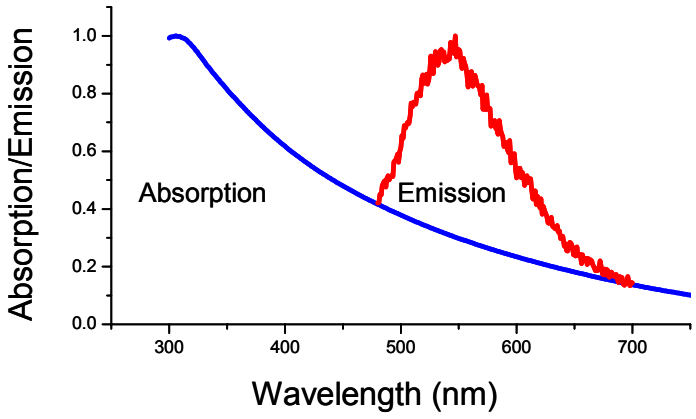

Figure 2.a: Absorption and emission spectra of melanin in DMSO.

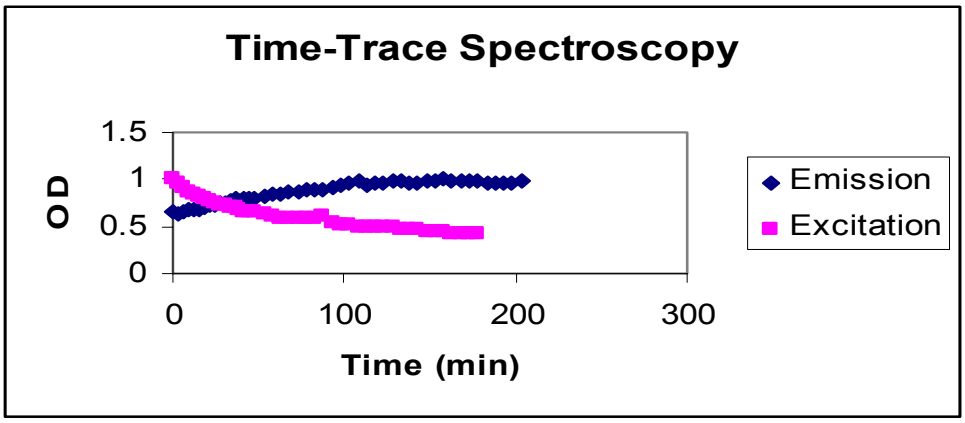

Figure 2.b: Time traces reveal oxidation effects on the absorption (squares) and emission (diamonds) of melanin.

Furthermore, the fluorescence emission (~540 nm) is also enhanced upon oxidation (see Figure 2.b for absorption and emission as a function of the oxidation time-traces). This is consistent with the argument that oxidation of *in vitro* melanin degraded the melanin polymer, resulting in an efficiently fluorescent molecule (Kayatz et al, 2001). This means that polymeric melanin causes fluorescence quenching of the non-oxidized fluorophores. To test this argument, we measured the excited-state fluorescence lifetime, which is independent of the fluorophores concentration and also directly proportional to the fluorescence quantum yield.

III.b Oxidation Affects on the Excited State Dynamics of Melanin: The fluorescence of melanin decays (under 495 nm excitation) as a triple exponential, independent of the solvent (HP, DMSO, or PBS) as shown in Figure 3 and Table 1. However, the average excited state lifetime of oxidized melanin is distinctively different from the non-oxidized melanin by at least a factor of 2. The incubation time of melanin was long enough to guarantee that oxidation reaction was complete. Under the same excitation conditions, the fluorescence was negligible in water.

The multiexponential fluorescence decays (see Table 1) suggest the presence of multiple species or electronic-state transitions of melanin, independent of the solvent or oxidation (Figure 3). However, the slower fluorescence decay of the oxidized melanin suggests an enhancement of the fluorescence quantum yield, which is consistent with our steady state spectroscopy measurements. By comparison, observed the faster decays observed in DMSO and PBS (non-oxidizing solvents) our argument that the polymerization of melanin causes fluorescence quenching, under non-oxidizing conditions, which compete with fluorescence.

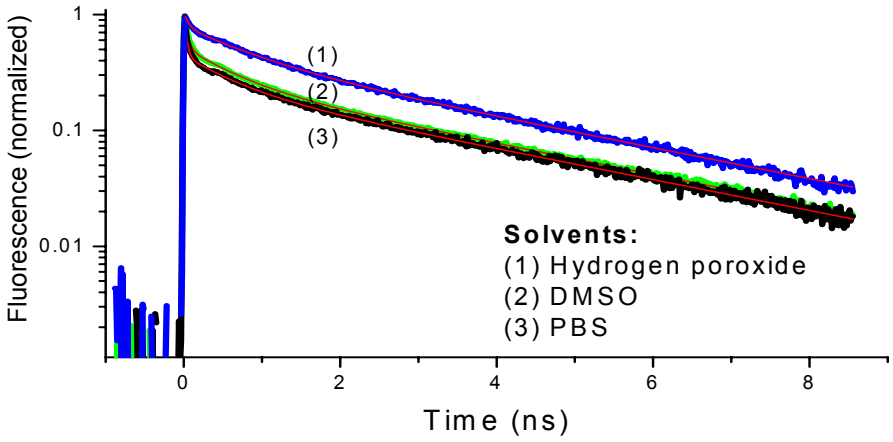

Figure 3. Excitation dynamics with no affects of polarization. A measure of the normalized fluorescence lifetime decay of the oxidized and non oxidized conditions. For the fitting parameters, please see Table 1.

To further test our hypothesis that there is a correlation between the observed fluorescence enhancement and the conformational structure of melanin, we measured time-resolved fluorescence anisotropy of melanin as a function of solvent type (oxidizing versus non-oxidizing).

Table 1. Fitting Parameters of the fluorescence lifetime decays of melanin in hydrogen peroxide (HP), DMSO, and PBS solvents.

Fitting Parameters	**HP**	**DMSO**	**PBS**
Time constant (τ_1)	51 ps	38 ps	29 ps
Amplitude (a_1)	0.21	0.48	0.52
Time constant (τ_2)	794 ps	616 ps	606 ps
Amplitude (a_2)	0.34	0.25	0.20
Time constant (τ_3)	3.26 ns	3.24 ns	3.26 ns
Amplitude (a_3)	0.45	0.26	0.24
Average Lifetime $<\tau_{fl}>$	1.75 ns	0.84 ns	0.92 ns

III.c Oxidation Effects on the Rotational Diffusion of Melanin: To further understand the mechanism that underlies both solvent and oxidization effects on the absorption and emission properties, we have measured time-resolved fluorescence *I(t)* and anisotropy *r(t)* using time-correlated single photon counting technique. We used the measured parallel $I_{//}(t)$ and perpendicular $I_{\perp}(t)$ fluorescence polarizations to calculate the anisotropy decay and, therefore, the rotational time of melanin, which is a sensitive

probe to both the environmental viscosity (η) and the hydrodynamic volume (V) of the fluorophores:

$$r(t) = \frac{I_{//}(t) - I_{\perp}(t)}{I_{//}(t) + 2I_{\perp}(t)} = r_0 \exp(-t/\phi), \qquad \text{and} \qquad \phi = \eta V / RT$$

where the denominator in the r(t)-function equal to the magic angle fluorescence decay and r_0 is the initial anisotropy. Also *R* and *T* are the gas constant and temperature (in Kelvin) of the solvent.

Figure 4 exhibit the oxidation effect on the rotational diffusion of melanin (see the figure caption for the fitting parameters). The anisotropy decays of oxidized melanin demonstrate clearly the degradation of melanin polymerization, which is present in non-oxidized melanin when dissolved in DMSO or PBS. Oxidation of Melanin affects its structural conformation. The anisotropy of melanin decays as a biexponential but the decay parameters are dependent on oxidation. Anisotropy of melanin in DMSO (*Black curve*) decays with two rotational time constants: fast component with ϕ_1= 640 ps (a_1 = 0.25) and slow component ϕ_2 = 2.03 ns (a_2 = 0.06). Anisotropy of melanin in hydrogen peroxide (*Blue curve*) decays also as a biexponential, but with two different rotational time constants: ϕ_1 = 95 ps (a_1 = 0.12) and slow component ϕ_2 = 443 ps (a_2 = 0.17). While the initial anisotropy (r_0) is about the same, the rotational time of oxidized melanin is ~1.6 time faster than the non-oxidized fluorophore. This small ratio of rotational time of polymerized-to-non-polymerized melanin might suggest a smaller polymerization extension.

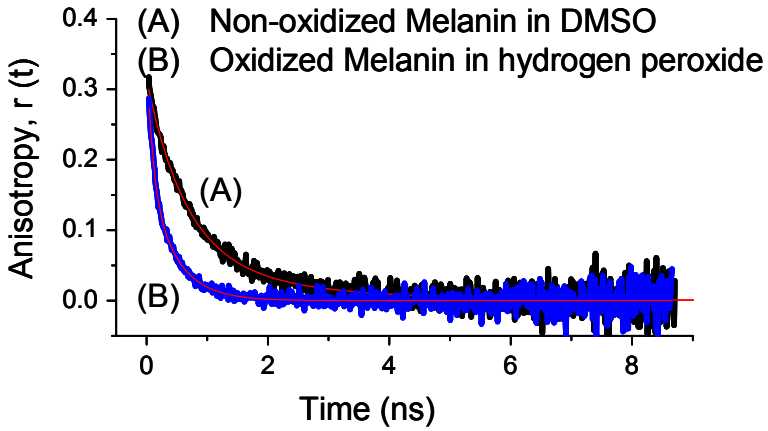

Figure 4. Oxidation of Melanin affects its structural conformation. The anisotropy of melanin decays as a biexponential but the decay parameters are dependent on oxidation (Curves A and B are for non-oxidized and oxidized melanin, respectively). See Table 2 for the fitting parameters.

Table 2. Fitting Parameters of the fluorescence anisotropy decays of melanin in hydrogen peroxide (HP) and DMSO.

Fitting Parameters	DMSO	HP
Rotational Time (ρ_1)	640 ps	95 ps
Amplitude (a_1)	0.25	0.12
Rotational Time (ρ_2)	2.03 ns	443 ps
Amplitude (a_2)	0.06	0.17
Initial Anisotropy (r_0)	0.31	0.29

These results confirm our assertion that the enhancement of melanin fluorescence properties upon oxidation can be understood in terms of structural conformation changes.

CONCLUSION

We investigated the oxidation effects of melanin fluorescence properties using steady-state and time-resolved fluorescence techniques. The enhanced fluorescence of oxidized melanin is attributed to the degradation of melanin polymerization. The longer excited state lifetime of oxidized melanin suggest that melanin stacking in a polymer causes fluorescence quenching. This argument is supported by the time-resolved fluorescence anisotropy, which suggest a much slower rotational time of melanin under non-oxidizing conditions (i.e., polymerized melanin). However, the multiexponential decays also suggest multiple structural conformers of melanin under oxidizing or non-oxidizing conditions. Our ultimate goal is to use the preliminary results and understanding of the sensitivity of melanin to the local environment to distinguish between cancerous and non-cancerous cases of skin.

ACKNOWLEDGEMENTS

I would like to express sincere thankfulness to the McNair Scholars Program Staff for all of their support and help. I would also like to extend thanks and appreciation to Dr. Ahmed Heikal, my FRA in the Bioengineering Department at The Pennsylvania State University, Laboratory for Functional Imaging of Biomolecules. Last but not least, the help and support of the graduate students in the lab, Ronn Walvick and Deepti Mudaliar, as well as the other 2004 summer research internship students in Dr. Heikal's group.

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