Dark- or Light-Colored Melanins: Generating Pigments Using Fe$^{2+}$ and H$_2$O$_2$.

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Abstract We have studied the formation of melanin-like pigments from catechol or pyrogallol and a wide range of other phenolic compounds using Fe$^{2+}$ and H$_2$O$_2$. Combining UV_Vis spectroscopic measurements and size-exclusion chromatography analyses we evaluated the impact of the intensity of the oxidation reaction by varying the concentration of H$_2$O$_2$ present in the reaction mixtures. All compounds tested, except tyrosine, reacted readily leading to mixtures that were black, brown or yellow-orange in color. For many compounds tested, the use of increasing concentrations of H$_2$O$_2$ resulted in either precipitation of the pigment or the formation of a soluble, lighter-colored pigment. With catechol or pyrogallol as model compounds, and using different concentrations of H$_2$O$_2$, several materials were synthesized, purified and dried. The physical-chemical properties of these materials were compared to the properties of melanin-like pigments synthesized from the same precursors using air-oxidation in an alkaline environment. For both precursors, a distinct chemical change, as judged from FT-IR spectroscopy, was introduced in the melanin structures when using H$_2$O$_2$ as the oxidizing agent and the relative intensity of this distinct signal strengthened with increasing concentration of H$_2$O$_2$ used in the reaction. In general, our results suggest that depending on the precursor molecule and the intensity of the oxidizing reaction conditions involved, light- or dark-colored melanin-like pigments can be generated. This may be an important factor when evaluating the visible outlook of histological or archeological specimens: the presence of a lighter color or the absence of a dark color may not necessarily mean the absence of melanin-like biomolecules.

Keywords: Melanin; peroxide; catechols; size exclusion chromatography; FT-IR spectroscopy

1. Introduction

Melanins (MNs) are darkly colored pigments ubiquitously found in nature and excellent reviews regarding their biosynthesis, chemistry, classification and functions have been written. The precise chemical structure of these pigments is not firmly established. Based upon degradation reactions involving oxidation in an alkaline environment, various model structures have been proposed and it has been suggested that MNs should be described as “heterogenous polymers derived by the oxidation of phenols and subsequent polymerization of intermediate phenols and their resulting quinones”.

The most common forms of MN in animals are eumelanin, derived from L-DOPA, and pheomelanin, derived from a combination of L-DOPA and L-cysteine. Certain areas of the brain contain neuromelanin (NM). NM are pigmented substances with unknown function found in select portions of the brain and NM-containing neurons are associated with neurodegenerative diseases like Parkinson’s (PD) or Alzheimer’s disease. Catecholamines like dopamine ([3] in Fig.1) or norepinephrine ([4] in Fig.1) could serve as precursors for the NMs. In addition to the catecholamines mentioned above, serotonin ([7] in Fig.1) has been investigated as a potential precursor for the formation of NMs. Allomelanins are nitrogen-free MNs found in plants derived from precursors like catechol ([1] in Fig.1) or pyrogallol ([2] in Fig.1). Some MNs produced by fungi or bacteria are referred to as pyomelanin. Pyomelanin is derived from homogentisic acid ([8] in Fig. 1) and this compound is also the precursor for the pigmentation observed in alkaptonuria (AKU) in humans. Tyrosine ([6] in Fig.1) could be considered an indirect precursor of MN-like pigments as it is the direct precursor for DOPA, which in turn is the precursor for ([3], [4]) and epinephrine ([5]) in Fig.1).

Although L-DOPA is the primary precursor for MN pigments, this compound was not included in the studies presented in this report. Separate studies have been conducted with L-DOPA and the results of these will be presented elsewhere. Fig.1 presents the chemical structures of all the compounds involved in the current report. Some of these compounds have been the subject of previous studies on the formation of MN-like pigments performed in our laboratories.
Fig. 1: Chemical structures of compounds used in this study: catechol (1), pyrogallol (2), dopamine (3), norepinephrine (4), epinephrine (5), tyrosine (6), serotonin (7) and homogentisic acid (8).

In biological samples, the MN pigments may present themselves as particulate matter of different sizes and shapes. The physic-chemical properties of MN particles, e.g., aggregation, have been studied and reviewed extensively before.\(^1\) For example, it has been shown that the MN particles present in ink sacs of cuttlefish are aggregates of much smaller MN particulate units.\(^7\) Although this model of MN-macroaggregates composed of MN-microparticles may not be applicable to all MN that are present in vivo or the MN synthesized in the lab.\(^8\) A key physical property of the MN class of pigments is their capacity to absorb light over a broad range of the electromagnetic spectrum giving the MN materials their dark brown or black appearance. MN is considered to have poor fluorescence properties, although treatment of MN with \(\text{H}_2\text{O}_2\) resulted in a markedly enhanced fluorescence that was attributed to the degradation of the pigment.\(^9\)

Similarly, the fluorescence of MN granules from human retina was studied as a function of age.\(^10\) The fluorescence intensity increased with age, a phenomenon that was attributed to the partial degradation of the pigment granules.\(^16\)

The biosynthesis of MN from DOPA involves a sequence of oxidation, cyclization and polymerization reactions most commonly described by the Raper-Mason scheme.\(^15\) MNs can be synthesized in the laboratory using air- or peroxide-mediated oxidation in an alkaline environment.\(^1\) In the case of (8), we have compared the physic-chemical differences of MN-like pigments generated when using air- or peroxide-mediated oxidation.\(^17\) We observed that when using more intense oxidation conditions, by using high concentrations of \(\text{H}_2\text{O}_2\), MN-like materials were generated exhibiting a lighter color, enhanced fluorescence and a distinct signal in their FT-IR spectra.\(^15\) In this report we expand these earlier observations by evaluating the effect of the intensity of the oxidative reaction conditions on the formation of pigments from the compounds shown in Fig.1. In addition, a range of MN-like materials were synthesized, purified and characterized from (1) and (2) by using different concentrations of \(\text{H}_2\text{O}_2\) as the oxidizing agent in the presence of \(\text{Fe}^{2+}\). This allowed us to evaluate the effect of the varying oxidation conditions on the physic-chemical properties of MN-like materials. We chose (1) and (2) as the simplicity of their chemical structures, e.g., the absence of carboxylate and nitrogen-containing functional groups, makes the interpretation of spectral data easier. For comparison purposes, we generated MN materials from (1) and (2) using air-oxidation in the presence of \(\text{NaOH}\).

2. Experimental Section

2.1. Materials

Catechol (1), pyrogallol (2), dopamine (3), norepinephrine (4), epinephrine (5), tyrosine (6), serotonin (7) and homogentisic acid (8) were obtained from Sigma-Aldrich (St Louis, MO). \(\text{FeCl}_2\cdot2\text{H}_2\text{O}\) was obtained from Fisher Scientific. \(\text{H}_2\text{O}_2\) solution at 3% (v/v) was obtained from Kroger Co (Cincinnati, OH). Test compound and \(\text{Fe}^{2+}\) solutions were prepared in distilled water just prior to the start of the reactions. Solutions containing 3% v/v \(\text{H}_2\text{O}_2\) were used within one month of their purchase.

2.2. Small scale experiments: Part 1

In a first set of experiments, mixtures containing 5mM (1), (2) or (3), and varying concentrations of \(\text{Fe}^{2+}\) and \(\text{H}_2\text{O}_2\) were prepared in wells of a 96-well microplate and kept at room temperature (RT) for multiple days. Occasionally, absorbance readings at wavelengths between 400 and 600 nm were taken. Details on the concentrations involved in these experiments are provided in section 3.1.

2.3. Small scale experiments: Part 2

In a second set of experiments, in small test tubes, compounds (1) through (8) at a fixed concentration of 5mM were mixed with \(\text{Fe}^{2+}\) at a fixed concentration of 0.3mM. \(\text{H}_2\text{O}_2\) was immediately added such that its final concentration varied between 0 and 0.1% v/v or 0 and 1% v/v (6 only). Details on the concentrations involved in these experiments are provided in section 3.2. Solutions containing the test compound at 5mM without any added \(\text{Fe}^{2+}\) and \(\text{H}_2\text{O}_2\) were prepared and immediately diluted 20-fold and analyzed using SEC. All reaction mixtures were kept at RT. After three days, aliquots from the reaction mixtures were diluted 20-fold and analyzed by SEC. UV-Vis absorbance readings were made on separate, undiluted aliquots from all the reaction mixtures. All the experiments were set up and analyzed in triplicate.

2.4. Large scale experiments

Reaction mixtures (total volume 25mL) containing about 100mg (1) or (2), 0.3mM \(\text{Fe}^{2+}\) and between 0 and 1.2% v/v \(\text{H}_2\text{O}_2\) were kept at RT for one week. All reactions were performed in water as the solvent.
Details on the concentrations involved in these experiments are provided in section 3.3.2.

Alternatively, about 100mg of (1) or (2) was dissolved in 5mL 0.15M NaOH and left at RT for up to one month. Occasionally, aliquots from the mixtures were diluted 50-fold with SEC solvent, centrifuged and analyzed using SEC. When the reactions were deemed complete, the mixtures were dialyzed using Spectrum Spectra/Por RC dialysis membranes with molecular-weight-cut-off of 3.5kDa obtained from Fisher Scientific (Suwanee, GA, USA) against water (up to 3.5L) for three days with up to four changes of water each day. The dialyzed mixtures were kept at -20°C for 24 hours and dried for three days using a Labconco FreeZone Plus 4.5L benchtop freeze-dry system obtained from Fisher Scientific (Suwanee, GA, USA).

2.5. UV_Vis spectroscopy

UV/Vis spectroscopic measurements were made in wells of a 96-well microplate using the SynergyHT microplate reader from Biotek (Winooski, VT). For measurements involving absorbance readings below 300nm, UV-transparent microplates were used.

2.6. Fluorescence

Fluorescence measurements were made in wells of an opaque 96-well microplate using the SynergyHT

![Image of absorbance readings at 400nm as a function of H_2O_2 concentration for mixtures containing (1) (panel A), (2) (panel B) and (3) (panel C) and various concentrations of Fe^{2+} after three days of reaction.](image)

Fig. 2: Effect of H_2O_2 concentration on color formation from (1), (2) or (3). Absorbance at 400nm as a function of H_2O_2 concentration of reactions mixtures containing 5mM (1) (panel A), (2) (panel B) or (3) (panel C) and various concentrations of Fe^{2+} after three days at RT.

When using Fe^{2+} and H_2O_2 as the oxidizing agents, darkly colored solutions were initially observed for (1), (2) or (3) at all concentrations of Fe^{2+} or H_2O_2 used. The results presented in Fig.2 indicate that for (1) the intensity of the color of the reaction mixtures increased with increasing concentration of Fe^{2+} or H_2O_2. However, it can not be excluded that during the course of these experiments some of the darkly-colored pigment that was generated adhered to the plastic material of the wells of the microplate and as such artificially increasing the absorbance readings. In the cases of (2) or (3), the use of higher concentrations of H_2O_2 (0.1% v/v or higher) resulted in a decrease in intensity of the color of the reaction mixtures during the

2.7. Size exclusion chromatography (SEC)

SEC analyses were performed as described elsewhere.

2.8. FT-IR spectroscopy

FT-IR spectroscopic scans were made as described elsewhere.

3. Results & Discussion

3.1. Small scale experiments: Part 1

In a first set of experiments, mixtures containing 5mM (1), (2) or (3) and various concentrations of Fe^{2+} (0, 0.08, 0.15, 0.23, 0.38, 0.76, 1.5 or 3.0mM) and H_2O_2 (0, 0.01, 0.03, 0.05, 0.1 or 0.17 % v/v) were prepared in wells of a 96-well microplate and kept at RT for multiple days. Occasionally absorbance readings at wavelengths between 400 and 600nm were taken. Fig.2 presents absorbance readings at 400nm as a function of H_2O_2 concentration for mixtures containing (1) (panel A), (2) (panel B) and (3) (panel C) for select concentrations of Fe^{2+} after three days of reaction.
latter parts of the experiments. When using lower concentrations of H$_2$O$_2$ (0.01-0.05 % v/v) the reaction mixtures involving (2) or (3) were darkly colored and remained as such throughout the experiments. This pattern of results for (2) or (3) appeared to be independent of the concentration of Fe$^{2+}$ that was present. The range of absorbance values shown in Fig. 2 runs high (up to 4.0). However, calibration graphs relating the concentration of purified pigments obtained from (1) and (2) as a function of absorbance at any wavelength between 230 and 900nm were linear ($r^2 > 0.999$) for absorbance values between 0.0 and 4.0 (see Fig.13 and results discussed further in this report). The pattern of results presented in Fig.2 are comparable to the pattern of results obtained from similar experiments involving (8) as reported elsewhere.  

3.2. Small scale experiments: Part 2

In the second set of small scale experiments, the effect of H$_2$O$_2$ on the pigment formation from a wide variety of compounds (see Fig. 1) was investigated. The reaction mixtures contained 5mM compound, 0.3mM Fe$^{2+}$ and 0, 0.004, 0.008, 0.015, 0.038, 0.075 or 0.11 % v/v H$_2$O$_2$. An exception was made for (6), for which all H$_2$O$_2$ concentrations used were 10-fold higher compared to the reactions involving all the other compounds. Initial experiments had indicated that (6) did not react at all when H$_2$O$_2$ concentrations of 0.1% v/v or lower were used. All mixtures were kept at RT for three days and all sets of experiments were set up and analyzed in triplicate. The reactions involving (6) produced mixtures that had a slight yellow color independent of the H$_2$O$_2$ concentrations present. For all other compounds, brown to black mixtures were obtained for the lower concentrations of H$_2$O$_2$ (between 0.004 and 0.038% v/v) used. At the higher concentrations of H$_2$O$_2$ tested (0.075 and 0.11% v/v), most mixtures had turned light to dark orange or showed clear signs of dark precipitations ((4), (5) or (7)). The extent of color formation, measured through UV-Vis spectroscopy, was evaluated together with an estimation of the reactivity of the compound, determined through SEC analyses. At the end of each set of reactions, the absorbance at 400, 500 or 600nm was measured of undiluted, 200μL aliquots from the reaction mixtures. Other aliquots from the reaction mixtures were diluted 20-fold and analyzed by SEC. For each compound, the area-under-the-curve (AUC) of the peak corresponding to the compound was estimated and compared to the AUC of the compound from the blank mixtures set up at the start of each set of reactions and analyzed immediately after the start of the reactions. Thus, by comparing the AUC of the compound at the start of the reaction and the AUC of the compound at the end of a reaction, an estimate was made of the % compound remaining at the end of the reaction. For all compounds shown in Fig.1, Fig.3 presents a comparison of the absorbance at 400nm of the reaction mixtures and the % compound remaining at the end of the reaction as a function of H$_2$O$_2$ concentration present in the reaction mixtures. All results are presented as average ± standard deviation (n=3).

![Fig.3](image)

Fig.3: Effect of H$_2$O$_2$ on color formation and reactivity for all compounds shown in Fig.1. Reaction mixtures containing 5mM compound, 0.3mM Fe$^{2+}$ and varying concentrations of H$_2$O$_2$ were kept at RT. After three days, the absorbance at 400nm of each mixture was measured using UV-Vis spectroscopy and the % compound remaining was estimated using SEC analyses as detailed inside the manuscript. All results are presented as average ± standard deviation (n=3). The '*' in some charts refers to the fact that, due to clearly visible precipitations, no UV-Vis measurements were made for the reaction mixtures containing the highest concentration of H$_2$O$_2$. Panel A = catechol (1), panel B = pyrogallol (2), panel C = dopamine (3), panel D = norepinephrine (4), panel E = epinephrine (5), panel F = tyrosine (6), panel G = serotonin (7) and panel H = homogentisic acid (8).
The “*” shown in some of the panels of Fig. 3 indicates that for (4), (5) and (7) precipitations were clearly visible in the reaction mixtures containing the highest concentration of H$_2$O$_2$ and no UV-Vis readings were performed on aliquots from these reaction mixtures. For (1), (2) and (7), SEC analyses were not performed on all reaction mixtures of the series of small scale experiments set up involving these compounds as their SEC peak retention times range between 55 and 75 minutes; making the SEC analyses much longer compared to the SEC analyses involving the other compounds.

With the exception of (6), all compounds tested reacted readily and exhibited a steady decline in % compound remaining as a function of H$_2$O$_2$ present in the reaction mixture with varying degrees of light or dark colors present in the crude reaction mixtures. The three panels presented in Fig. 4 illustrate different representations of the results shown in Fig. 3. The results presented in Fig. 4 are the averages of the three experiments, but the standard deviations have been omitted for clarity purposes as the reproducibility of the experimental results are illustrated in Fig. 3. Fig. 4, panel A, presents a comparison of the % compound remaining as a function of H$_2$O$_2$ concentration present for all compounds tested except (6). Fig. 4, panel B, presents a comparison of the absorbance at 400nm of the reaction mixtures as a function of H$_2$O$_2$ concentration present for all compounds tested except (6). Fig. 4, panel C, presents a correlation between the % compound remaining and the color of the reaction mixture as estimated from absorbance readings at 400nm for all compounds tested except (6).

Fig. 4: Overlay of experimental results illustrated in Fig.3 for reaction mixtures containing 5mM compound, 0.3mM Fe$^{2+}$ and varying concentrations of H$_2$O$_2$ after three days of reaction at RT. Panel A illustrates the effect of H$_2$O$_2$ concentration on the reactivity of the various compounds, panel B illustrates the effect of H$_2$O$_2$ concentration on the absorbance at 400nm of the reaction mixtures and panel C illustrates the correlation between the % compound remaining and the color of the reaction mixtures as estimated from absorbance readings at 400nm.

The results presented in Fig. 4, panel A, indicate that all compounds shown in Fig. 1, except (6), had a similar level of reactivity towards the Fe$^{2+}$/H$_2$O$_2$ oxidation conditions. For all the compounds involved in Fig. 4, panel A, nearly 100% of the compound had reacted away at H$_2$O$_2$ levels of 0.075% v/v or above. For the reaction mixtures containing 0.038% v/v or below, all were brown or black in color with varying degrees of intensity. Lighter colors, light brown to orange, were obtained for some compounds, e.g., (2), (3) or (8), at the higher concentrations of H$_2$O$_2$ used. Black precipitations combined with orange-colored supernatants were observed in the mixtures containing
(4), (5) or (7) at the highest concentration of H$_2$O$_2$ used. Although for many reaction mixtures no visible precipitations were observed and the aliquots of the reaction mixtures could be analyzed using SEC, we can not exclude the possibility that the pigment materials generated through the oxidation reactions may exist as aggregates of finely-dispersed small molecules as is discussed for MN pigments. Fig. 4, panel C, correlates the extent of the reactivity of the compound and the intensity of the color of the reaction mixture as evaluated through absorbance readings at 400 nm. For all compounds, with the exception of (8), the absorbance increased with declining % of compound remaining up to the point when about 0% of the compound remained; typically when 0.075% v/v H$_2$O$_2$ was present. The use of higher concentrations of H$_2$O$_2$ (0.11% v/v H$_2$O$_2$) often resulted in a decline of the absorbance of the reaction mixtures or in a precipitation of the material generated. These results suggest that only when excess levels of H$_2$O$_2$ are present, the darkly-colored pigment generated initially is subjected to additional oxidation leading to the observed physical-chemical changes; precipitations or decline in absorbance. Peroxide treatment of pre-prepared MN materials has been shown to alter their physical-chemical properties. These changes have been attributed to oxidative degradation of the pigments. However, SEC analyses of the reaction mixtures (see results and discussions below) involving the highest concentration of H$_2$O$_2$ used, consistently showed the absence of the starting compound and the presence of a single peak with a retention time below 15 minutes; corresponding to a material with a hydrodynamic volume associated with high molecular mass materials. No peaks were ever observed that would suggest the presence of “degradation products” derived from the initially formed pigment. In the case of (8), the intensity of the absorbance reading appeared to decline before 100% of the compound had reacted away. This would suggest that in the case of (8), the pigment material generated in the initial phase of the reactions may be more sensitive to further oxidation leading to a lighter colored material.

The value of SEC analysis to monitor the formation of colored pigments from catecholic or phenolic compounds has been discussed in previous reports. In SEC, molecules are separated on the basis of differences in their hydrodynamic volume (often related to the molecular mass of the molecule); the lower the retention time, the higher the hydrodynamic volume of the analyte. In our SEC analyses, the lower limit of exclusion, as determined by the injection of water, was about 15 minutes. Peaks with a retention time lower than 15 minutes are typical for high molecular mass compounds, while peaks with retention times of 15 minutes or more are typical for low molecular mass compounds. As illustrated in Fig. 3, SEC was used to evaluate the reactivity of the various compounds towards the Fe$^{2+}$ and H$_2$O$_2$ combination. In addition, the SEC analyses of the reaction mixtures offered a window into the formation of high molecular mass materials and the possible presence of low molecular mass reaction intermediates. This aspect of the SEC analyses is exemplified in Fig. 5 for two reaction mixtures involving (7).

![Graph](image-url)

**Fig. 5**: SEC analyses, traces at 275 and 400 nm, of reaction mixtures involving 5 mM (7), 0.3 mM Fe$^{2+}$ and 0.015% or 0.075% v/v H$_2$O$_2$ after three days of reaction at RT.

In the SEC profile shown in Fig. 5, the peak with retention time of about 55 minutes corresponds to (7). In the presence of the lower concentration of H$_2$O$_2$ (0.015% v/v), compound (7) had only minimally reacted away and a multitude of peaks associated with low molecular weight compounds were present in the SEC profile. In the presence of the higher concentration of H$_2$O$_2$ (0.075% v/v), compound (7) appeared to have completely reacted away and the SEC profile showed a single dominant peak with a retention time of about 13.6 minutes and absorbance in the visible range of the electromagnetic spectrum. The SEC profiles of partially reacted compounds (3) through (5) did exhibit peaks associated with low molecular mass other than the peak corresponding to the starting compound. This was not observed for SEC profiles of partially reacted (1), (2) or
(8). For all compounds, the SEC profiles of the reaction mixtures whereby the starting compound had completely reacted away, showed a single peak with a retention time below 15 minutes and with absorbance in the UV and visible range of the electromagnetic spectrum (results not shown).

For all compounds, except (6), using increasing concentrations of H$_2$O$_2$ resulted in the formation of a peak with retention time below 15 minutes (typically between 12 and 14 minutes) and this peak exhibited absorbance in the visible range of the electromagnetic spectrum. This includes the reaction mixtures involving (4), (5) or (7) at the highest concentration of H$_2$O$_2$ used. Although these reaction mixtures exhibited clearly visible precipitations, the soluble fraction of these reaction mixtures did contain a peak with retention time below 15 minutes in their SEC profiles. Fig.6 presents the peak retention time (average ± standard deviation; n = 3) of the high molecular mass material with absorbance in the visible range present in the reaction mixtures for all compounds tested (except (6)) containing 0.3mM Fe$^{2+}$ and 0.038, 0.075 or 0.11% v/v H$_2$O$_2$.

![Fig. 6: SEC peak retention time (average ± standard deviation; n = 3) of the high molecular mass material with absorbance in the visible range present in the reaction mixtures containing 5mM compound, 0.3mM Fe$^{2+}$ and 0.038, 0.075 or 0.11% v/v H$_2$O$_2$ after three days of reaction at RT.](image)

For (2) and (8) there was little to no variation in these peak retention times with the varying concentrations of H$_2$O$_2$ used. For the other compounds evaluated in Fig.6, there appeared to be a decline in peak retention time, associated with an increase in hydrodynamic volume, with increasing concentration of H$_2$O$_2$ present in the reaction mixtures. This decline appeared to be linear with regression coefficients ranging between 0.84 (for (1)) to 0.989 (for (4)). The results presented in Figs. 3 and 4 indicate that for the various compounds involved, reaction mixtures containing 0.11% v/v H$_2$O$_2$ represented an excess amount of H$_2$O$_2$. The fact that the peak retention time of the pigment material generated tended to decline (associated with an increase in hydrodynamic volume) when the highest concentration of H$_2$O$_2$ was used is another indication that no physical degradation of the pigmented material occurred under these circumstances.

Overall, the range of the peak retention times for all the materials of high hydrodynamic volume with absorbance in the visible range of the electromagnetic spectrum included in Fig.6 is fairly narrow. The average (± standard deviation) peak retention time for all data points shown in Fig.6 was 13.1 ± 0.6 (n=21). These observations suggest that the hydrodynamic volume of the colored pigment generated when using different compounds and/or concentrations of H$_2$O$_2$ are not substantially different from one another. In addition, the SEC peak retention times shown in Fig.6 are similar to the SEC peak retention times obtained for MN-like pigments generated from DOPA or (8) through air- or Fe$^{2+}$/H$_2$O$_2$-mediated oxidation. Such results suggest that the hydrodynamic volume of the pigments generated may be independent from the chemical nature of the precursor or the reaction conditions employed to generate the material. As discussed earlier, at this stage it is unclear whether the pigments synthesized exist as polymeric molecules in solution or as finely-dispersed, particulate aggregates of small molecules.

### 3.3. Large scale experiments

#### 3.3.1 (1) or (2) and air-oxidation in the presence of NaOH

Large scale reaction mixtures containing about 100 mg (1) or (2) dissolved in 5mL 0.15M NaOH solution were kept at RT for up to one month. Both mixtures consisted of black solutions with no signs of precipitation and remained as such throughout the dialysis purification process. SEC analyses of purified materials revealed a single peak with a retention time of about 10 minutes for the reaction involving (1) and 12.7 minutes for the reaction involving (2) (results not shown). FT-IR spectra were recorded for the dried materials and these are included in Figs. 8 and 9, panels A and B.

#### 3.3.2. (1) or (2) and Fe$^{2+}$/H$_2$O$_2$-mediated oxidation

Aqueous reaction mixtures (total volume 25mL) containing about 100 mg (1) or (2), 0.3mM Fe$^{2+}$ and 0.012, 0.03, 0.06, 0.22, 0.32, 1.2 or 2.2% v/v H$_2$O$_2$ were kept at RT for up to one week. The visual observations made for the small scale experiments (see Section 3.1) were mirrored in the large scale experiments. All reaction mixtures involving (1) quickly turned black and remained as such throughout the experiments and purification processes. Reaction mixtures involving (2), in the presence of the lower concentrations of H$_2$O$_2$ used, quickly turned black and remained as such throughout the experiment and purification process. However, the large scale reaction mixtures of (2) in the presence of 1.2 or 2.2% v/v H$_2$O$_2$ turned black instantly, but turned yellow-gold over the course of multiple days and remained as such during the purification process.

#### 3.3.2.1 SEC analyses

Fig. 7, panels A and B, present SEC profiles of select reaction mixtures (50-fold diluted) after two days of reaction and after the dialysis of the reaction mixtures (20-fold diluted).
In Fig. 7, panel A, the peak with a retention time of about 78 minutes corresponds to (1). The peak with a retention time of about 13 minutes corresponds to the high molecular mass pigment generated. This peak exhibited absorbance in the UV and visible range of the electromagnetic spectrum. However, as the peak corresponding to the pigment is broad, it is possible that the peak associated with the pigment partially overlaps with the peak typically observed when H$_2$O$_2$ is present in the reaction mixtures (peak retention time about 15 minutes). In Fig. 7, panel B, the peak with a retention time of about 58 minutes corresponds to (2). The peak with retention time of about 15 minutes corresponds to H$_2$O$_2$. The peak with a retention time of about 13 minutes corresponds to the high molecular mass pigment generated. This peak exhibited absorbance in the UV and visible range of the electromagnetic spectrum. During dialysis, the unreacted (1) or (2) and H$_2$O$_2$ were washed away, as confirmed through SEC analyses, and the purified materials were lyophilized. Given that the molecular weight cut-off of the dialysis tubing used is 3.5kDa, there is the possibility that some lower molecular mass pigment oligomers were removed during this dialysis process. Thus, the final, purified materials obtained may represent fractions that are enriched in high molecular mass species. SEC analyses did reveal that, when using lower concentrations of H$_2$O$_2$, more unreacted precursor remained in the reaction mixtures, while when using the higher concentrations of H$_2$O$_2$, the precursors reacted away nearly completely (results not shown). These observations were mirrored in the fact that, following the purification and drying processes, more pigment material was recovered from the reaction mixtures using the higher concentrations of H$_2$O$_2$.

Various pigments were thus prepared from (1) or (2) using varying concentrations of H$_2$O$_2$. The peak SEC retention times of all the materials thus made from (1) fell within a range of 12.8 to 13.4 minutes. The peak SEC retention times of all the materials thus made from (2) fell within a range of 12.2 to 13.3 minutes. These results suggest again that the hydrodynamic volume of the pigment generated through Fe$^{2+}$/H$_2$O$_2$-mediated oxidation is independent of the precursor compound and/or the intensity of the oxidation reaction conditions.

3.3.2.2 FT-IR spectroscopic analysis

FT-IR spectra were recorded of all lyophilized materials generated from the large scale experiments involving (1) or (2). Select spectra of pigments generated from (1) are presented in Fig.8, panel A (total spectrum) and panel B (fingerprint region). These figures include a spectrum of (1). Select spectra of pigments generated from (2) are presented in Fig. 9, panel A (total spectrum) and panel B (fingerprint region). These figures include a spectrum of (2).
The spectra of the pigments derived from (1) or (2) do not exhibit any of the well-defined, sharp peaks visible in the spectra of (1) or (2) indicating that the materials are more heterogeneous and do not exist as a single, well-defined structure. The pigment prepared from (1) through air oxidation in the presence of 0.15M NaOH exhibited peaks at wavenumbers of 1,230, 1,390, 1,485 and 1,580 cm$^{-1}$ and a broad peak at wavenumber 3,320 cm$^{-1}$. The pigments generated from (1) in the presence of H$_2$O$_2$ exhibited peaks with wavenumbers in the range of 1,230-1,270 cm$^{-1}$, 1,370-1,400 cm$^{-1}$, 1,570-1,600 cm$^{-1}$ and, 1,700-1,705 cm$^{-1}$ (for only a select few pigments) and a broad peak around a wavenumber of 3,300 cm$^{-1}$. The pigment prepared from (2) through air oxidation in the presence of 0.15M NaOH exhibited peaks at wavenumbers of 1,250, 1,380 and 1,575 cm$^{-1}$ and a broad peak at wavenumber 3,370 cm$^{-1}$. The
pigments generated from (2) in the presence of H₂O₂ exhibited peaks with wavenumbers around 1,050, 1,220, 1,300, 1,605 cm⁻¹ and 1,700-1,705 cm⁻¹ (for only a select few pigments) and a broad peak at a wavenumber between 3,200 and 3,300 cm⁻¹. Overall, the FT-IR spectra of the pigments generated from (1) or (2) exhibit a similar pattern of peaks as the FT-IR spectra of MN specimen reported elsewhere. The spectra of both (1) or (2) exhibit multiple sharp peaks at wavenumbers below 1,250 cm⁻¹. These peaks are associated with aromatic C-H in-plane or out-of-plane bends. These absorbance peaks are not present in the pigments made from (1), while some spectra of the pigments materials made from (2) exhibit a broad absorbance in this region. The broad absorbance for wavenumbers between 1,230 and 1,400 cm⁻¹ present in the spectra of all materials can be attributed to phenolic C-O stretch or OH bend. The spectra of (1) and (2) exhibit three to four sharp peaks with wavenumbers between 1,450 and 1,650 cm⁻¹. Such absorbances are associated with C=C-C aromatic ring stretching. In the spectra of the pigments derived from (1), two absorbance peaks can be observed in this wavenumber range, while in the spectra of the pigments derived from (2) only a single, broad absorbance peak could be observed in this wavenumber range. In addition, the position of this broad absorbance peak appeared to shift to higher wavenumbers with increasing H₂O₂ used to generate the pigments from (2) (results not shown). The significance of this observation is not clear. The strong, sharp peaks at wavenumbers above 3,000 cm⁻¹ present in the spectra of both (1) or (2) are not present in the spectra of any of the pigments made from (1) or (2). The spectra of the pigments made from (1) or (2) exhibit broad absorbance bands in the region above 3,000 cm⁻¹ and exhibit a lower relative intensity. This pattern can be attributed to the presence of hydrogen bonds within the final structures. The peak maxima of the broad absorbance band at wavenumbers above 3,000 cm⁻¹ fluctuated among the various pigments generated from (1) or (2), but no specific relationship between, e.g., the position of this peak and the concentration of H₂O₂ used to generate the pigment, could be observed.

For both (1) and (2), the FT-IR spectra of the pigments prepared in the presence of the higher concentrations of H₂O₂ (0.12% to 1.2% v/v) exhibit a distinct, sharp peak at a wavenumber of about 1,705 cm⁻¹. A peak at that wavenumber can be attributed to the presence of a carbonyl entity (C=O); either from a ketone or a carboxylic acid functionality. This feature in the FT-IR spectra may be due to a chemical change whereby a phenolic functional group was converted into a ketone functional group or whereby an aromatic unit was opened and two carboxylic acids were generated into the structure. At this stage, no distinction between these two possibilities can be made. The C=O entity of aromatic carboxylic acid groups should exhibit IR absorbances at a wavenumber of about 1,750 cm⁻¹. However, hydrogen bonding between carboxylic acids and nearby phenolic functional groups will lead to a shift of this IR absorbance band to much lower wavenumbers. The IR absorbance of C=O entities belonging to unsaturated, endocyclic ketones is predicted to appear at wavenumbers similar to the ones of hydrogen-bonded carboxylic acid groups. Fig. 10 represents the relative absorbance of the peak between 1,705 and 1,710 cm⁻¹, compared to the absorbance of the peak between 1,575 and 1,650 cm⁻¹ for the MN materials made from (1) or (2).

The results indicate that the relative intensity of the peak between 1,705 and 1,710 cm⁻¹ increased with increasing concentration of H₂O₂ used to generate the pigment. A similar pattern of results was obtained when comparing the relative FT-IR absorbance of the peak between 1,705 and 1,710 cm⁻¹ to the absorbance of the peak between 3,200 and 3,400 cm⁻¹ for the pigments made from (1) or (2) (results not shown). A similar observation has been made for Fe²⁺/H₂O₂-mediated oxidation reactions involving (8). Overall, the results suggest that, independent of the precursor used, the use of H₂O₂ as the oxidizing agent introduces a distinct chemical functional group (ketone or carboxylic acid or both) in the materials and this chemical functional group is more prominently present when higher H₂O₂ concentrations are used. In this context, it is worth noting that, for MN materials made from L-tyrosine or L-DOPA, it has been discussed that the presence or absence of carboxylic acid functionalities in the MN materials may affect the physical properties of these MNs.

FT-IR spectroscopy is commonly used to characterize MN specimens from various sources. In some of these spectra a distinct peak between 1,700 and 1,720 cm⁻¹ can be observed. However, it is not always clear whether the presence of this peak is the result of the chemical process employed in the synthesis of the MN material or because such a peak is present in the FT-IR spectrum of the precursor of the MN material. UV-Vis spectroscopy and fluorescence emission

Stock solutions of the purified and dried materials generated from (1) or (2) were redissolved in water at 1 mg/mL and a dilution series with concentrations ranging from 0.02 to 0.4 mg/mL was prepared from each stock solution. The stock solutions of the pigments made from (1) were black in color while stock solutions of the pigments made from (2) were black or gold in color.
Fig. 11, panels A and B, present photographs of select dried and redissolved materials generated from (2).

![Photographs of purified and dried materials](image)

For all dilutions prepared, UV-Vis spectra between 230 and 900nm were recorded and fluorescence emissions were measured for 150μL aliquots. Fig.12 presents typical UV-Vis profiles of select pigments thus obtained.

![UV-Vis spectra of select pigments](image)

All materials exhibited the typical smooth UV-Vis absorbance curves of MN-like pigments, showing sharply increasing absorbance values with decreasing wavelengths. Fig. 13 shows, for one select material, the relationship between the concentration of the pigment in water and a) its absorbance at 250 or 400nm and b) its fluorescence emission.

![Relationship between pigment concentration and absorbance at 250 or 400nm](image)

For the UV-Vis absorbance, linear relationships were obtained over the entire pigment concentration range tested. For the fluorescence emission, linear relationships were obtained in the lower concentration range tested. The patterns shown in Fig. 13 were obtained for all pigments made from (1) or (2). Given a) that SEC analyses revealed that the materials had similar hydrodynamic volumes and thus possibly similar molecular masses and b) that the relationship between the pigment concentration and UV-Vis absorbance was linear; the slopes of these lines can be used and compared as an estimate of the extinction coefficient or absorptivity of the pigments according to the Lambert-Beer law. Fig. 14, panel A, presents the relationship between the H₂O₂ concentration used to prepare the pigments from (1) or (2) and their estimated extinction coefficients for the absorbance at 250 or 400nm. Fig. 14, panel B, presents the relationship between the H₂O₂ concentration used to prepare the materials from (1) or (2) and their fluorescence emission measured for the dilution at 0.05 mg/mL.

![Relationships between the estimated values of the extinction coefficients and H₂O₂ concentration](image)

For the materials made in the presence of the lower concentrations of H₂O₂, the extinction coefficients of the MNs made from (1) or (2) appear to be similar. However, for the materials made in the presence of the higher concentrations of H₂O₂, the extinction coefficients of the MNs made from (2) appear to be much lower than the extinction coefficients of the MN materials from (1). The fluorescence emission of the MN materials made from (1) appeared to be
independent of the concentration of H₂O₂ used to prepare the MNs. On the other hand, the fluorescence emission of the MN materials prepared from (2) showed a marked decrease for those MNs prepared in the presence of the highest concentrations of H₂O₂. Other reports have described the effects of H₂O₂ on the physical properties of MNs and detailed the loss of color or enhanced fluorescence.  However, these reports involved the treatment of pre-existing MN materials with H₂O₂ and the physico-chemical changes were attributed to oxidative degradation.

The chemical structures of both precursors are very similar. The chemistry of the MN generated from both precursors using H₂O₂ appears to be similar as judged from the FT-IR analyses. Despite these chemical similarities, their physical properties, color, UV-Vis absorbance profiles and fluorescence emissions, are markedly different. Thus, depending on the starting compound and the concentration of H₂O₂ used to generate the MN material one can obtain dark-colored pigments with little fluorescence emission or light-colored materials with enhanced fluorescence emission. A similar observation has been made for MN-like materials generated from (8).  Additional studies involving compounds (3) through (7) and DOPA are ongoing in our laboratory and will be reported upon elsewhere.

Whether our observations have any relevance to any MN-related in vivo situations remains to be seen. However, it is worth pointing out that for greying of hair or vitiligo, conditions that are associated with the loss of pigmentation, the presence of increased levels of H₂O₂ have been discussed as a potential contributing factor to the changes in pigmentation. Peroxides or other reactive oxygen species (ROS) are routinely generated in various cells and tissues. Thus it is likely that the generation of ROS can be an oxidative potential that can convert catecholic or other phenolic precursors into MN-like pigments. Our results do suggest that, depending on the intensity of the oxidative reaction, chemically-distinct, dark- or light-colored MN-like pigments could be generated depending on the type of precursor that is present. Such physically and chemically distinct MN materials could find different applications or possess different cell-biological properties. In addition, our results suggest that the absence of a dark color does not necessarily mean the absence of MN-like materials which may have implications for the evaluation of histological or fossil specimens.

4. Conclusions

In conclusion, our observations suggest, that depending on the precursor molecule and the oxidation conditions applied, melanin-like pigments can be generated that are chemically similar, but exhibit very different physical properties. Thus, melanin-like pigments can be generated with different absorbancies towards UV or visible light and with different fluorescence emission intensities. This may be an important factor when evaluating the visible outlook of histological or archeological specimens: the presence of a lighter color or the absence of a dark color may not necessarily mean the absence of melanin-like biomolecules.

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