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Article

# The "Unconventional" Effect of Cysteine on the *In Vitro* Synthesis of Melanin

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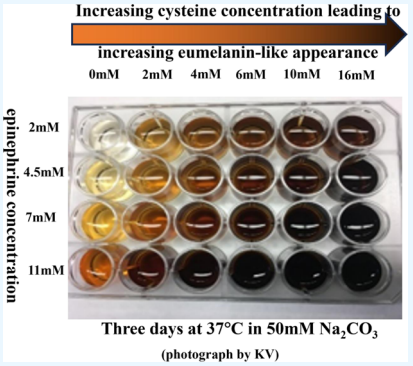
**ABSTRACT:** This report details some of our observations regarding the impact of cysteine on the air-mediated oxidation of catecholamines, particularly epinephrine. The intent was to synthesize light-colored, pheomelanin-like materials. Pheomelanin is commonly described as a material generated from a mixture of catecholamines and cysteine. However, we observed that (1) the presence of cysteine resulted in a concentration-dependent delay in the onset of color formation and (2) the presence of cysteine resulted in darker, more eumelanin-like materials. These effects were particularly impactful in the case of epinephrine. More elaborate studies involving other amino acids or scaled-up reactions were conducted with epinephrine as the precursor. These studies show that other amino acids, *e.g.*, methionine or serine, could lead to darker materials, but none were as impactful as cysteine. Although our results are in contrast to typical descriptions regarding the impact of cysteine on the synthesis of melanin, they may reflect crucial differences between the *in vitro vs in vivo* synthesis of pheomelanin.




## 1. INTRODUCTION

Despite decades of research, melanins (MNs), a ubiquitous class of darkly colored pigments, are still poorly defined in terms of their chemical structure and some of their physical properties. Many reviews have been written about this enigmatic class of biomolecules detailing these unresolved issues. In general, there is a consensus that MNs are formed through the oxidation of phenolic precursors and a wide variety of such precursors can be used to generate MN-like materials.[1−4] In human and other animal species' physiology, two distinct classes of MNs are responsible for the coloration of skin and hair, eumelanin (EuMN) and pheomelanin (PhMN).[5,6] EuMN is typically described as a brown to black-colored material built from L-DOPA as the precursor. PhMN is typically described as a yellow to red pigment formed by a combination of L-DOPA and the amino acid cysteine (C). The biochemical pathways that lead to these two different classes of MN are described by the classic Raper-Mason scheme. In this scheme, L-DOPA undergoes oxidation and cyclization to intermediates like 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) which then yield the final EuMN material. In the presence of C, this pathway is diverted to a different set of intermediates, *e.g.*, 5-S-cysteinyl-DOPA, 1,4-benzothiazine (BTZ), or 1,4-benzothiazine-3-carboxylic acid (BTZCA), which then lead to the final PhMN material.[3,7−9] The presence of elevated levels of PhMN in an individual's skin is a risk factor making the individual more prone to sunlight-induced skin damage, potentially leading to an increased risk of skin cancer.[10,11] As for other MN, PhMN is poorly defined in its chemistry and its physical properties. PhMN is typically distinguished by its appearance as a lighter-colored material and the presence of C during its synthesis.

MN materials absorb light over a broad range of the electromagnetic spectrum leading to monotonic absorbance profiles over the entire ultraviolet to near-infrared region.[12] Thus, spectroscopic analyses are frequently used to quantify MN preparations. In addition, spectroscopic analyses can be used for qualitative comparisons, *e.g.*, distinguishing EuMN *vs* PhMN, as will be discussed in this report. MNs can be generated from a wide variety of precursors through: (a) oxidation using enzymes, (b) oxidants like $H_2O_2$ or others, (c) infusion with $O_2$, or (d) exposure to air under basic conditions.[13,14] The results and discussions described in this report involve the air-mediated oxidation of catecholic precursors in the presence of $Na_2CO_3$. The report is a combination of results collected over several years which have been detailed elsewhere.[15−19]

## 2. MATERIALS AND METHODS

**2.1. Materials and Solutions.** Dopamine.HCl and L-cysteine.HCl (C) were obtained from Alfa Aesar (Tewksbury, MA). Epinephrine.HCl, norepinephrine.HCl, L-DOPA, and all other amino acids (AA): serine (S), threonine (T), proline (P), 4-hydroxyproline (4-OH P), glycine (G), methionine (M),

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tryptophan (W), glutamine (Q), and leucine (L), were obtained from Sigma-Aldrich (St Louis, MO). All other chemicals used were purchased from Fisher Scientific (Waltham, MA) and were of analytical grade.

**2.2. Analyses and Techniques.** Ultraviolet−visible (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 350 nm, UV-transparent microplates were used. Fluorescence measurements were made in wells of an opaque 96-well microplate using the SynergyHT microplate reader from Biotek (Winooski, VT) with excitation filter set at 360 nm, emission set at 460 nm, and sensitivity factor set at 75. Fourier transform infrared (FT-IR) analyses were performed using the Spectrum Two FT-IR spectrometer from PerkinElmer (Waltham, Massachusetts). Scans were made using the universal attenuated total reflection (ATR) accessory between 650 and 4000 $cm^{-1}$ with a resolution of 4 $cm^{-1}$ and using the OptKBr beam splitter and $LiTaO_3$ detector. For each sample, 24 scans were accumulated. Dialysis was performed using Spectrum Spectra/Por RC dialysis membranes with a molecular-weight cutoff (MWCO) of 3.5 kDa obtained from Fisher Scientific (Suwanee, GA). Samples were lyophilized using a Labconco FreeZone Plus 4.5 L benchtop freeze-dry system obtained from Fisher Scientific (Suwanee, GA).

**2.3. Preliminary Experiments.** For all precursors, four stock solutions were prepared in 50 mM acetate buffer (pH = 6.4) with concentrations at 4, 9, 14, or 22 mM. Five stock solutions of C were prepared in 50 mM $Na_2CO_3$ solution at concentrations of 4, 8, 12, 20, and 32 mM. For each stock solution of all four precursors, six reactions were prepared by mixing 1000 $\mu$L of precursor stock solution with 1000 $\mu$L of 50 mM $Na_2CO_3$ or 1000 $\mu$L of all five stock solutions of C. These mixtures were set up in wells of a 24-well plate, and the plates were incubated at 37 °C for 3 days. After 3 days of reaction, photographs were taken of the plates. Spectra in the visible region were recorded for each reaction mixture after 4-fold dilution with water.

**2.4. Kinetic Studies.** For each precursor, a 1.5 mM stock solution was prepared in 50 mM acetate buffer (pH = 6.4). A stock solution of C at 2 mM was prepared in 50 mM $Na_2CO_3$. To a 40 $\mu$L precursor stock solution, varying volumes of 50 mM $Na_2CO_3$ or C stock solution were added such that the total volume was 250 $\mu$L, the concentration of precursor was 0.24 mM, and the concentration of C ranged between 0 and 0.9 mM. All mixtures were kept in wells of a 96-well microplate and the absorbance at 250, 280, 315, 350, and 400 nm was monitored for 3 h at 37 °C. All reactions were set up in triplicate.

**2.5. Reactions with Epinephrine and C or Other AA.** Stock solutions of epinephrine were prepared in 50 mM acetate buffer (pH = 6.4) at a concentration of 2 mM. Stock solutions of C or other AA were prepared in 50 mM $Na_2CO_3$ solution. Reactions were initiated by the addition of 1 mL epinephrine stock solution to solutions containing 50 mM $Na_2CO_3$, with or without AA, to a total volume of 2 mL. All reactions were set up in test tubes and contained 1 mM epinephrine, 25 mM $Na_2CO_3$, and up to 10 mM of AA and were kept at room temperature for at least 2 days.

**2.6. Large-Scale Reactions.** Epinephrine.HCl (150 mg) was dissolved in 150 mL of 50 mM $Na_2CO_3$ to a concentration of about 3 mM. The reactions contained no AA, 510 mg S, or 570 mg C (both about 30 mM). The mixtures were left stirring at room temperature for up to 3 days. The reactions were monitored by RP-HPLC to ensure that the precursor had reacted away. At the end of the reaction, 15 mL of 0.5 M $CaCl_2$ was added to initiate precipitation as discussed elsewhere.[17] After overnight standing at room temperature, the mixtures were centrifuged. The supernatants were dialyzed and lyophilized and termed the dispersible fraction ($F_{disp}$). The remaining precipitates were washed twice with water, with 1 N HCl until no gas evolution could be observed and twice more with water. The washed precipitates were lyophilized and termed the precipitated fraction ($F_{prec}$).

## 3. EXPERIMENTAL RESULTS

**3.1. Preliminary Observations.** Figure S1A−D illustrates photographs of the reaction mixtures described in Section 2.3. The following visual observations, consistent for all four precursors, were made: (1) the presence of C delayed the onset of color formation and (2) the presence of C resulted in darker reaction mixtures compared to the mixtures with no C, an effect that was particularly noticeable in the case of epinephrine (see panel D in Figure S1). MNs are typically described as insoluble in any solvent. However, our *in vitro* synthesis of MNs leads to mixtures in which the MN products appear to be dissolved. An argument has been made that the "dissolved" reaction products exist as dispersed, stabilized colloidal particles.[20] Thus, absorbance spectra of MN materials dispersed in the buffer/$Na_2CO_3$, aqueous environment employed in our studies were readily obtained. Quantitative estimates of the "darkness" of any sample were calculated by integrating, between 400 and 900 nm, the absorbance spectrum of the sample fitted with an exponential function and calculating the area under the curve (AUC) as outlined elsewhere and shown in eq 1.[15]

$$\mathrm{AUC} = \int_{400}^{900} A_0 \times e^{-k\lambda} d\lambda = \frac{A_0}{-k} \times (e^{-k\times 900} - e^{-k\times 400}) \quad (1)$$

In this equation, $k$ is the decay constant of the exponential profile of the absorbance ($A$) as a function of the wavelength ($\lambda$). In addition, to distinguish EuMN-like materials from PhMN-like materials, the ratio of the absorbance at 650 nm over the absorbance at 500 nm ($A_{650}/A_{500}$) was evaluated. Although these two wavelengths are arbitrarily chosen, it does follow precedent as a suggested way to differentiate dark-colored EuMN from light-colored PhMN.[21,22] Given the exponential relationship between $A$ and $\lambda$, an exponential relationship exists between $k$ and $A_{650}/A_{500}$, as shown in eq 2.

$$\frac{A_{650}}{A_{500}} = \frac{A_0 \times e^{-k\times 650}}{A_0 \times e^{-k\times 500}} = \frac{e^{-k\times 650}}{e^{-k\times 500}} = e^{-k\times(650-500)} = e^{-k\times 150} \quad (2)$$

Figure 1 provides a graphical representation of eq 2, and the "fluidity" of the visual appearances MN materials may exhibit independent of the presence of C.

Figure S2 illustrates the application of eq 1 for the calculation of AUC and obtaining the parameters $k$ and $A_{650}/A_{500}$ for two typical absorbance profiles.

Figure 2A shows the relative AUC (see eq 1) as a function of C concentration for the mixtures shown in Figure S1D. In addition, the impact on the EuMN- or PhMN-like character of the mixtures by C is shown in Figure 2B, with the results obtained from the five reaction mixtures containing C grouped


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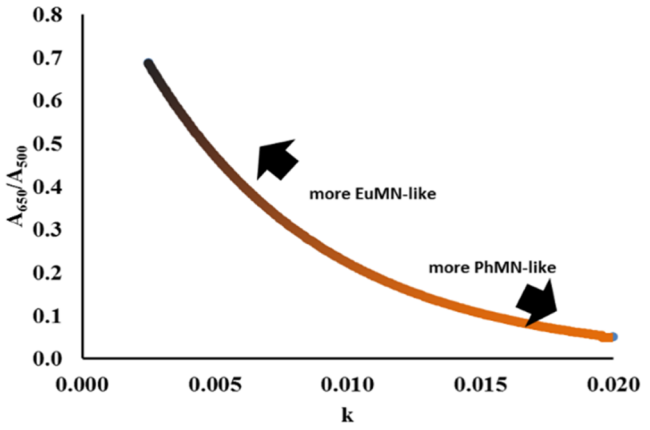


**Figure 1.** Relationship between decay constant $k$ from eq 1 and the ratio of absorbance at 650 nm over 500 nm ($A_{650}/A_{500}$) to distinguish EuMN-like materials from PhMN-like materials according to eq 2.

together. The results associated with Figure S1A−C, are shown in Figures S3−S5.

The results presented in Figure 2 objectively illustrate the quantitative and qualitative differences in the appearances of the reaction mixtures shown in Figure S1D. The presence of C in the reaction resulted in markedly darker appearances (up to nearly 800%) and tilted these appearances from PhMN-like to more EuMn-like.

**3.2. Kinetic Studies.** The impact of C on the kinetics of the oxidation of the catecholamines is illustrated in Figure 3 for the case of L-DOPA and in Figures S6−S8, for the cases of dopamine, norepinephrine, and epinephrine. For all experiments, the absorbance changed in a reproducible manner. In the presence of C, there was a significant lag time between the start of the reaction and the observed changes in absorbance. This lag time was independent of the wavelength monitored but was dependent on the concentration of C present as shown in Figures 3B and S6−S8. Although the pattern of results was consistent for all four precursors, more variability was observed for reactions involving norepinephrine and epinephrine.

**3.3. Effect of C and Other AA on the Oxidation of Epinephrine.** Of all of the precursors investigated, the oxidation of epinephrine presented some unique results. Compared to the other precursors tested, the presence of C had the most impact in terms of increasing the darkness of the mixtures and the shift from pheomelanin-like appearance to eumelanin-like appearance as shown in the photographs of Figure S1D and in Figure 2A,B. The oxidation of epinephrine, in the absence of any other substance, typically results in yellow to rusty-orange reaction mixtures (see Figure S1D). In the presence of AA, particularly M or C, the reaction mixtures took on darker appearances when molar excess amounts of AA were used. Figure 4 presents values of $k$ *vs* $A_{650}/A_{500}$ obtained from crude reaction mixtures containing epinephrine oxidized in the presence of C, M, or other AA.

Depending on the type of AA and its concentration, colors ranging from very light to very dark could be obtained. In addition, the presence of AA changed the values of $k$ and $A_{650}/A_{500}$ such that the entire range from PhMN-like to EuMN-like materials could be generated from epinephrine as shown in Figure 4.

**3.4. Large-Scale Reactions Involving Catecholamines and Cysteine.** Given the unique observations made in the case of epinephrine, large-scale reactions with this precursor without any AA or in the presence of S or C were evaluated, processed, and characterized similarly as for previous large-scale reactions.[16,17,23] S was chosen because of its close structural similarity to C. A fractionation involving a coprecipitation process using $Ca^{2+}$ was performed on the crude reaction mixtures as discussed in Section 2.6. For all reactions, a $F_{disp}$ material was isolated. However, only for the reaction containing epinephrine and C could a dark-colored $F_{prec}$ be obtained. Figure S9A presents photographs of the mixtures at the end of the reaction and of purified and dried $F_{prec}$ obtained from the reaction containing epinephrine and C. $F_{disp}$ materials were dispersed in water, and UV−vis and fluorescence profiles were recorded. FT-IR spectra were collected for all dried materials. Figure 5A−D shows typical results thus obtained. Panel A shows UV−vis profiles of solutions at 0.2 mg/mL of the $F_{disp}$ materials. Panel B shows the concentration-dependent fluorescence of the $F_{disp}$ materials. Panels C and D show the FT-IR spectra of the dried materials.

The UV−vis profiles of $F_{disp}$ from reactions without AA or in the presence of S are very similar. Both show strong absorbance bands around 280 and 340 nm. In the case of S, a stronger absorbance is to be observed at wavelengths above 400 nm which correlates with the darker color of the reaction mixture (see Figure S9A). The UV−vis profile of $F_{disp}$ from the reaction of epinephrine with C lacks the above-mentioned absorbance

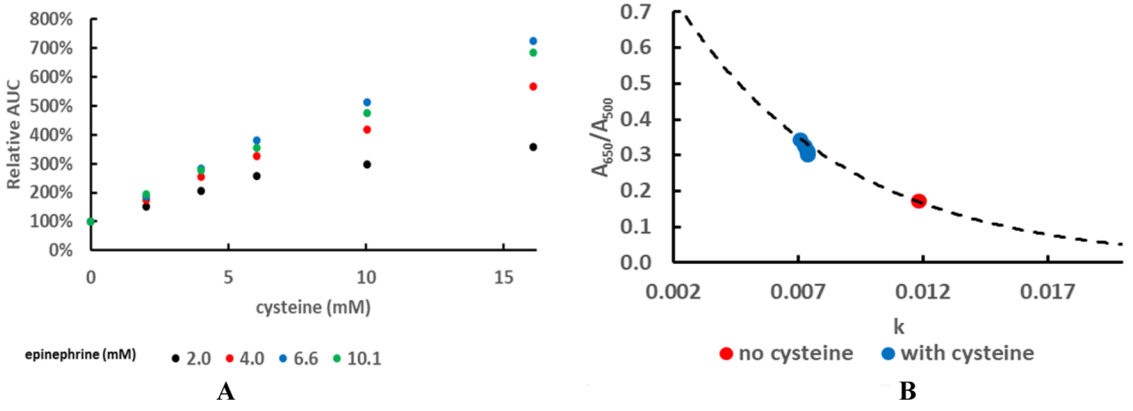


**Figure 2.** (A): Relative AUC, compared to the reaction without C, as a function of C (mM) for the reactions involving epinephrine at various concentrations of C from Figure S1D. (B): Relationship between $k$ and $A_{650}/A_{500}$ for the reaction mixtures involving epinephrine, with or without C, from Figure S1D.



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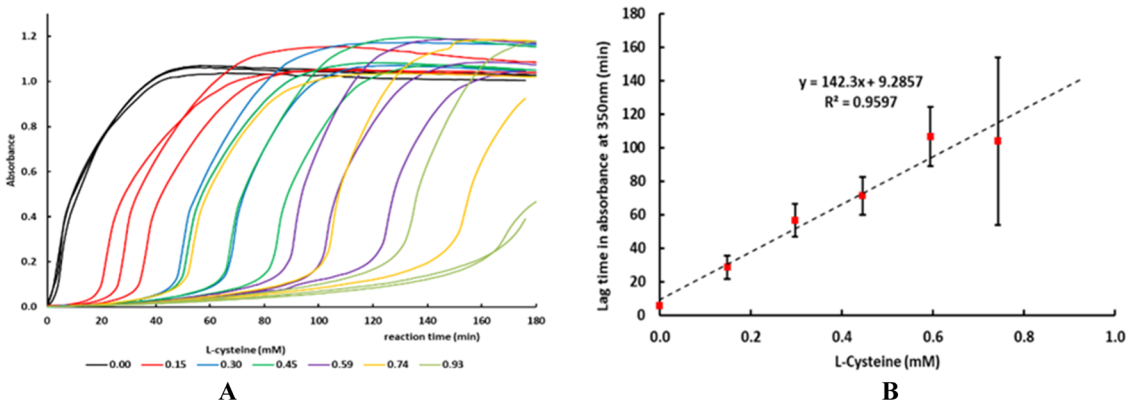


**Figure 3.** Results of the kinetic experiments ($n$ = 3) of the oxidation of epinephrine (0.25 mM) in the presence of C (between 0 and 0.9 mM). (A): Change in absorbance at 350 nm as a function of reaction time. (B): Correlation between the concentration of C and the average lag time ± standard deviation ($n$ = 3) between the start of the reaction and the observed increase in absorbance at 350 nm.

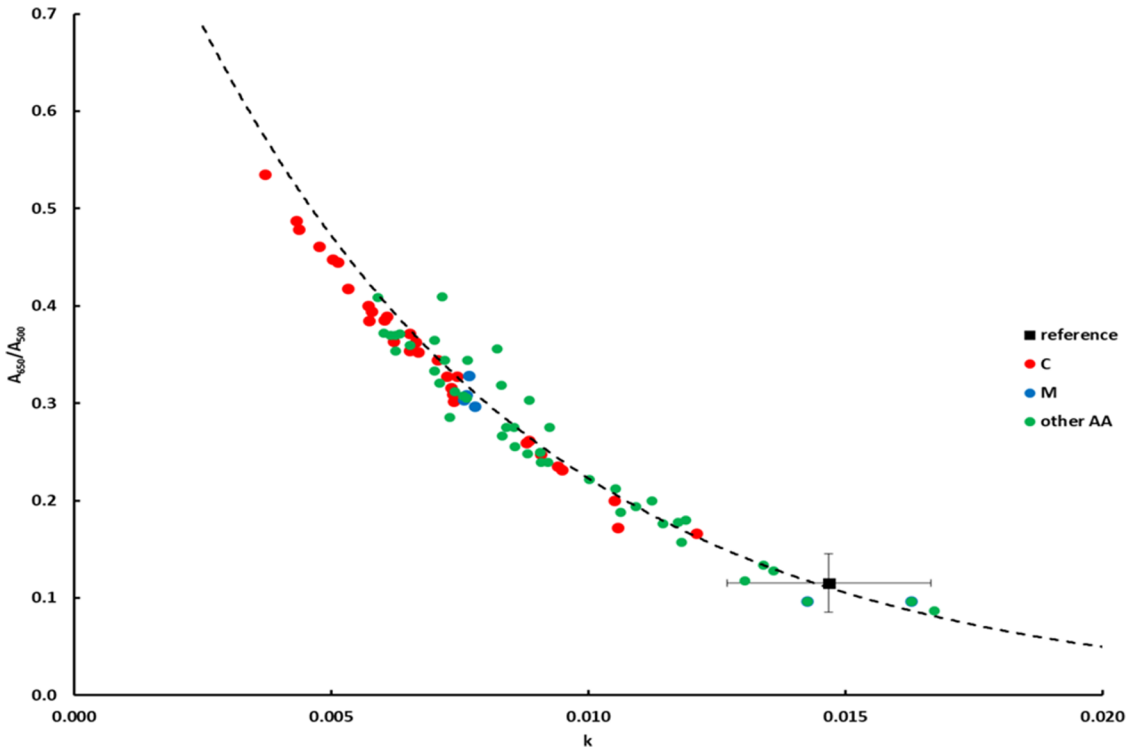


**Figure 4.** Values of $k$ *vs* $A_{650}/A_{500}$ for reactions involving epinephrine and C, M, or other AA (S, T, P, 4-OH P, G, W, Q, or L). Experimental details are discussed in the Section 2. The dotted line represents the theoretical relationship between $k$ and $A_{650}/A_{500}$ according to eq 2; "reference" represents the average value ± standard deviation ($n$ = 6) obtained for the reactions involving epinephrine without any AA present in this set of experiments.

bands and displays much stronger absorbance values at wavelengths above 400 nm. However, any specific absorbance bands could be masked as the absorbance profile of the entire mixture may be dominated by the absorbance profile of the EuMN-like material generated. The fluorescence profiles presented in Figure 5B do suggest that the presence of AA like S or C during the reaction affected the fluorescent properties of $F_{disp}$. However, not enough data has been collected thus far to confirm a pattern of results. The FT-IR spectra of the three $F_{disp}$ fractions shown in Figure 5C are qualitatively similar. They show absorbance bands that are consistent for MN-like materials as discussed in various other reports.[24] The FT-IR spectrum obtained for $F_{prec}$ of the reaction involving epinephrine and C (see Figure 5D) is qualitatively different from those obtained from the $F_{disp}$ fractions. The absorbance in the 1000−1200 $cm^{-1}$ region is relatively stronger, and a prominent absorbance band around 1450 $cm^{-1}$ can be observed. However, we can not exclude the possibility that the $F_{prec}$ fraction was contaminated with precipitated cystine that may have formed during the reaction. When C is left stirring in an alkaline environment exposed to air, a white precipitate forms that, in its FT-IR spectrum, resembles cystine (results not shown). Such a side reaction could occur in reaction mixtures containing C and could have an impact on the synthesis of the MN material as is discussed below.

## 4. DISCUSSION

The results presented in this report are in contrast to the notion that the presence of C during MN synthesis would promote the formation of PhMN-like materials as outlined in the Section 1. It



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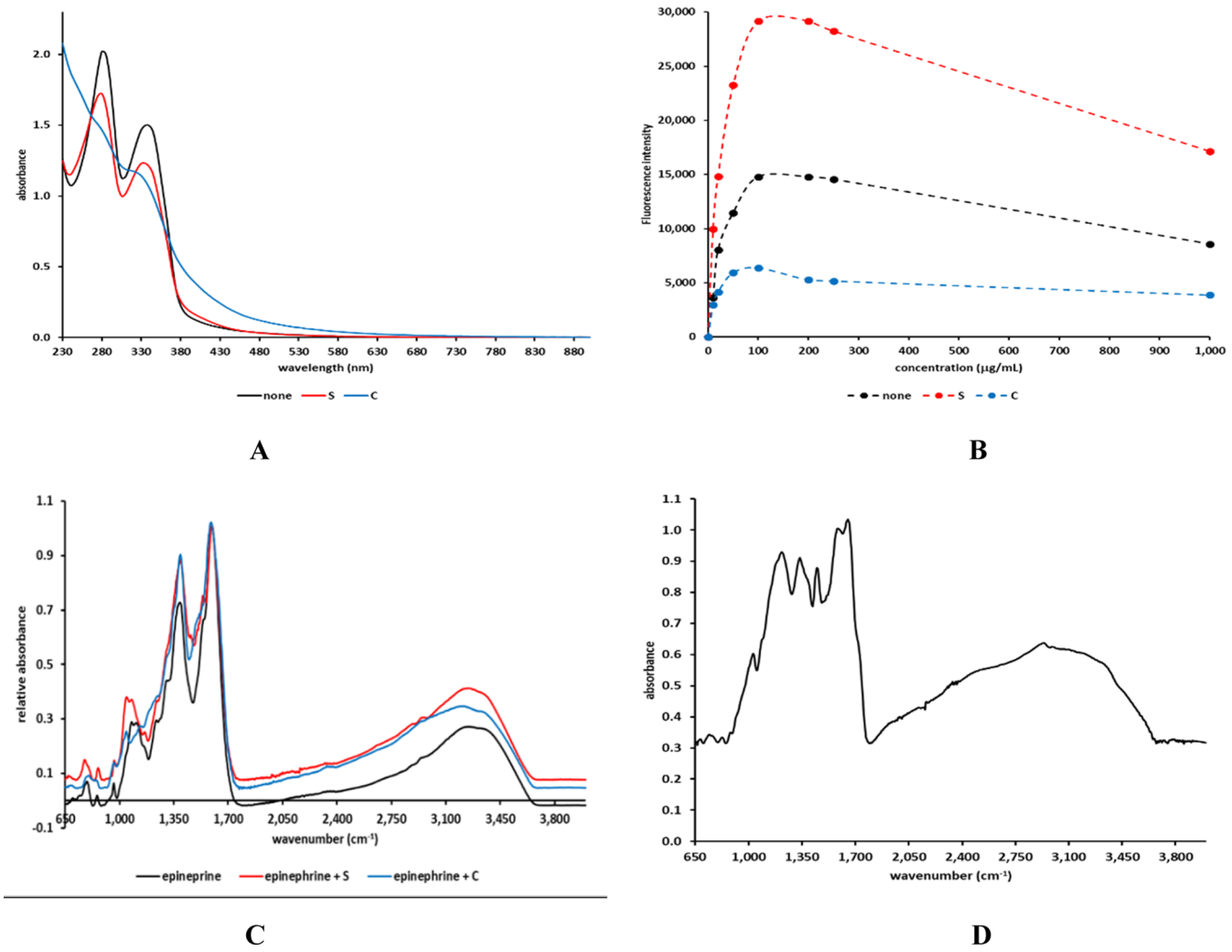


**Figure 5.** (A): UV−vis spectra of $F_{disp}$ at 0.2 mg/mL obtained from reaction mixtures containing epinephrine without any AA or containing S or C as outlined in the Section 2. (B): Concentration-dependent fluorescence profiles of solutions of $F_{disp}$ obtained from reaction mixtures without AA or in the presence of S or C. (C): FT-IR spectra, normalized for their absorbance at 1600 $cm^{-1}$, of $F_{disp}$ from the reactions involving epinephrine, epinephrine and S and epinephrine and C. (D): FT-IR spectrum, normalized for the absorbance at 1600 $cm^{-1}$, of the $F_{prec}$ from the reaction involving epinephrine and C.

is important to emphasize some of the critical differences between our experiments and those from other reports on PhMN. Our reactions involve air-mediated oxidation while other PhMN synthesis strategies often involve the use of enzymes like tyrosinase.[25−27] In addition, we monitored our reactions over much longer time periods, days *vs* hours, compared to other published reports. Anecdotal observations reported elsewhere provide support to some of the results presented in this report. In their study of the effect of C on the oxidation of tyrosine, L-DOPA, or cysteinyldopas, Agrup et al. noted a marked darkening of the reaction mixture upon prolonged reaction times.[26] In addition, they noted the suppression of the oxidation of cysteinyldopas by C in a concentration-dependent fashion. In their study of PhMN, Deibel and Chedekel observed the formation of a dark brown solution and brown solid when reacting L-DOPA with tyrosinase in the presence of C.[27] They noted that the yield of pigment increased with prolonged reaction times and also noted lag times in the formation of colored products. Ozeki et al. observed an increased intensity in color in later stages of the reaction when L-DOPA was oxidized by tyrosinase in the presence of C.[28] They attributed this to a shift to a more oxidized state of the PhMN structure. The product of the reaction of L-DOPA and C in the presence of tyrosinase was described by Ito as a dark brown precipitate.[29] Our and these anecdotal observations suggest that simply mixing L-DOPA in the presence of C will not lead to the synthesis of a PhMN-like material with yellow-to-red appearance. Some of the discussions presented here are echoed in the discussions presented by others regarding the discrepancies in the attempts to synthesize PhMN *in vitro*.[1,30,31] Figures 3B and S6B−S8Bprovide detailed evaluations of the delay in onset of color formation in the presence of C. This delay in color formation could be attributed to the antioxidant properties of C. In a mixture of precursor and C, C may be oxidized to cystine before any oxidation of precursor occurs. *In vivo*, where enzymes mediate the (biochemical) reactions, an enzyme like tyrosinase could selectively bind both precursor and oxygen and preferably promote the oxidation of the precursor leaving any C present unoxidized. The presence of the unreacted C, with its reactive thiol functionality, could then affect the chemistry of the developing MN-like material leading to PhMN rather than EuMN. *In vitro* approaches to the synthesis of PhMN may


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require conditions whereby in a mixture of precursor and C, the precursor is preferably oxidized without the oxidation of C.

The discussions thus far provide an explanation for the failure to produce PhMN-like materials but fail to explain why the presence of C would result in darker, more EuMN-like materials (see Figures S1D and S9). Our objective evaluation of the nature of the MN material, EuMN-like *vs* PhMN-like, is based upon the suggestion that the ratio of the absorbance at 650 nm over the absorbance at 500 nm ($A_{650}/A_{500}$) can be used as a parameter to distinguish both types of MN. Values of $A_{650}/A_{500}$ between 0.25 and 0.30 are considered typical for EuMN-like materials, while values between 0.10 and 0.14 are considered typical for PhMN-like materials.[8,21,22] The classification into EuMN- or PhMN-like materials we present in this report is based upon eq 2 and shown in Figure 1. The plots shown in Figures 2B and 4 are derived from objective spectroscopic analyses from which $A_{650}/A_{500}$ and $k$ were obtained. However, these objective evaluations correlate with the subjective, visible appearances of the reaction products. It is important to note that our classification of EuMN-*vs* PhMN-like is independent of whether C was present during the reaction. In addition, it allows for an objective presentation of the wide range of colors that can be formed as seen in the photographs in Figure S1 and highlighted for the case of epinephrine in Figure 4. In previous reports, we have detailed results indicating the presence of light-colored, PhMN-like materials, hidden behind the dark-colored EuMN-like materials when precursors like L-DOPA, dopamine, or catechol are oxidized.[16,17] This phenomenon can also be observed in the report by Ma et al. on the oxidation of some catecholamines (see Figure 1 in ref 32).

Thus, it appears to us that many precursors can form PhMN-like materials when oxidized in an alkaline environment, but not all are capable of forming EuMN-like materials. We hypothesize that for any precursor involved, the generation of light-colored, PhMN-like materials may be the default. The presence of intrinsic or extrinsic factors may tilt the synthesis toward EuMN-like materials. Intrinsic factors could be the specific chemical features within the structure of the precursor that allows them to spontaneously form EuMN in addition to PhMN, *e.g.*, L-DOPA and dopamine. For these types of precursors, the PhMN-like materials generated would be "out of sight"; masked by the dark EuMN-like materials generated. Extrinsic factors could be differing reaction conditions that promote aggregation of the reaction products tilting the final products toward EuMN, *e.g.*, epinephrine reacted in the presence of C. As outlined before, when reactions consist of a mixture of precursor and C, there is the possibility that C is oxidized into cystine prior to the oxidation of the precursor. The presence of cystine may then have a pro-aggregating effect leading to darker, more EuMN-like materials. This is our current hypothesis regarding the EuMN-generating impact of C on the oxidation of precursors that typically yield PhMN-like materials as illustrated for the case of epinephrine in Figure 4 and elsewhere.[19] This hypothesis, and a comparison to what may happen *in vivo*, is shown in Figure S10. The notion that intrinsic/extrinsic and/or aggregating factors may determine some of the physicochemical properties of MN was observed and discussed by Micillo et al.[33] Their results and discussions are difficult to compare to our results as a different precursor (DHI) was used in their studies and different oxidizing conditions.

## CONCLUSIONS

Our study of the impact of cysteine on the *in vitro* synthesis of melanin yielded two impactful observations: (1) the presence of cysteine does not lead to the generation of a pheomelanin-like material; (2) on the contrary, the presence of cysteine leads to the generation of more eumelanin-like materials. These observations are in contrast to typical descriptions of pheomelanin and possible explanations are discussed.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c00889.

Photographs of reaction mixtures involving cysteine and different catecholamines; illustrative plot associated with eq 1; additional data similar to Figure 2; additional data similar to Figure 3; photographs of reaction products associated with experiments of Section 3.4; and figure illustrating current hypothesis to explain results (PDF)

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### Author Contributions

Conceptualization, methodology, analysis, writing, and editing by K.V.

### Notes

The author declares no competing financial interest.

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