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Hydration-Controlled X-Band EPR Spectroscopy: A Tool for Unravelling the Complexities of the Solid-State Free Radical in Eumelanin

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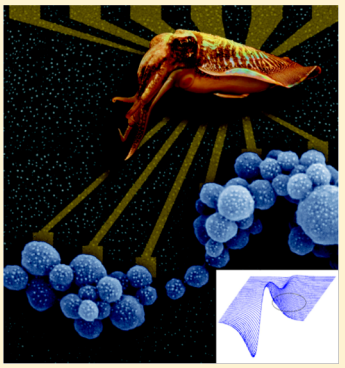
Supporting Information

ABSTRACT: Melanin, the human skin pigment, is found everywhere in nature. Recently it has gained significant attention for its potential bioelectronic properties. However, there remain significant obstacles in realizing its electronic potential, in particular, the identity of the solid-state free radical in eumelanin, which has been implicated in charge transport. We have therefore undertaken a hydration-controlled continuous-wave electron paramagnetic resonance study on solid-state eumelanin. Herein we show that the EPR signal from solid-state eumelanin arises predominantly from a carbon-centered radical but with an additional semiquinone free radical component. Furthermore, the spin densities of both of these radicals can be manipulated using water and pH. In the case of the semiquinone radical, the comproportionation reaction governs the pH- and hydration-dependent behavior. In contrast, the mechanism underlying the carbon-centered radical's pH- and hydration-dependent behavior is not clear; consequently, we have proposed a new destacking model in which the intermolecular structure of melanin is disordered due to $\pi-\pi$ destacking, brought about by the addition of water or increased pH, which increases the proportion of semiquinone radicals via the comproportionation reaction.



INTRODUCTION

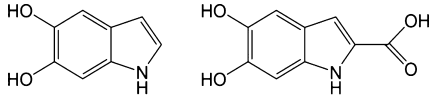
The melanins are an important class of multifunctional biomacromolecules found throughout the biosphere.[1] The main forms of melanin in humans are brown-black eumelanin and yellow-red pheomelanin.[1] Both eumelanin and pheomelanin are present to varying degrees in the skin being responsible for tissue pigmentation. While melanins are believed to be photoprotective pigments acting as our primary defense against ultraviolet radiation, one of the most ubiquitous and potent environmental carcinogens,[2] the efficiency of eumelanin and pheomelanin to act as photoprotectants, may differ significantly.[3,4]

Eumelanin is composed of two basic monomeric building blocks[1] (Scheme 1), 5,6-dihydroxyindole (DHI), and 5,6-dihydroxyindole-2-carboxylic acid (DHICA). The heterogeneous macromolecular system derived from these monomeric units contains variously cross-linked oligomers and quinone redox states, resulting in an overall macroscopic disordered solid-state material.[5] Incomplete polymerization of these monomeric building blocks produces unterminated carbon-centered free radicals.[6,7]

Of major interest is that solid-state eumelanin possesses a unique collection of physical and chemical properties[5] that include electrical and photoconductivity; broad, monotonic optical absorption across the UV, visible, and near IR regions; almost unity nonradiative conversion of absorbed photon energy into heat; and a persistent EPR-active free-radical species. Several of these properties are consistent with eumelanin's biological roles (particularly as a photoprotectant).

Lately, eumelanin has gained significant attention as a potential bioelectronic solid-state material. With the creation of electronic grade thin films,[8−12] investigations are underway to see whether its unique electronic properties (hydration-dependent conductivity, organic amorphous semiconductor) can be utilized in biomimetic devices.[11]

Scheme 1. Building Blocks of Eumelanin, 5,6-Dihydroxyindole (DHI), and 5,6-Dihydroxyindole-2-carboxylic acid (DHICA)



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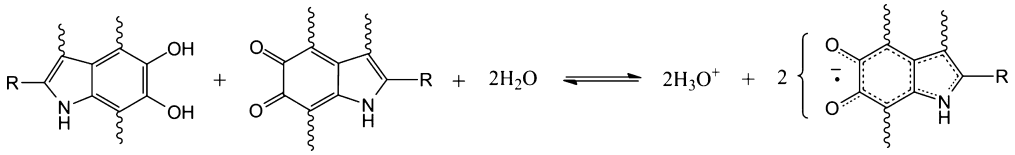
Scheme 2. Comproportionation Reaction



Advancing melanin as a bioelectronic solid-state material will require an understanding of its electronic/conductive properties in a wet/ambient atmosphere. The presence of a persistent free-radical species in eumelanin may be responsible for its electronic/conductive properties. Until recently,[13,14] the accepted charge-transport model for eumelanin was based on McGinness et al.'s contention that it is a Mott−Davis amorphous semiconductor[15,16] in which the Fermi surface of eumelanin is populated by unpaired electron spins (which gives rise to an EPR signal,[17]) all present in the trapped states within the density of states profile. It was argued that with the addition of water the free electron spin density will decrease as the localized trapped electrons will start to delocalize (due to the lowering of energy barriers from a high dielectric material) and pair up with one another.[17] Recently we utilized electrical photoconductivity, muon spin resonance (muSR), and photoconductivity-EPR measurements of solid-state eumelanin as a function of environmental humidity to show that hydration of melanin shifts the comproportionation equilibrium (Scheme 2) so as to dope electrons (semiquinone) and protons into the system.[14] However, this study highlights a strange dichotomy of the melanin EPR signal, as in solution the EPR spectra are typical of a carbon-centered radical at low pH and a semiquinone radical at high pH resulting from a shift in the comproportionation reaction (Scheme 2) toward the products,[18−20] whereas in the solid state the resonance has been attributed to radicals resulting from incomplete polymerization.[7]

Most EPR studies of eumelanin have been performed in the solution state[18−25] (although it should be noted that at all but very high pH values eumelanin is insoluble and is in fact suspended rather than dissolved[26]) and analysis of the EPR spectrum has been attributed to a semiquinone free radical that is regulated by the comproportionation reaction (Scheme 2),[5,18−20,25] although at low pH, the EPR resonance is centered around $g = 2.0034$,[19] characteristic of a carbon-entered radical.

Evidence of Scheme 2 was found in a series of EPR[18−20,25] and chemical studies. For example, the temperature-dependent intensity of the continuous-wave electron paramagnetic resonance (CW-EPR) signal can be changed reversibly if eumelanin is in solution.[20] (This is not always the case in the solid-state.[27]) Furthermore, the temperature dependence of the EPR signal of the semiquinone radical (Scheme 2) shows Arrhenius behavior (signal intensity $\propto e^{-E_A/kT}$), which is expected for a reversible chemical reaction. It should be noted that this is a unique observation because generally for a magnetically isolated spin 1/2 system one expects the temperature dependence of the magnetic susceptibility (χ_M) and the EPR signal intensity to exhibit Curie−Weiss behavior ($\chi_M \propto 1/T$). Regular, weak base titration curves have been obtained in pH-dependent solution studies as well with the requisite reversibility.[19,21−24] In one particular study the pH was varied under photoexcitation, and an increase in the EPR signal intensity was observed.[21,22] This has been interpreted as indicating the production of more semiquinone free radicals. Finally, metal ion studies demonstrated that when multivalent diamagnetic metal ions are added the intensity of the EPR signal also increases, a consequence of shifting the comproportionation reaction toward the products, through coordination of the catecholate moieties.[23−25,28] Similar observations have been found for another *o*-quinone system: the flavins.[29] However, Q-band (35 GHz) EPR experiments reveal that the radical signal observed at X-band frequencies may consist of up to four different free radical species, possibly related to whether the radical is anionic, neutral, or cationic.[30]

In contrast with the solution studies, there has only been a small number of EPR studies on solid-state eumelanin,[6,27,31,32] a state of eumelanin that is very difficult to control due to its hygroscopic properties.[33,34] The only definitive conclusion appears to be that the free-radical EPR signal shows Curie−Weiss temperature dependence (signal intensity $\propto 1/T$).[6,27]

Gonçalves et al.[27] examined the changes in intensity of the EPR signal upon wetting and drying the solid-state sample, suggesting that the solid-state free-radical species might be regulated by the comproportionation reaction. Our muSR results support this hypothesis.[14] This contrasts the assertion by Blois[7] that the EPR signal arises from unterminated free radicals formed during the free-radical polymerization of the eumelanin polymer.

Clearly, and in contrast with solution studies, there are a number of ambiguities relating to the identity of the free radical in solid-state melanin. Furthermore, it appears that water content has a strong influence on the solid-state EPR signal.[27,31]

Considering all of the above, it would appear that there is a great need to perform a systematic EPR study of solid-state eumelanin as a function of hydration. Thus far the only systematic presentation of a hydration-dependent EPR signal has been presented by ourselves,[14] but no attempt was made to analyze the EPR spectra (power dependence and radical composition) as a function of hydration and pH. Consequently, we present herein the first carefully controlled hydration power saturation (PS) EPR study on eumelanin in the solid state at low, neutral, and high pH to investigate the identity of the solid-state free-radical species.

EXPERIMENTAL METHODS

Sample Preparation. Eumelanin samples were synthesized and pellets made as described in an earlier article.[34] The pellets were then broken to fit within the EPR quartz sample tube. A neutral powder sample was also used.

To determine whether pH could affect the EPR signal, samples of differing pH values were also produced. Acidic samples were made by the same synthetic route described previously[34] for neutral powders with one exception: at the end of the synthesis, samples were filtered and then washed with water to neutralize the powder; the acidic samples were not washed, thus keeping the powders at a low pH. The acidic powder was then dried and pressed as previously described.

Basic samples were more difficult to obtain because eumelanin can be partially dissolved at a high pH. An

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alternative hydration procedure was employed to achieve the high pH, see Hydration Control below.

X-Band CW-EPR Power Saturation Measurements. CW-EPR spectra were measured with a Bruker Biospin Elexsys E500 CW-EPR spectrometer. The spectrometer consisted of a 10 in. electromagnet with a 12 kW power supply (enabling static fields up to 1.4 T), an X-band (ca. 9 to 10 GHz) SuperX microwave bridge, and a super high Q cavity. Furthermore, a Eurotherm 4131VT variable temperature controller was employed in conjunction with a liquid nitrogen gas flow through system, together ensuring that a constant sample temperature (298 K) was maintained. The setup also included a Bruker Biopsin teslameter (ER036TM) and an EIP 548B frequency counter, which provided calibration of the magnetic field and microwave frequency, respectively. A personal computer (PC) with the Mandriva Linux operating system and the Bruker Biopsin Xepr (v. 2.66.45) data acquisition software was used to control the spectrometer. Xepr allows the measurement of multidimensional EPR data sets.

The eumelanin sample was placed within a cylindrical quartz EPR tube (Wilmad 707-SQ) with an inner diameter of 3 mm. This tube was then inserted into the liquid nitrogen flow through quartz insert, which was placed in the super high Q cavity.

The sample tube was then connected to a vacuum line system (Figure S1, Supporting Information) designed to control the hydration levels of the eumelanin sample, in a manner similar to a previous adsorption study.[34]

It should be noted that for every set of experiments the vacuum line was carefully tested for potential leaks. The line was always evacuated to a measurement of 0 mbar (on the BOC-Edwards pressure gauge (GK series, 0−50 mbar)), at which point the line would be isolated from the rotary pump via use of the valve. The system was then allowed to stand for 1 h to see if the pressure increased. Any deviation necessitated a disassembly of the vacuum line to determine the origin of the air leak.

Once the above procedure indicated no increase in pressure, the system was deemed fit for the hydration-controlled experiments described below.

Hydration Control. The sample was hydrated as described in the aforementioned previous adsorption study.[34] In brief: the vacuum line was evacuated for 1 h using a rotary pump ($<10^{-3}$ mbar). The vacuum line was then isolated from the pump and a PS experiment (see below) was conducted. After the PS experiment, water vapor was bled into the vacuum line (using the degassed water vial) up to a pressure of 3 mbar to wet the eumelanin sample. From a previous study, we have shown that it takes ∼45 min for melanin pellets to absorb water and reach equilibrium.[34] Thus, the pellet was then left for 1 h to equilibrate, at the end of which another set of PS measurements was taken. Following the PS measurements, the water pressure in the line was increased by 3 mbar and the above procedure was repeated. Pressures in 3 mbar increments were taken up to a final value of 21 mbar. This hydration procedure was used for neutral powder and pellets of eumelanin as well as for acidic eumelanin pellets.

Given the aforementioned problems with creating a basic sample, a procedure was developed to hydrate the eumelanin sample with ammonia. The same experimental setup as that described above was employed, but an additional vial was attached to it containing ammonia solution (28% Univar, freeze−pump−thawed three times). After the vacuum line was checked for leaks, the sample was pumped for 1 h to ensure relative dryness. The sample was then exposed to the ammonia vial, which was kept open overnight (∼12 h). This enabled the eumelanin sample to reach equilibrium with the ammonia in the environment. The long equilibrium time was deemed necessary because previous experience with molecules of similar size like ethanol showed slow adsorption kinetics (∼5 h or greater).[34]

After ammonia saturation, the vacuum line was evacuated for 10 min to ensure removal of the ammonia vapor but at the same time ensuring that the maximum amount of ammonia remained in the sample. The hydration/PS procedure was then performed as described above.

Power Saturation Measurements. A 2D experiment (microwave power vs magnetic field) was performed using the Xepr data acquisition software. The initial spectrum (center field 351.4 mT, sweep width 4.0 mT, 4096 data points) was measured at 60 dB, with a 100 kHz modulation frequency and an amplitude of 0.036 mT. The spectrum was signal-averaged (two scans) to ensure reproducibility. The microwave power was then increased in 1 dB steps up to a power of 20 dB (2 mW), taking 41 steps.

Data Analysis. The water content in the eumelanin samples was determined using the same method as that outlined in the adsorption study.[34]

The first derivative EPR spectra were doubly integrated to determine the total spin density. Specifically, the first derivative spectra were integrated using the trapezoidal rule and then baseline corrected to account for the random noise before performing a second integration.

Spin density as a function of water content (for a given pH) was obtained by normalizing the entire PS curve at a given hydration level against the PS curve at a hydration level of 14% and then taking the average over all 41 points. This enables us to discount saturation effects and allows us to compare samples of different pH values to one another. Note, one would normally be inclined to use the dry data as the normalizing curve; however, ensuring that a sample is dry is extremely difficult. However, for a given vapor pressure, we can determine a sample to be at equilibrium. A value of 14% was taken as the normalizing position because this data point was common to all three data sets.

A $P_{1/2}$ analysis was conducted (see the Supporting Information) with the modeling of one radical species for the acidic sample (carbon-centered radical). Parameters obtained from this analysis were then used in the modeling of two species for the neutral and basic samples because it was clear from the analysis that the neutral and basic samples had the carbon-centered radical species and another species corresponding to the semiquinone radical. For the values of the parameters, see the Supporting Information, Figures S14−S16.

Deconvolution of the data employed two pseudovoigtian curves and was performed using matlab (v 13). Fitting parameters are given in the Supporting Information.

RESULTS AND DISCUSSION

X-band microwave frequencies are ideal for performing hydration-controlled EPR experiments because dielectric loss from water absorption is minimal and the limited *g*-value resolution may allow identification of multiple species or the determination of the anisotropic spin Hamiltonian parameters. Presented in Figures 1a−c and 2a−c are the X-band CW-EPR

spectra and PS curves for acidic, neutral. and basic solid-state pellet samples of eumelanin as a function of hydration.

The PS data are qualitatively similar to that reported by Stratton and Pathak[31] for eumelanin under atmospheric conditions for the same microwave power range. It should be noted, however, that these authors had no quantitative control of the hydration level of their samples. Essentially, they had only two levels of hydration: dry and wet (after soaking in water). The results described herein are far more reliable because we have systematically controlled the hydration level, which as discussed in the Introduction and shown from our results below, is a vital parameter.

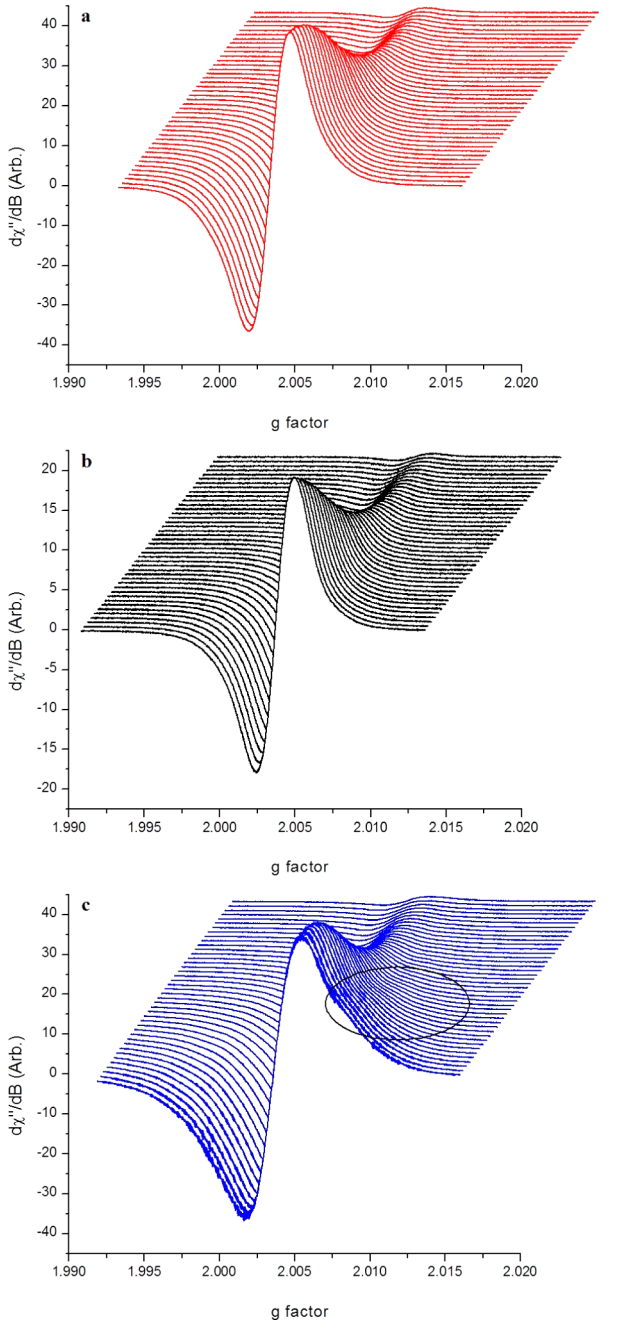

Figure 1. Power saturation EPR studies (a–c) at a water content of ~14% for samples at different pH. The third dimension (into the page) indicates decreasing microwave power (from 2 mW to 0.2 μW). (a) Acidic sample. (b) Neutral sample. (c) Basic sample. All spectra have a g_{iso} typical of a carbon centered radical. Circle in the basic sample data clearly reveals the presence of a shoulder at high microwave powers, although it is also present at lower microwave powers.

An examination of Figure 2d reveals a decrease in spin density (doubly integrated spectra) as the water content increases. To ensure this was not an experimental artifact, such as dielectric loss,[35,36] we undertook the following precautions. The first was to minimize dielectric loss by placing the pellets' largest surface in the nodal plane of the electric component of the microwaves in the super high Q cavity. Furthermore, the diode current of the spectrometer cavity (a proxy for the Q-factor and hence dielectric loss) was monitored constantly. It was observed that the diode current did not change over the course of an EPR hydration isotherm for a given sample, which could take up to 16 h at different water vapor pressures (ranges from 3 to 21 mbar). This indicates that the decrease in intensity as a function of water content is legitimately due to an actual free radical spin density change in eumelanin.

PS data were also obtained for a neutral powder of eumelanin to investigate morphological effects (Figure S2 in the Supporting Information). However, there was no difference in the behavior between the PS curves for the neutral powder and the pellets. Therefore, morphology is not a determining factor in the changes to the free radical spin density within solid-state eumelanin.

The average electronic g factors for the different samples over their hydration ranges can be seen in Table 1. (It should be noted that there was no trend as a function of hydration.)

As can be seen, the g values do not change significantly between samples. This indicates that the resonant field position of the EPR signal is not greatly affected by changes in pH or hydration level. This is the first indication that the solid-state EPR signal may not arise from a dominant semiquinone free radical. Furthermore, the values of the g factors are indicative of a carbon-centered free radical (~2.0036)[37] rather than a melanin semiquinone free radical (≈ 2.0045 to 2.005).[38]

It is worth investigating Gonçalves et al.'s study[27] closely because their proposition is highly original and was based on the first attempt to account for hydration effects on the EPR signal in solid-state eumelanin. They based their argument on solid-state EPR and Fourier transform infrared spectra, in which they observed an increase in COOH moieties and an increase in the EPR signal after the sample was dried. Essentially, their model proposes: (i) that there is a reservoir of hydronium ions trapped within the structure of melanin; (ii) when eumelanin is heated, the loosely bound hydronium ions are released and subsequently trapped by COO^- entities; and (iii) the loss of hydronium ions increases the pH, producing more semiquinone radicals via the comproportionation reaction (Scheme 2).

However, their model never accounted for a loss of water explicitly nor does it take into account the relative strengths of the equilibrium constants (i.e., pK_a/K_{eq} values). If accounted for, one should see a decrease in the semiquinone concentration because water will shift the comproportionation reaction toward more reactants. This will more than offset losses due to trapping of the hydronium ions by COO^- because the effect of a loss of water is greater. (The comproportionation reaction strongly favors reactants over products;[23] see the Supporting Information for a full explanation.)

Inspecting Gonçalves et al.'s published results[27] closely, it is noted that the eumelanin sample was heated to dry it. This may

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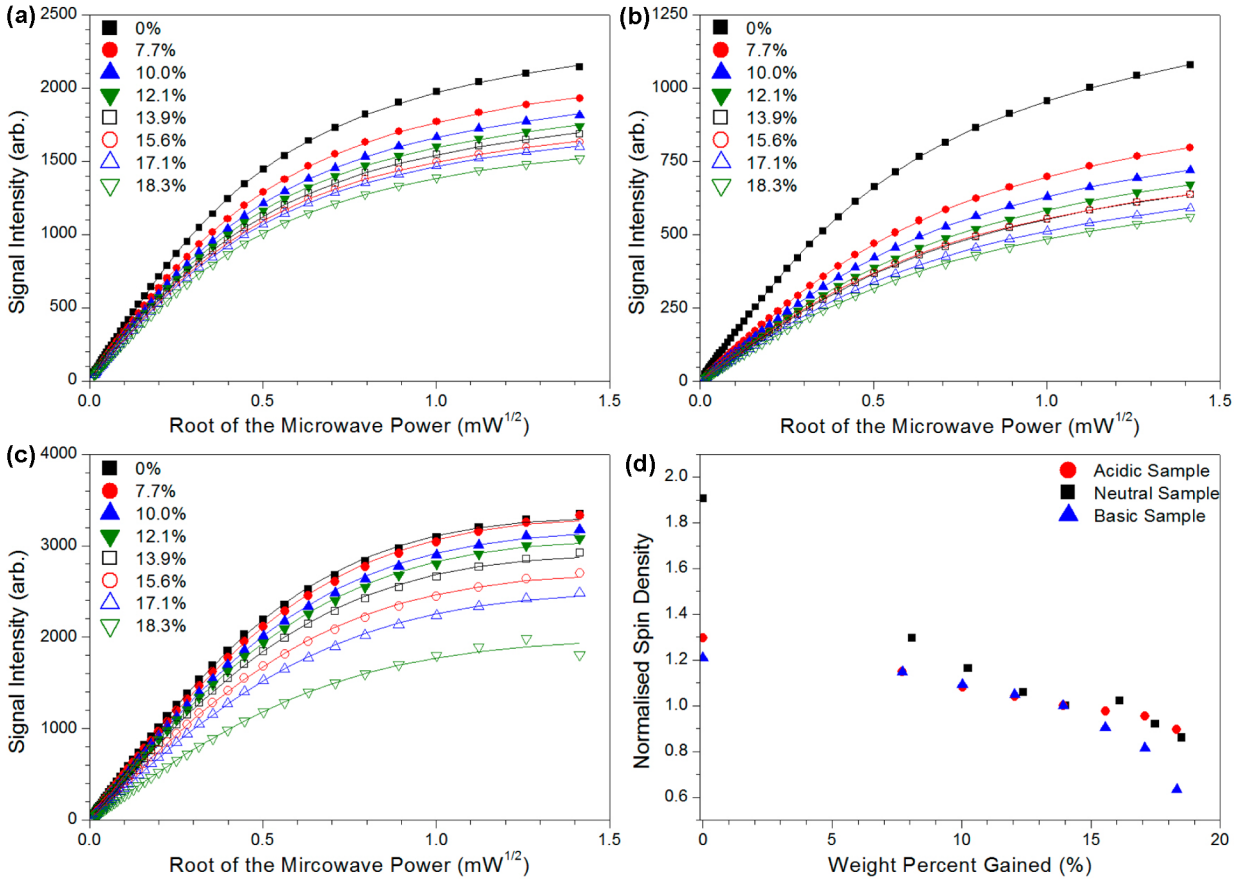

Figure 2. Power saturation data for (a) Acidic, (b) Neutral, and (c) Basic pellet samples of eumelanin. (d) Normalized free-radical spin density changes as a function of hydration for the different samples.

Table 1. Calculated Mean *g* Factors for the Electronic Spins in the Eumelanin Samples[a]

morphology	pH	mean *g* factors
powder	neutral	2.0036 ± 0.0003
pellet	neutral	2.0037 ± 0.0003
pellet	acidic	2.0033 ± 0.0002
pellet	basic	2.0035 ± 0.0003

[a]Morphologies and pH are indicated.

explain the anomaly because increased temperature should shift the reaction to the right, but their argument is difficult to sustain because the experiments were performed without good hydration control. Variation in one variable (water content) while experimentally changing another (temperature) and inspecting a third (COOH/EPR intensity) will always pose difficulties in elucidating a model. Furthermore, Gonçalves et al.'s. work[27] as well as other solid-state studies[6] showed a typical Curie–Weiss temperature dependence (signal intensity $\propto 1/T$) of the EPR signal (whether wet or dry, as long as water content stayed the same[27]) rather than the Arrhenius temperature dependence observed for the melanin semiquinone in solution.[20] Clearly the solid-state EPR signal cannot arise from a purely semiquinone free radical. EPR spectra of acidic, neutral, and basic samples (Figure 1) consist of a dominant resonance with a *g* value (Table 1) consistent with a carbon-centered radical.

A comparison of Figure 1a,b with Figure 1c reveals the presence of a shoulder at a higher g value (circled area in 3D curve, Figure 1c). Inspection of the second derivative EPR spectra (not shown) as a function of microwave power shows that the shoulder is present over the entire range of microwave powers.

Attempts to fit either a single Gaussian or Lorentzian function to the integrated experimental spectra from the neutral and basic samples failed. Assuming the presence of two radical species (carbon-centered radical and semiquinone radical), each with a single isotropic resonance as a minimalistic model, we utilized two pseudovoigtian curves to fit the integrated experimental spectra (Figure 1). The pseudovoigtian curves were fixed at optimized *g* factors of 2.0032 (carbon-centered radical) and 2.0045 (semiquinone free radical), which afforded the best fit (as judged by performing a least-squares analysis of all spectra) of the modeled data to the experimental spectra (representative example shown in Figure 3). For parameter values, see the Supporting Information. The *g* values of 2.0032 and 2.0045 are consistent with carbon-centered and semiquinone radicals, respectively.[37,38]

Similar results were obtained for the neutral pellet sample but with a less pronounced semiquinone peak (see the Supporting Information for all fitting parameters). EPR spectra of the acidic sample were satisfactorily modeled assuming a single resonance arising from a single radical, namely, the carbon-centered radical. Essentially, the data indicate that the majority of the solid-state EPR signal is due to a radical with a *g* factor typical of a carbon centered radical but that a significant fraction of the signal is associated with a semiquinone free radical in the neutral and basic samples. These results are

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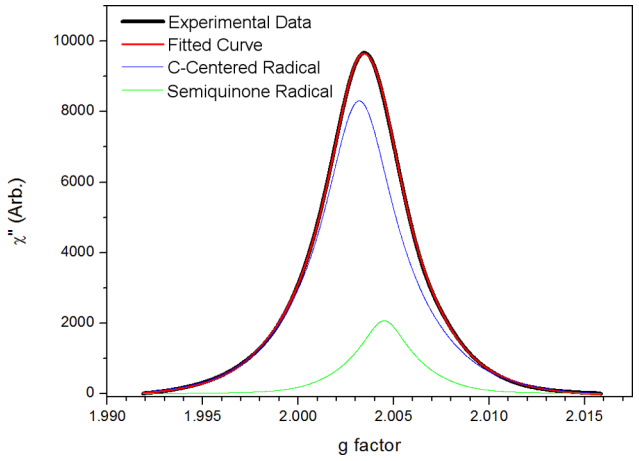


Figure 3. Deconvolution of the integrated EPR spectrum associated with the basic sample. The spectrum is best explained by the presence of two free radicals, the more dominant carbon-centered signal (g = 2.0032) and the small semiquinone signal (g = 2.0045). Data at a water content of 14.0% and microwave power of 0.71 $mW^{1/2}$.

consistent with those found in solution, where at low pH a carbon-centered radical predominates and upon increasing the pH the comproportionation reaction produces increasing concentrations of semiquinone radicals. However, it should be emphasized that the isotropic EPR spectra of the carbon-centered and semiquinone free radicals deduced from the experimental spectra (Figure 3) are likely to be anisotropic[39] and indeed may be composed of multiple subspecies[30] that together form the overall pseudovoigtian line shape. Inclusion of all of these affects in the data analysis would over-parameterize the problem; consequently, we have restricted the analysis to two isotropic species, which as Figure 3 shows provides an excellent fit at X-band frequencies.

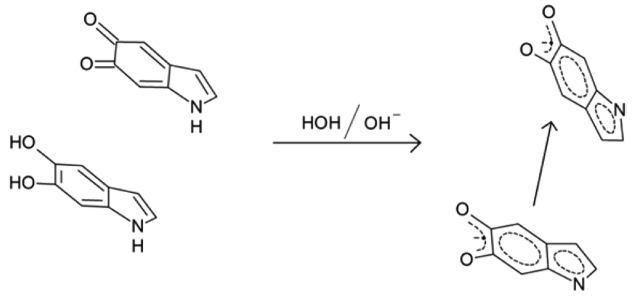
Returning to the minimalist model, there appears to be two free-radical species giving rise to resonances that overlap at X-band frequencies producing a subtle shoulder (See Figure 1c) on the dominant resonance. An accurate analysis of the $P_{1/2}$ dependence of each component is not straightforward. However, we did attempt such an analysis, the majority of which can be seen in Figures S14–S16 in the Supporting Information. For the acidic sample, only one spin was assumed (the carbon-centered radical) and it would appear that the $P_{1/2}$ values remain constant around 0.4 mW across the entire hydration range. For the neutral and basic samples, it was assumed that there are two spins contributing to the signal (as discussed above). In addition to the $P_{1/2}$ for the carbon-centered radical (0.4 mW), the semiquinone signal's $P_{1/2}$ appeared to also remain constant (bar the dry point) at ~1.85 mW. The contribution to the intensity of the EPR signal from each of the free radicals is consistent with our analysis above; essentially, the number of carbon-centered free radicals decreases with water content while the semiquinone free radical comprises a significant fraction of the signal and may be increasing with water content. This is also consistent with previously observed hydration-dependent, solid-state muSR data,[14] in which the number of semiquinone free radicals was observed to increase as a function of water content.

We now ask the question as to why the EPR spectra of the basic sample reveal an increase in the intensity of the minor, semiquinone component of the signal as a function of increasing pH. A simple explanation is that with an addition of base, the comproportionation reaction (Scheme 2) is shifted toward the right-hand side (products), producing more semiquinone free radicals. Thus, even though the total intensity of the signal is decreasing due to a destruction of the carbon centered radical, it is being slightly offset by an increase in semiquinone free-radical spin density.

If this hypothesis holds, then it not only resolves the ambiguities in the literature as to the identity of the solid-state radical species, but also provides a consistent picture across both solution and solid-state samples. The addition of water will have a similar effect as the addition of a base in the comproportionation reaction (which has been observed in the previously mentioned solid-state muSR study[14]), so we can expect that as melanin is taken from the solid state to dilute solution, at high pH the carbon-centered radical will be minimized and the semiquinone free radical will be maximized but with an overall decrease in the total signal intensity. This explains why the semiquinone signal is predominantly seen in solution (even though there are carbon-centered radicals present), while solid-state samples, typically neutral, yield the carbon-centered radical with a small proportion of the semiquinone radical.

A significant question remains: why does the main carbon-centered radical signal change with water content? To answer this vexing question, we propose a phenomenological model to enhance future discussions. To understand what we are proposing, we need to briefly review the underlying microscopic structure of melanin. It has been shown that melanin is composed of small units of stacked, chemically disordered[40,41] oligomers where the stacking is determined by face-on $\pi-\pi$ interactions.[26] A convenient location for the carbon-centered free radical to reside would be in the center of the stacked oligomeric units, which would stabilize the radical. With the addition of water, semiquinone units are formed, which donate an extra electron to its aromatic structure. This will have the effect of changing the effective negative charge experienced on each atomic species within the molecule. In turn, the oligomeric sheets will destack because the $\pi-\pi$ stacking arrangement will be disrupted according to Hunter and Sanders[42] (Scheme 3).

Scheme 3. Destacking Model



With the oligomer units destacking, the carbon-centered radicals would be more exposed to the wet environment, resulting in their breakdown to diamagnetic adducts, thereby leading to a reduction in the EPR signal intensity.

Such a destabilizing effect should be enhanced by increasing the pH of the sample; that is, as the pH is increases, more semiquinones are effectively produced per water molecule because of the addition of OH^- ions, which will also significantly shift the comproportionation equilibrium (Scheme 2) toward the products. This should lead to an increased rate in

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the reduction of the EPR signal intensity with water content, which is what is seen for the high pH sample at high water contents. (Compare the acidic and neutral spectra with the basic spectra in Figure 2d.)

Furthermore, this destacking model would explain the semiquinone dominance in the solution state because most (but not all) of the carbon-centered radicals should have been eliminated by interaction with a ubiquitous water environment via the increased production of semiquinone free radicals. Therefore, there should be a transition point as eumelanin is fully hydrated where the carbon-centered EPR spin signal decreases and where the semiquinone spin signal starts to dominate. Finally, with the destacking model one would expect the eumelanin pellet sample to swell. This has been observed in the adsorption isotherm experiments,[34] where data could not be obtained above 0.8 relative vapor pressure because significant warping of the sample occurred.

Herein, we have proposed a minimalist model in which two free radical species exist within eumelanin coexisting side by side: A potential carbon-centered radical (g = 2.0032, Table 1 and Figure 3)[6,27,31] and a semiquinone free radical (g = 2.0045, Figure 3 and literature[5,18−24,28,29]) whose intensity increases as the pH is increased.[19,22] The electronic structure (g and hyperfine anisotropy) of these free radicals in the solid state remains ill-defined and will require more sophisticated hydration-controlled pH-dependent EPR studies, such as multifrequency (L-, S-, Q-, W-, D-band or higher) CW EPR or pulsed EPR and pulsed ENDOR experiments at one or more of these frequencies.[43] Sample size and dielectric loss at the higher frequencies will become more critical parameters. In addition, solution studies at Q-band frequencies[30] have indicated the presence of four species, which will further complicate the analysis. Clearly a global analysis of all of these experiments is required, and such a study is being undertaken to test the hypothesis presented herein.

CONCLUSIONS

In conclusion, we have presented the first hydration-controlled PS X-band EPR study on solid-state eumelanin in Figures 1 and 2. X-band microwave frequencies are ideal for performing hydration-controlled experiments, as dielectric loss from water absorption is minimal when suitable precautions are undertaken and may also provide g-value resolution. Analysis of the spectra (Figure 3) with a minimalistic model involving two radical species and a review of the literature[5,6,14−16,18−20,27] indicates that the solid-state EPR signal in eumelanin is dominated by a carbon-centered free radical, with a significant contribution from another free radical, which is believed to be a semiquinone radical from the comproportionation reaction (Scheme 2). These results are consistent with those found in solution, where at low pH carbon-centered radicals predominate and upon increasing the pH semiquinone radicals are produced from the comproportionation reaction (Scheme 2).

Importantly, the spin density of these free radicals can be changed with the addition of either water or base. The explanation for the increase in spin density for the semiquinone is straightforward; water/OH^- perturbs the comproportionation reaction (Scheme 2), increasing the concentration of semiquinone free radicals. As for the decrease in the spin density of the dominant carbon-centered spin, the picture is not as clear, and thus we have proposed a new model: the destacking model. Essentially the intermolecular structure of melanin is disordered due to $\pi-\pi$ destacking, brought about by the addition of water or increased pH, which increases the proportion of semiquinone radicals via the comproportionation reaction (Scheme 2). This then leads to exposure of the carbon centered spin and its destruction due to the wet environment.

The results presented in this article have demonstrated that a systematic, hydration-controlled EPR study of melanin can be accomplished with an X-band CW-EPR setup. This should open the way to a systematic hydration-controlled multifrequency EPR study, which is the next step in unraveling the nature of the carbon-centered free radical within eumelanin and potentially understanding melanin's physical response to the environment.

ASSOCIATED CONTENT

Supporting Information

Additional EPR analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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