Free-Radical Scavengers

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Atypical Structural and π-Electron Features of a Melanin Polymer That Lead to Superior Free-Radical-Scavenging Properties**

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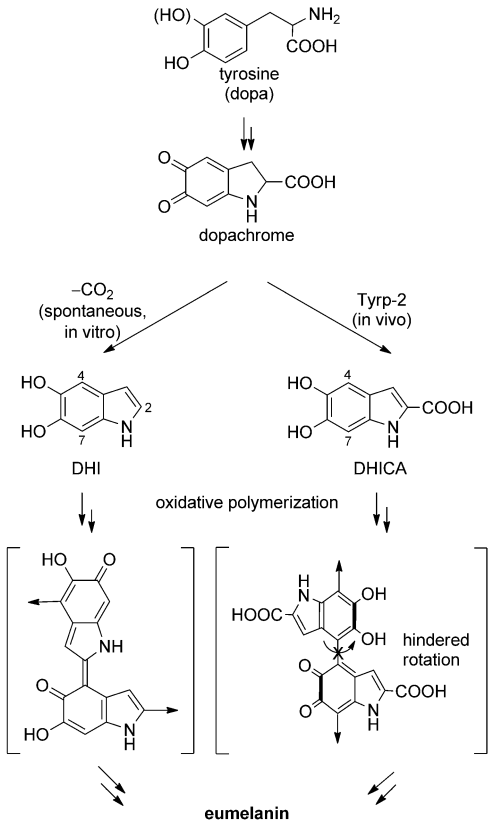
Black and insoluble eumelanin biopolymers are the key determinants of skin and hair pigmentation in dark human phenotypes, and are believed to play a major antioxidant and photoprotective role.[1] This latter property is attributed to the peculiar physicochemical properties of eumelanins, including a broad-band UV/Vis absorption, a distinct electron paramagnetic resonance (EPR) signal,[2] and hydration-dependent electronic–ionic-hybrid conductor behavior.[3] The peculiar bio-optoelectronic, dielectric, metal-binding and free-radical properties of eumelanins have recently spurred intensive research that is aimed at their exploitation as soft, bioinspired, and biocompatible multifunctional (nano)materials for a diverse range of technological applications, for example, in organoelectronics to prepare nanoparticles with free-radical-scavenging properties, and as antioxidants for the thermooxidative stabilization of polymers.[4] In human melanocytes, eumelanin synthesis involves enzymatic oxidation of tyrosine or dopa to give dopachrome, which undergoes tyrosinase-related protein (Tyrp2)-assisted isomerization to 5,6-dihydroxyindole-2-carboxylic acid (DHICA), a major circulating melanogen.[5] In the absence of enzymatic assistance, a spontaneous decarboxylation occurs instead to give mainly 5,6-dihydroxyindole (DHI; Scheme 1). Thus, whereas natural eumelanins consist of DHICA-derived units of more than 50%, synthetic dopa melanin contains mainly DHI, with only 10% of DHICA.[6]

To date, most of the application-oriented studies of melanins have been performed on commercial materials or on dopa melanins with a low DHICA content. Surprisingly, possible differences between the synthetic polymers from various precursors have been disregarded. Recently, it has been reported that DHICA melanin exhibits potent hydroxyl radical-scavenging properties in the Fenton reaction, whereas DHI melanin does not.[7] Moreover, photophysical studies of DHICA oligomers suggested efficient deactivation of the excited state that is mediated by inter-unit interactions within the oligomeric molecular scaffold.[8]



Scheme 1. Eumelanin synthesis. Numbers and arrows on the structures indicate the main bonding patterns in DHI and DHICA melanins.

Notwithstanding these observations, the actual potential of DHICA melanin as an efficient biocompatible antioxidant, and the underlying structure–property relationships have remained unexplored. Our current knowledge of DHI and DHICA homopolymers is limited and can be summarized as follows:

1) Oxidative polymerization of DHICA mainly leads to atropisomeric 4,4′- and 4,7′-linked structures with hindered inter-unit rotation, whereas DHI forms 2,4′- and 2,7′-linked oligomers.[9]
2) Upon oxidation, DHI dimers generate predominantly planar species that strongly absorb in the visible region,[10] whereas DHICA oligomers do not show significant absorption above 400 nm; this is due to inter-unit dihedral

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angles of circa 47° with localized *o*-quinone moieties, and significant interruption of inter-unit π-electron delocalization.[11]

3) DHI polymerization leads to two-dimensional structures with an estimated thickness of 55 ± 2 nm.[12] Similar sheets of protomolecules that stacked to form onion-like nanostructures with an interlayer distance of 3.3 Å have been characterized by scanning electron microscopy (SEM), transmission electron microscopy (TEM), and theoretical studies of synthetic dopa melanins.[13]

Several studies indicate that the properties of eumelanin are intrinsically defined by the degree of electronic delocalization across the planar oligoindole components, rather than by their supramolecular organization.[13b] This view has been widely used as the basic principle that underlies structure–property relationships of synthetic eumelanins. Recently, however, a comparative investigation of the UV/Vis spectra of solubilized eumelanins in a buffer that contained polyvinylalcohol (PVA; 1%)[14] showed that in solution, the visible absorption spectrum of DHICA melanin, but not of the DHI polymer, varies significantly and in a non-linear fashion with dilution, which suggests aggregation-dependent contributions. This unexpected finding prompted us to undertake the first comparative investigation of DHICA, DHI, and dopa melanins. The main goals of the study were: 1) to assess the relative free-radical-scavenging capacities of the melanins with different standard assays, and 2) to characterize the optical, paramagnetic, and morphological properties of the synthetic polymers to identify the key structural and electronic factors that influence the response to aggregation and antioxidant capacities.

The free-radical-scavenging properties of the synthetic melanins were determined by scavenging assays with 1,1-diphenyl-2-picrylhydrazyl (DPPH),[15] 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS),[16] and nitric oxide (NO; Figure 1).[17] DHICA melanin proved to be a far more efficient scavenger than DHI or dopa melanin in all assays.

To determine the origin of the superior free-radical-scavenging properties of DHICA melanin, further experiments were conducted to investigate its π-electron properties and its aggregation mechanisms, in comparison with the DHI polymer.

The absorption spectra of DHI, DHICA, and dopa melanins, which were finely suspended at pH 7.5, are shown in Figure 2 (see also the Supporting Information, Figure S1). Whereas DHI melanin gave a typical monotonic profile, the

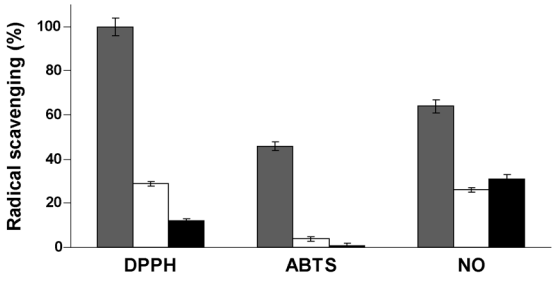

Figure 1. Free-radical-scavenging properties of DHICA (■), DHI (□), and dopa (■) melanin (mean values ± SD for 3 experiments).

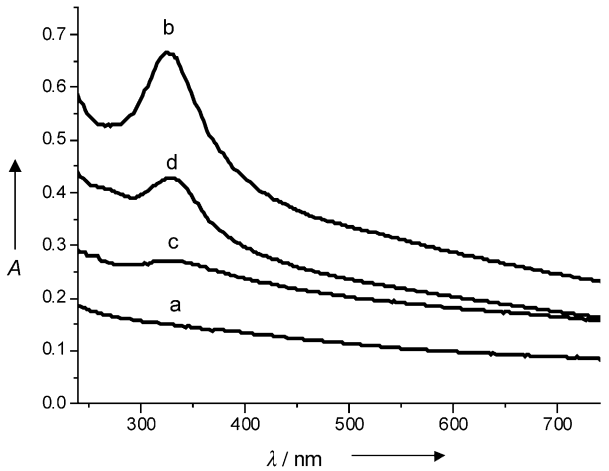

Figure 2. UV/Vis spectra of synthetic eumelanins, as suspensions in Tris buffer (2.5 mg/100 mL, pH 7.5). a) DHI melanin; b) DHICA melanin; c) dopa melanin; d) DHICA melanin (acetate buffer, pH 3).

DHICA polymer displayed an intense absorption band in the UV region at around 320 nm that persisted at acidic pH values. This characteristic feature of DHICA melanin can be attributed to reduced monomer-like chromophoric components that co-exist with quinonoid units, and persist during the polymerization process as a consequence of hindered inter-unit π-electron delocalization within oligomer/polymer scaffolds. This observation has previously been described for measurements of the monomer to the full polymer.[18]

Consistently, reductive treatment of DHICA melanin with $NaBH_4$ did not affect the band at 320–350 nm, but caused a ca. 30% decrease in the visible component, which suggests a contribution to the latter from reducible quinonoid chromophores (not shown).[19] The small but distinct band at around 320 nm in the spectrum of dopa melanin can likewise be attributed to the minor DHICA-derived component.

The EPR spectra of dry samples of DHI and DHICA polymers, along with power saturation curves, are shown in Figure 3. Despite very similar *g* values, quantitative determination of the signal amplitudes (ΔB) indicated a narrower signal for DHICA melanin. A remarkable difference was also apparent from the power saturation curves. Both data sets together suggest greater homogeneity of the free-radical components in DHICA melanin.[2,3,20] Recently, two main free-radical components of synthetic eumelanins (a major component assigned to carbon-centered radicals localized at the center of the stacked units, and a minor component likely attributed to semiquinone-type species) have been identified.[20] Based on these results, the EPR signals of samples of dehydrated melanin reported herein are likely to be mainly due to carbon-centered radical species rather than to semiquinone-type components.

Furthermore, the spectra of eumelanin solutions that were prepared by oxidative polymerization of DHICA and DHI in a phosphate buffer–1% PVA[14] were compared with those of solid samples that were prepared under the same conditions, but in the absence of PVA. As previously shown, PVA inhibits the precipitation of melanin during the polymerization, but has no effect on the preformed polymer. Data obtained from

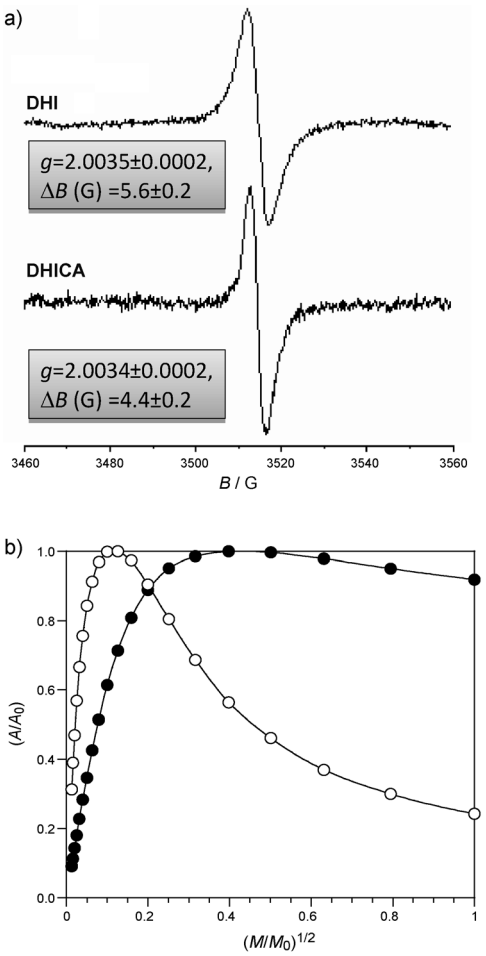

Figure 3. a) EPR spectra of DHI and DHICA melanins. Spectra were recorded on thoroughly desiccated samples. $T = 25\,°C$, microbridge power = 0.6 mW. b) Power saturation profiles for DHI (●) and DHICA (○) melanins. Quite similar values were obtained for lyophilized samples recorded under the same conditions (see the Supporting Information, Table S1).

dynamic light scattering indicated that it acts by physically hindering the growth of aggregates beyond the threshold limit that corresponds to the onset of precipitation.[12] A significantly lower ΔB value is obtained in PVA solution for DHICA melanin, but not for DHI melanin (Table 1). This experiment revealed that the free-radical population of DHICA melanin is significantly affected by aggregation, which increases dishomogeneity. Notably, the g factor was not affected by aggregation for both melanins.

Analysis of the EPR spectra of dopa melanin and of whole intact black human hair (DHICA content ca. 50 %)[6] gave ΔB values of 5.1 ± 0.2 and 4.9 ± 0.2 G, respectively, which is midway between those for the DHI and DHICA homopolymers (Figure 3).

Table 1: Differences in the EPR spectral parameters for DHI and DHICA melanins prepared in the presence and in the absence of 1 % PVA.

	Δg	$\Delta(\Delta B)$ [G]
DHI melanin	-0.0001 ± 0.0002	0.1 ± 0.2
DHICA melanin	-0.0001 ± 0.0002	-0.8 ± 0.2

The mode of aggregation of the oligomer species that are formed during the initial stages of the oxidative polymerization of DHICA and DHI was investigated by TEM. The images of DHICA polymers taken after a reaction time of two hours (Figure 4) revealed relatively large elongated structures that are more than 100 nm long and significantly different from the onion-like aggregates with an approximate diameter of 50 nm that were generated by the DHI polymer and other typical eumelanins.[13b,c]

Major differences in the mode of aggregation of dopa, DHI, and DHICA melanins were also apparent by SEM analysis[13a,21,22] (see also Figures S2, S3).

The peculiar properties of DHICA melanin are controlled by the carboxylate group, which diverts reactivity from the pyrrole 2 position towards the 4 and 7 benzenoid positions, and forces the inter-ring dihedral angles to twist to minimize electrostatic interactions. The resulting oligoindole chains are not amenable to π-stacking, and may give rise to atypical weak intermolecular interactions. The UV/Vis absorption profile of DHICA melanin would thus be due to both electronically isolated monomer-like units in the UV region and chromophores in the visible region, which are partially generated by intermolecular π-electron perturbations.[19] The comparatively low ΔB value and the power saturation profile of DHICA melanin would suggest a relatively homogeneous free-radical species that is spatially confined within restricted segments of the polymer, in contrast to the broader variety of free-radical species that could be generated within the delocalized π-electron systems of the DHI polymer. Correspondingly, a less effective chemical disorder for DHICA eumelanin with respect to equivalent DHI macromolecular ensembles has previously been predicted by computational and spectrophotometric studies.[18] An overall view of these concepts is given in Figure 5.

The somewhat counterintuitive conclusion is that the efficient antioxidant, redox, and (photo)protective properties of DHICA melanin are primarily determined by the destabilizing effects of hindered inter-unit conjugation, which lead to non-planar structures with monomer-like behavior and weak aggregating interactions. Moreover, the formation of weak aggregates may account for a greater accessibility of free radicals to DHICA compared to the compact π-stacked DHI and dopa melanins. Extrinsically defined properties would thus reflect an adaptive mechanism that compensates for low electron delocalization and intramolecular stabilization. An analysis of these results in light of the recently proposed hydration-dependent destacking model of structural disorder in dopa eumelanin may thus be worthwile.[20]

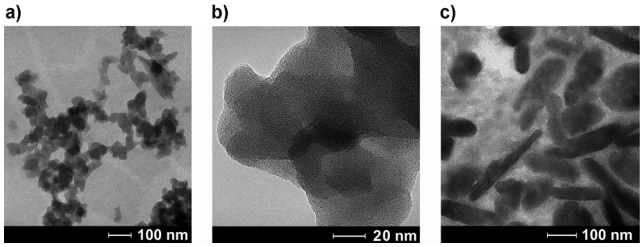

Figure 4. TEM images of DHI (a, b) and DHICA (c) polymers taken at an oxidation time of 2 h.

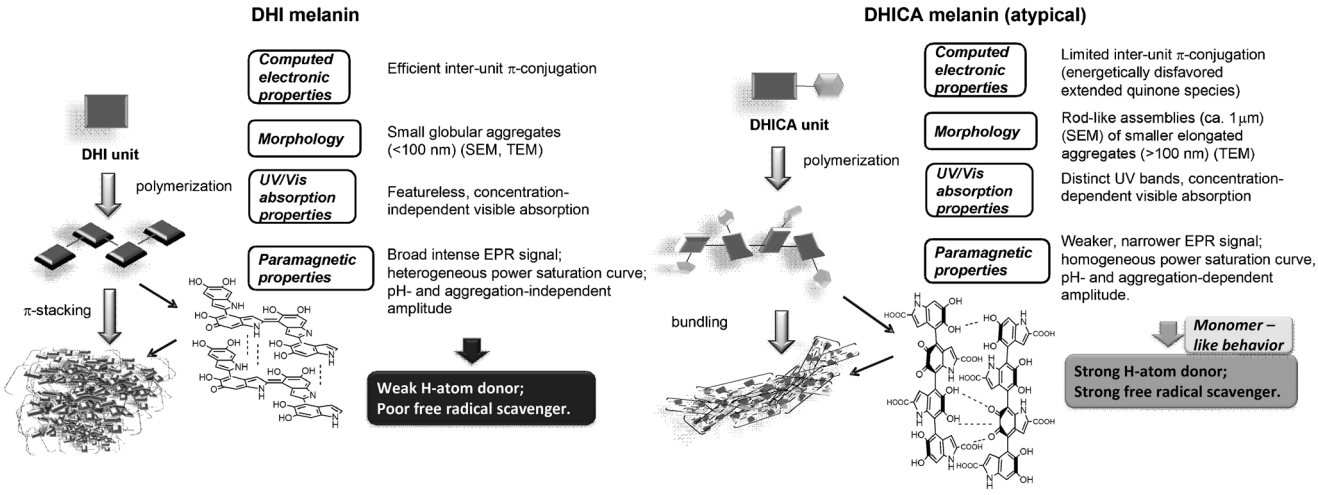

Figure 5. Structure–property relationships proposed for DHI and DHICA melanins.

In conclusion, the results reported in this paper 1) suggest the use of DHICA melanin as a superior free-radical scavenger compared to the melanins that have thus far been used for technological applications, 2) expand on current paradigms for eumelanin biopolymers to include aggregation-dependent properties, and 3) offer a plausible chemical explanation of why nature selected DHICA to confer (photo)protective properties to skin melanins, which is a crucial issue of human pigmentation.[5,23] Although the proposed model remains to be fully confirmed, it may stimulate further work aimed at assessing melanin properties from a biological and technological perspective.

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