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Authors: Paola Manini, Valeria Lino, Paola Franchi, Gennaro Gentile, Teresa Sibillano, Cinzia Giannini, Emanuela Picardi, Alessandra Napolitano, Luca Valgimigli, Cinzia Chiappe, and Marco d'Ischia

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A Robust Fungal Allomelanin Mimic: An Antioxidant and Potent π-Electron Donor with Free-Radical Properties that can be Tuned by Ionic Liquids

Paola Manini,[a]* Valeria Lino,[a] Paola Franchi,[b] Gennaro Gentile,[c] Teresa Sibillano,[d] Cinzia Giannini,[d] Emanuela Picardi,[a] Alessandra Napolitano,[a] Luca Valgimigli,[b] Cinzia Chiappe,[e] Marco d'Ischia[a]

Abstract: Developing effective strategies to increase the chemical stability and to fine tune the physico-chemical properties of melanin biopolymers by rational control of π-electron conjugation is an important goal in materials science for biomedical and technological applications. Herein we report that poly-1,8-dihydroxynaphthalene (pDHN), a non-nitrogenous, catechol-free fungal melanin mimic, displays a high degree of structural integrity on MALDI-MS and CP/MAS ^{13}C NMR analysis, a strong radical scavenging capacity (DPPH and FRAP assays) and an unusually intense EPR signal (g = 2.0030). Morphological and spectral characterization of pDHN, along with de-assembly experiments in ionic liquids, indicated amorphous aggregates of small globular structures with an estimated stacking distance of 3.9 Å and broadband absorption throughout the visible range. These results indicate that DHN-based melanins exhibit a high structural integrity and enhanced antioxidant and free radical properties of potentially greater biomedical and technological relevance compared to typical indole-based eumelanins.

Introduction

The production of black, insoluble and highly π-conjugated melanin pigments is part of the adaptive response of man, mammals, birds, fish, cephalopods, plants and fungi, to various cues, such as UV irradiation, oxidative stress and highly damaging radiations. Melanins are usually defined on the basis of their biogenetic origin.[1,2] Animal pigments consist mainly of black nitrogenous eumelanins, which arise from the oxidative polymerization of tyrosine via 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA).[1] In plants and microorganisms, different kinds of melanin-type pigments are produced, originally termed allomelanins, which arise from non-nitrogenous phenolic substrates.[3,4] All melanins exhibit almost complete insolubility in all solvents, a high degree of structural and supramolecular disorder and an antioxidant capacity associated with efficient H-atom donor behavior.[5] All these properties are associated with a characteristic set of physicochemical properties, including broad band visible light absorption, accounting for their dark colors, an intrinsic free radical character associated with a permanent electron paramagnetic resonance (EPR) signal and water-dependent hybrid ionic-electronic conductor behavior.[6,7]

The peculiar properties of natural eumelanins have prompted intense research efforts aimed at exploiting synthetic mimics[8] as tailorable biomaterials for nanosized functional systems, polymer stabilization, surface functionalization and coating, signal transducing biointerfaces for organic (bio)electronics,[9,10] drug delivery and other biomedical and technological applications.

Despite considerable progress in the understanding of melanin properties and the definition of basic structure-property-function relationships for polymer design,[5,11] there are still a number of gaps that hinders the definitive maturation of a eumelanin-based technology.

Rational selection of monomer building blocks has recently emerged a sa valuable strategy for controlling and tailoring π-electron properties of melanins for antioxidant, optoelectronic and biomedical applications.[12] In support of this view, in a recent study we showed that replacing nitrogen by sulfur markedly affects the mode of polymerization of DHI leading to novel melanin-type materials with a high degree of regioregularity.[13]

Fungal pathways of melanin synthesis have so far been little considered as a source of bio-inspiration in the field of functional materials.[14] In *Ascomyces* a black melanin pigment is produced by the oxidative polymerization of 1,8-dihydroxynaphthalene (DHN).[15] An interesting property of *Ascomyces* melanin is its reported ability to shield organisms from ionizing radiation.[16] In addition, fungal melanin synthesis has been related to energy transduction mechanisms and biological properties such as virulence.[17]

[a] Prof. P. Manini, Dr E. Picardi, Prof. A. Napolitano, Prof. M. d'Ischia
Department of Chemical Sciences
University of Napoli Federico II
Via Cintia 4, I-80126 Napoli, Italy
E-mail: paola.manini@unina.it
[b] Prof. P. Franchi, Prof. L. Valgimigli
Department of Chemistry "G. Ciamician"
University of Bologna
Via S. Giacomo 11, I-40126 Bologna, Italy
[c] Dr G. Gentile
Institute for Polymers Composites and Biomaterials
National Research Council of Italy
Via Campi Flegrei 34, I-80078 Pozzuoli, Italy
[d] Dr T. Sibillano, Dr C. Giannini
Istituto di Cristallografia (IC) CNR
via Amendola 122/O, I-70126 Bari, Italy
[e] Prof. C. Chiappe
Department of Pharmacy
University of Pisa
via Bonanno Pisano 6, I-56126 Pisa, Italy

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Intrigued by these and other properties of *Ascomyces* melanins, we have recently investigated the black melanin polymer produced by the oxidative polymerization of DHN[18] in comparison with DHI- and DHICA-melanins as reference nitrogen-containing biomimetic polymers. MS analysis indicated the presence of regular patterns of oligomers with intact structural units (see SI section). This feature revealed a marked difference from DHI melanins in which extensive breakdown of quinone units during polymerization is usually observed.[19]
Chemical studies indicated that DHN tends to polymerize via 2,2'-, 2,4'- and 4,4'-bondings to give regular patterns of oligomers that may exhibit extended quinone-methide moieties.[20]

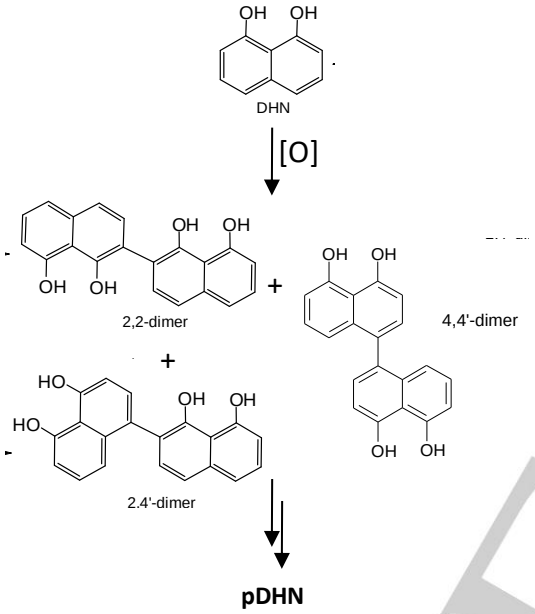

Figure 1. First stages of the oxidative polymerization of DHN.

The symmetry and the meta-like *peri* dioxygenation pattern of DHN, precluding labile *o*-quinone formation in the first step of the process, could drive the generation of relatively more regular melanin-type polymers and their efficient assembly into aggregates. This reactivity can account for the different properties exhibited by the DHN melanin compared to DHI-based eumelanins.
To verify this prediction, we undertook a detailed spectral and morphological characterization of pDHN with a view to elucidating the impact of extended quinone-methide formation within catechol- and *o*-quinone-free scaffolds on the degree of π-conjugation and stacking in the polymer aggregates. Electron paramagnetic resonance (EPR) data were coupled with de-stacking experiments in ionic liquids (IL) for probing aggregation dependent effects on the EPR signal. Finally the radical scavenging properties of pDHN were preliminarily assessed in comparison with those of reference eumelanins.

Results and Discussion

Structural and morphological characterization of pDHN. pDHN was synthesized using the enzymatic system horseradish peroxidase/H_2O_2 in phosphate buffer, pH 7.4, as previously reported.[18] Under these conditions, the reaction led to the transient formation of a greenish blue species which rapidly evolved toward the formation of a black insoluble precipitate. This latter was isolated by centrifugation and washed for structural and spectroscopic characterization.
Preliminary solubility tests were performed by suspending pDHN in different solvents (isopropyl alcohol, *N*-methylpyrrolidone, *N*,*N*-dimethylformamide (DMF) and dimethylsulfoxide at a nominal concentration of 1 mg/mL, then exposing suspensions to ultrasounds for 30 minutes and measuring the final absorbance value following filtration. The most homogeneous suspension was obtained in DMF (Figure 2, inset). The UV-visible spectrum of the DMF suspension showed broadband absorption throughout the whole spectrum, typical of eumelanin polymers, with an intense absorption in the visible region, denoting a high degree of polymerization. Moreover, the presence of shoulders at 390, 460 and 580 nm suggested the presence of extended quinonoid moieties, as reported in a previous work,[20] indicating a high π-electron conjugation.

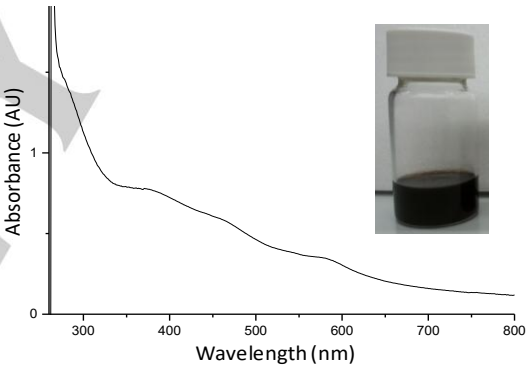

Figure 2. UV-vis spectrum of a pDHN suspension in DMF.

A typical melanin-type spectrum was observed by ATR analysis (see SI section). MALDI-MS analysis confirmed the previously reported regular polymeric nature of pDHN.[18]
^{13}C cross-polarization/magic-angle-spinning (CP/MAS) NMR analysis (see SI section) revealed the presence of a wide overlap of bands in the range of δ 100-150, attributable to the aromatic carbons, with a small peak around 160 ppm, attributed to OH-bearing carbons, and a broader band at 190 ppm consistent with carbonyl-type carbons. These resonances overall were compatible with extended quinone-methide moieties framed within more or less large oligomeric scaffolds. No detectable aliphatic resonances, denoting oxidative breakdown of quinonoid moieties, were observed, unlike with most DHI melanins.[21]
Transmission electron microscopy (TEM) analysis(Figure 3) revealed that pDHN is constituted by porous, quasi-spherical primary particles, similar to those observed in the case of melanins from DHI,[5] connected in loose aggregates with approximate size in the range 200 nm - 2 mm. The statistical

analysis of the lateral dimensions of the primary particles revealed a narrow unimodal size distribution, well fitted with a lognormal function(Figure 4c, adj R-square = 0.9726, shape parameter σ = 0.33 ± 0.02), with median value ρ = 21.1 ± 0.5 nm.

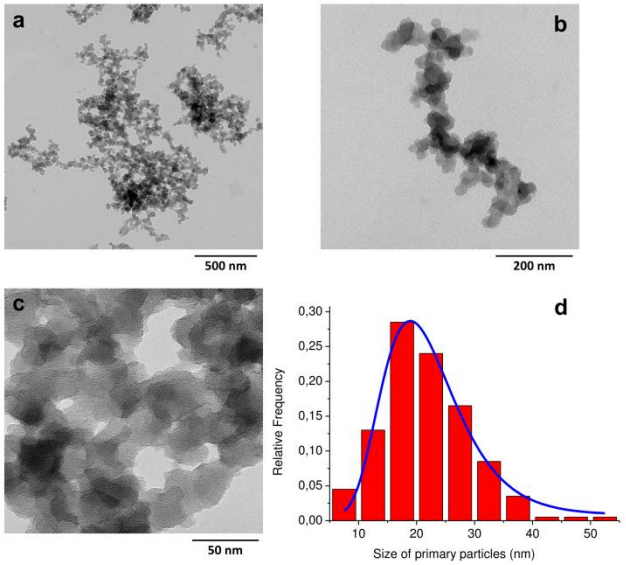

Figure 3. TEM images and size distribution of the nanostructures from pDHN.

The X-ray powder diffraction (XPD) spectrum of pDHN, shown in Figure 4, presents the typical pattern of an amorphous material with a major peak at q = 1.6 $Å^{-1}$, corresponding to a stacking distance d = 2π/q = 3.9 Å. This spectrum is compared in the same figure with the XRD pattern collected on DHI-melanin, with the major diffraction peak at q = 1.8 $Å^{-1}$ (i.e. d = 3.5 Å). Fungal melanins have recently been reported to produce diffraction spectra with a peak at approximately q = 1.4 $Å^{-1}$ (corresponding to a stacking distance d = 2π/q = 4.5 Å),[22] which is at an angular position smaller than that measured for polymerized L-DOPA and other eumelanins reported previously.[23,8]

On this basis, stacking distances in the 4.1–4.5 Å range were deduced for fungal melanins, which are similar to the distance determined for our pDHN, and quite larger from those of DHI-melanin (3.5 Å).[8]

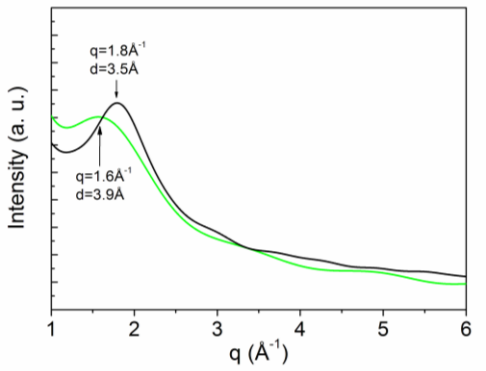

Figure 4. X-ray powder diffraction spectra of pDHN (green trace) and DHI-melanin (black trace).

EPR investigations and effect of pDHN de-assembly in ionic liquids. Electron paramagnetic spectra (EPR) recorded on solid pDHN revealed an unusually intense melanin-type signal.

The g-factor value determined for pDHN (g = 2.0030) was smaller than that measured for nitrogenous melanins from DHI and DHICA (2.0034),[5] but in line with that measured for polyphenolic polymers;[24] this suggested a more pronounced C-centered character of unpaired spins in the fungal melanin mimic (Table 1, Figure 5).

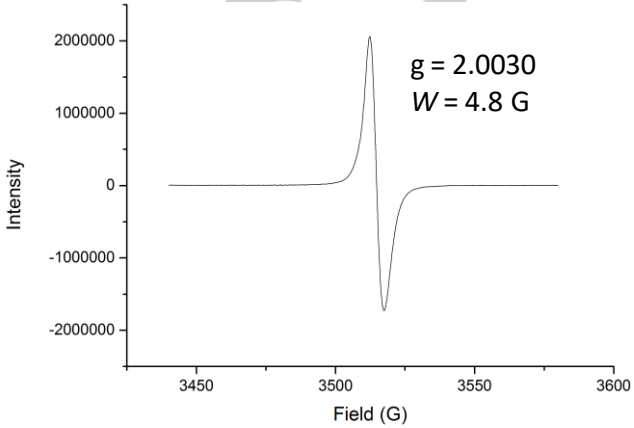

Figure 5. EPR spectrum of pDHN in the solid state.

Table 1. EPR spectral parameters (*W* = line width in Gauss; g-factor) recorded for pDHN and other synthetic melanins and polyphenols in the solid state.

Sample	*W* (G)	*g*-factor
pDHN	4.8	2.0030
DHI melanin[5]	5.6	2.0035
DHICA melanin[5]	4.4	2.0034
Caffeic acid-polymer[24]	3.7	2.0033
Gallic acid-polymer[24]	3.3	2.0030

A reasonable explanation may be based on the lack of *o*-semiquinone free radicals, which typically account for g-factor values in eumelanins higher than 2.0040.[10] This observation would be compatible with extended quinone-methide moieties ensuring larger spin delocalization across planar segments of polymeric carbon platforms. The line width (*W*) of the signal, 4.8 G, proved to be intermediate between the DHI melanin value (5.6 G) and the DHICA melanin value (4.4 G). This data confirmed that the pDHN structure allowed a tridimensional spin delocalization not only along the polymer chain in the conjugated quinonoid planar regions, but also through π-stacking interactions between the aromatic units, as suggested also for DHI melanin.

Recently, we discovered that ionic liquids (ILs) can cause extensive de-assembly of polydopamine aggregates up to the nanosize level, allowing unprecedented insights into the role of aggregation in melanin paramagnetic properties.[25] Accordingly, in further experiments the effect of ILs on pDHN EPR properties was investigated to inquire into the origin of the intense signal and for probing possible structure-property relationships. To this aim, two sets of ILs (based on cation structure) were synthesized (Figure 6). IL1-10 were characterized by an imidazolium moiety and differed only in the anion counterpart,

while IL11-12 featured a tetraalkylammonium moiety. IL1, IL4, IL10 and IL11 were all characterized by a dimethylphosphate anion component and different cation counterparts. Special interest was focused on the effect of *N*-methyl-*N*,*N*,*N*-trioctylammoniumoleate (IL12), which proved highly effective in de-assembling polydopamine by means of the long alkyl chains on both the cationic and anionic portions.

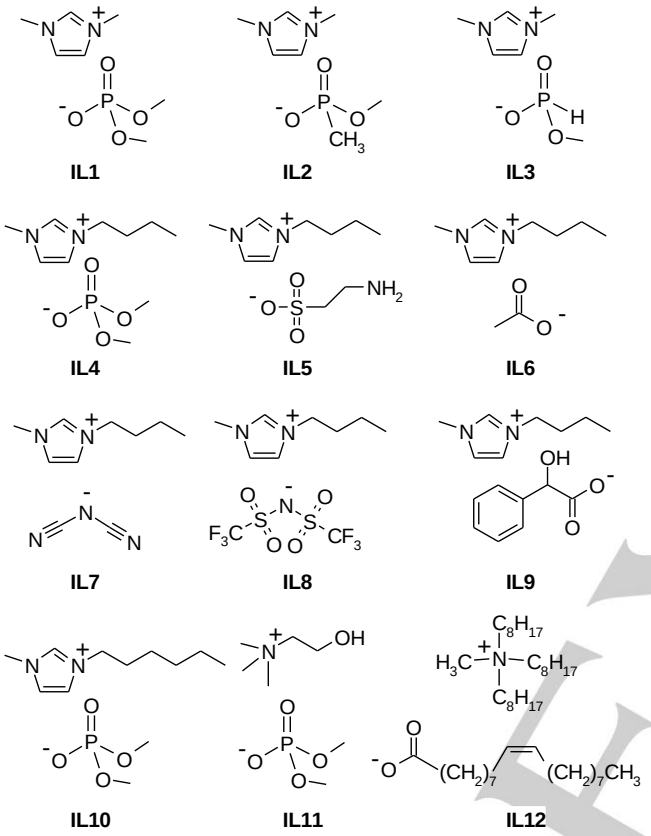

Figure 6. Formulas of the ILs synthesized in this work

pDHN was added to ILs at 1 mg/mL concentration and subjected to ultrasound treatment for 30 min in an ice bath. The suspensions thus obtained were centrifuged and the supernatants subjected to EPR spectroscopy. As reported in Tables 2, pDHN-containing IL samples gave signals with variable g-factor values and line widths. This behavior would suggest aggregation-dependent inhibition ("freezing" effect) to spin delocalization in the solid state. Upon de-aggregation in ILs, an increased proportion of the spin population becomes exposed to the IL, with consequent removal of aggregation dependent constraints to delocalization. Data reported in Table 2 indicated that g-factors varied in most of the tested ILs compared to the solid state, with values mainly ranging 2.0037-2.0047, typical of phenoxyl *O*-centered radicals.

Worthy of note is the case of IL-5 and IL-12 inducing the most significant shift of the g-factors. This suggested the determinant role played by hydrophobic (IL-12) and H-bond accepting (IL-5) properties of ILs in tuning the spin delocalization via disassembly of the polymer.

The same effect can be observed also by examining the band width (*W*) of the signals of the pDHN in ILs and in the solid state. In particular, all ILs induced a marked signal broadening (high *W*) suggesting a higher degree of spin delocalization in the melanin backbone due to the disassembly of the polymer. This effect can be rationalized considering the Kamlet-Taft solvent parameters reported for some of the ILs tested (Table 3).[26-28] In detail, the signal broadening proved to be more pronounced for ILs with low polarity/polarizability (π*) and high hydrogen-bond acceptor basicity (β).

Table 2. EPR spectral parameters (*I* = signal intensity in arbitrary units; *W* = line width in Gauss; g-factor; *N*×spins/*L*) recorded for pDHN suspended in different ionic liquids.

Ionic liquid	*I* (A.U.)	*W* (G)	*g*-factor	*N*×spins/*L*[a]
IL1	1.05 E+05	5.1	2.0038	1.09 E-05
IL2	1.40 E+05	5.3	2.0038	1.52 E-05
IL3	1.14 E+05	5.3	2.0037	1.24 E-05
IL4	4.60 E+04	7.4	2.0032	1.05 E-05
IL5	1.10 E+04	5.4	2.0047	1.34 E-06
IL6	1.03 E+04	6.2	2.0039	1.60 E-06
IL7	3.80 E+04	5.4	2.0030	4.63 E-06
IL8	5.05 E+04	5.7	2.0035	6.78 E-06
IL9	2.20 E+05	7.5	2.0040	5.17 E-05
IL10	1.80 E+04	5.2	2.0031	2.03 E-06
IL11	1.20 E+04	6.4	2.0036	2.05 E-06
IL12	4.88 E+04	6.3	2.0043	8.09 E-06
None	3.01 E+06	4.8	2.0030	-

[a] *N*×spins/*L*= concentration of radicals (unpaired electrons) in the IL suspension (corresponding to molar concentration in solution).

Table 3. Kamlet-Taft solvent parameters(π*= polarity/polarizability, β = hydrogen-bond acceptor basicity) measured for ILs.

Ionic Liquid	π*	β	Reference
IL4	0.98	1.13	[26]
IL6	1.04	1.09	[26]
IL2	1.05	1.10	[27]
IL8	1.09	0.24	[26,28]
IL7	1.13	0.62	[26,28]

From these data it was concluded that ILs can give rise to variable, structure-specific interactions with free radical centers in pDHN aggregates, at variance with the case of polydopamine,[25] for which constant shifts in g-factor were observed with destacking with all ILs. It appears, moreover, that the hydrophobic and basic character of the IL plays an important role in promoting the disassembly of the polymer with consequent tuning of spin delocalization.

Antioxidant properties. DHI and related diffusible eumelanin precursors[29]as well as their polymers[5]display marked antioxidant properties of potential relevance for applications as functional materials. Recent studies indicated a correlation between the paramagnetic properties of synthetic eumelanins and (poly)phenolic polymers and their antioxidant activity.[5,24] Accordingly, in a final group of experiments the potential antioxidant behavior of pDHN were preliminarily explored. To this aim, two commonly used chemical tests were used, namely the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and the ferric

reducing antioxidant power (FRAP) test.[31,32] The first one accounts for the H-donor ability of a given species, whereas the second one measures the iron(III) reducing power. pDHN was assayed as fine suspension obtained by homogenization in a glass/glass potter.

Data obtained from DPPH and FRAP assays on pDHN are reported in Figure 7 and Table 4 and compared with those reported in the literature from other melanin and polyphenolicpolymers.

Table 4. EC_{50} values from DPPH assay and ferric reducing power expressed as Trolox equivalents of pDHN and other melanin and polyphenol samples.

Sample	DPPH-EC_{50} (μg/mL)	FRAP (Trolox eqs)
pDHN	6.5±0.8	1.82±0.3
DHICA melanin[30]	14.4± 0.7	0.90± 0.06
Caffeic acid-polymer[24]	21.5 ± 0.7	0.65 ± 0.05
Gallic acid-polymer[24]	10.5 ± 0.7	0.49 + 0.05

Data are expressed as mean ± SD of three independent experiments.

Both assays showed that pDHN has a strong reducing capacity, higher than that exhibited by DHICA melanin and by the polymers obtained from catechols (e.g. caffeic acid) and triphenols (e.g. gallic acid). Indeed, in DPPH-EC_{50} test lower values indicate higher reducing capacity, while in FRAP test this is indicated by higher values. It should be noted, however, that the two tests are not simply inversely proportional, as they are based on different reaction mechanisms: the first investigates the formal H-atom transfer ability of the potential antioxidant, while FRAP assesses its ability to give electron-transfer.[33] Both tests converge promising higher antioxidant activity for pDHN, which could be based on both mechanisms. This behavior can be in part explained by considering the efficient antioxidant properties exhibited by the monomer precursor, 1,8-DHN. This is due to the stabilizing effect exerted on the aryloxyl radical both by intramolecular hydrogen bonding and by the naphthalene ring system.[34]

Conclusions

pDHN, a synthetic mimic of fungal melanin from *Aspergillus*, has been shown to display typical features of a eumelanin-type polymer but a greater structural stability, stronger antioxidant properties and a marked paramagnetic behavior. When viewed in terms of structure-property relationships, these results demonstrate that most of the characteristic properties of eumelanins, i.e. a broadband absorption spectrum, an intrinsic EPR signal and H-atom/electron donating capacity, are not specifically due to catechol-containing building blocks, nor to nitrogenous centers. Actually, DHN-derived structures can provide stronger stabilization to C-centered radicals rather than to *O*-centered ones, providing useful probe systems for studies of the origin of free radical properties in melanin type polymers. pDHN can be incorporated into various materials polymers and systems, and its presence and fate can be efficiently probed by EPR.

Non-nitrogenous fungal melanin mimics devoid of catechol functions are thus proposed as innovative prototypes of melanin-based functional materials with exceptionally robust π-electron properties and radical scavenging ability[35,36] suitable for applications in biomedicine and materials science. In addition, these results may provide an improved background to inquire into the fate of PAH-derived carbon-containing species in potential astrochemical and prebiotic environments.[37]

Experimental Section

Synthetic procedures for ILs. IL1-IL4 and IL10 were prepared by addition of the alkylating agent (trimethyl phosphate or dimethyl−methylphosphonate or dimethyl phosphite, 1.1 molar equivalents) to a molar equivalent of the proper alkylimidazole (methyl or butylimidazole), according to the previously reported procedure.[25] *N*-Methyl-*N*,*N*,*N*-trioctylammonium oleate (IL12) was prepared by addition of a molar equivalent of oleic acid to a methanolic solution of *N*-methyl-*N*,*N*,*N*-trioctylammonium methylcarbonate (43%), using the same experimental conditions described in the literature.[38] Analogously, ionic liquids IL5-IL9 were prepared by addition of an equimolar amount of the desired acid (2-aminoethanesulfonic acid, acetic acid, dicyanamide, bis(trifluoromethane)sulfonimide, mandelic acid) to the commercial 1-butyl-3-methylimidazolium methylcarbonate methanol solution, at room temperature. The mixture was gently heated to 50 °C. After 2 h, the mixture was concentrated in vacuo at 50 °C to remove the methanol and then was dried under high vacuum and continuous stirring at 50 °C for 8 h. In the case of IL5, due to the low solubility of this reagent in methanol, a water solution was used. Finally, choline dimethylphosphate (IL11) was obtained by reaction of choline chloride with a small excess of trimethyl phosphate (10% mol) at 80 °C for 12h. Any volatile compound was removed by vacuum evaporation. The pure product was subject to $AgNO_3$ analysis to confirm the absence of a starting material.[39] The structure and purity of the recovered liquids (yields 95−97%) were checked by NMR analysis.

Synthesis of poly-1,8-dihydroxynaphthalene (pDHN) and treatment with ILs. The oxidation of 1,8-DHN was carried out according to a previously reported procedure.[18] In brief, a solution of 1,8-DHN (500 mg,3.12 mmol) in acetonitrile (20 mL) was added to a 0.1 M solution of phosphate buffer, pH 7.4 (300 mL) and treated under vigorous stirring with HRP (15 U/mL) and hydrogen peroxide (375 μL, 1.2 molar equivalents). After 24 h the reaction was acidified with a solution of HCl (2 M) until pH 2 was reached. The reaction mixture was centrifuged at 4 °C (7000 rpm for 15 minutes) to collect the dark precipitate that was washed twice with water and then dried under reduced pressure. In separate experiments, pDHN samples have been suspended in the proper ionic liquid at a concentration of 1 mg/mL and subjected to ultrasound treatment for 30 min in an ice bath (amplitude 30%).[25] The resulting suspensions have been carefully centrifuged to remove a small residue which was not treated further, and the clear supernatants, exhibiting dark colorations have been used for EPR measurements.

X-ray powder diffraction analysis. X-ray powder patterns are collected at room temperature by means of a Rigaku RINT2500 rotating anode laboratory diffractometer (50 KV, 200 mA) equipped with the silicon strip RigakuD/teX Ultra detector. An asymmetric Johansson Ge(111) crystal is used to select the monochromatic Cu *K*α1 radiation (λ = 1.54056 Å).

Measurements were executed in transmission mode, by introducing the powders in Lindemann capillaries.

TEM analysis. TEM analysis was performed in bright field mode using a FEI-Tecnai G12 Spirit Twin (LaB6 source) operating at120 kV. TEM images were collected on a FEI Eagle 4 k CCD camera. Before the analysis, pDHNwas suspended in ethyl alcohol and collected by dipping a carbon coated copper grid in the suspension. TEM micrographs were analysed by means of the software ImageJ (release 1.43u) and the size of primary pDHN particles was measured. The size distribution of the particles was statistically analysed using the software Origin Pro (release 8.5).

EPR spectroscopy. X-Band EPR spectra were recorded at 298 K on a Bruker Elexsys E500 spectrometer equipped with an ER 049 Super X MW bridge and VT 1000 variable temperature unit. Spectra consisted of two accumulated scans set at MW power = 2.5 mW, modulation amplitude = 1.0 G, and modulation frequency = 100 kHz. Samples were placed in a200 µL calibrated glass tube sitting inside a 4 mm i.d. quartz tube, accurately positioned inside the cavity.[40] For sample in ILs,35 µL of solution was analyzed, so as to cover the entire sensitive area in the cavity. For solid state samples, 0.2−2.5 mg was analyzed. Measured g-factors were corrected with respect tothat of a standard sample of perylene radical cation in concentrated H_2SO_4 (g = 2.00258) and checked against the measured value for 2,2-diphenyl-1-picrylhydrazyl radical(DPPH•) at the solid state (g = 2.0036).[41] For quantitative analysis, the double integral of the EPR signal was compared with that of different samples of DPPH• dissolved in the corresponding ILs, and checked with a reference solution of tetramethylpyperidine-*N*-oxyl radical (TEMPO•) in acetonitrile and in ionic liquids IL1 and IL4 (g = 2.0061).[42] For higher relative accuracy, only two reference EPR signals corresponding to IL5 and IL12 were calibrated using the double integral, while spin/L values for samples in the other ILs were determined onthe basis of samples in IL5 and IL12 by comparing the signal intensity normalized on the line width.[25]

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Keywords: allomelanins•antioxidants•electron paramagnetic resonance spectroscopy•free radicals•ionic liquids

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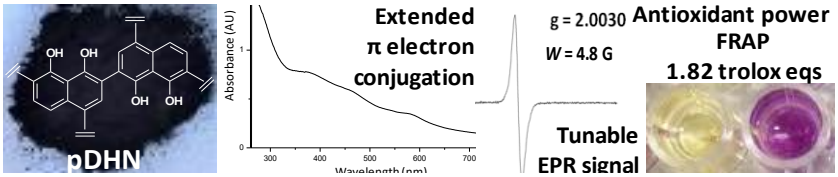

Biomimetic oxidation of 1,8-dihydroxynaphthalene (1,8-DHN), the monomer precursor of *Aspergillus* allomelanin, leads to a black and insoluble melanin-like polymer with potent antioxidant activities and an unusually intense EPR signal subject to modulation by de-assembly in ionic liquids.

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A Robust Fungal Allomelanin Mimic: An Antioxidant and Potent π-Electron Donor with Free-Radical Properties that can be Tuned by Ionic Liquids