

Dihydroxynaphthalene-Based Allomelanins: A Source of Inspiration for Innovative Technological Materials

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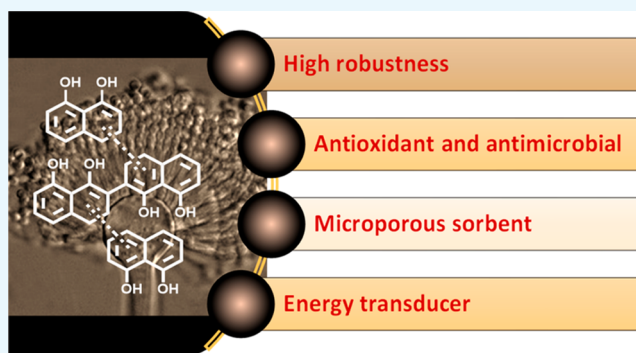
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ABSTRACT: Melanins are a wide class of natural pigments biosynthesized by different kinds of living organisms throughout all of the life domains, from bacteria to fungi, plants, and mammals. The biological functions played by these natural pigments are different (i.e., camouflage, radioprotection, thermoregulation) and ascribable to a peculiar set of physical–chemical properties making melanins a unique class of biopolymers. Among these, allomelanins from 1,8-dihydroxynaphthalene (1,8-DHNmel) produced by some Ascomycetes have recently attracted particular interest for their robustness and ability to protect fungi against both hostile (i.e., attack from fungicidal agents) and extreme (i.e., high energy radiations) environments. Starting from this background, in this mini-review we offer a panorama of the recent advances on the oxidative chemistry of 1,8-DHN leading to the formation of allomelanin mimics with tailored structural and functional properties for technological applications.



1. INTRODUCTION

The term “melanins”, from the ancient Greek *μελας* (black), was introduced for the first time by the Swedish chemist Berzelius in 1840 to address the human dark pigments extracted from eye membranes. Nowadays, the term melanins generally refers to a wide group of dark pigments originating from the enzymatic oxidative polymerization of phenolic derivatives and differing from composition, occurrence, and function.¹ The diversity exhibited by these pigments reflects the need of each species to adapt to a specific environment with the more robust nitrogen-free allomelanins found mainly in plants, fungi, and bacteria (Figure 1).

The modern classification of melanins sets these pigments into five groups on the basis of the structural features of the key monomeric units: (a) eumelanins, pheomelanins, and neuromelanin, found mainly in mammals and characterized by the presence of 5,6-dihydroxyindole and/or benzothiazine units; (b) allomelanins found in plants and fungi, characterized by the presence of phenolic, catecholic, and 1,8-dihydroxynaphthalene (1,8-DHN) units; (c) pyromelanins found mainly in bacteria, characterized by the presence of homogentisic acid units (Figure 1).¹

Despite the structural diversity exhibited by these pigments, which reflects the different chemistry of the starting monomers in the oxidative polymerization process, all melanins exhibit almost complete insolubility in any solvent, except in the case of pheomelanins and of some bacterial melanins that are slightly alkali soluble, a consequence of the high degree of

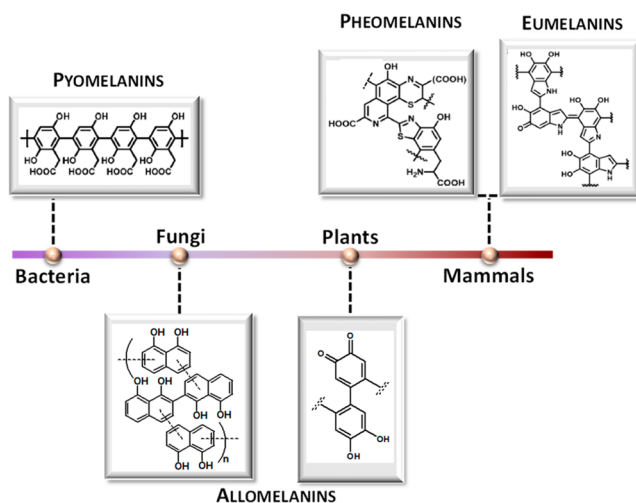
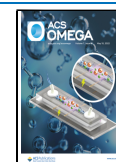


Figure 1. Structures of the melanin pigments found in Nature.

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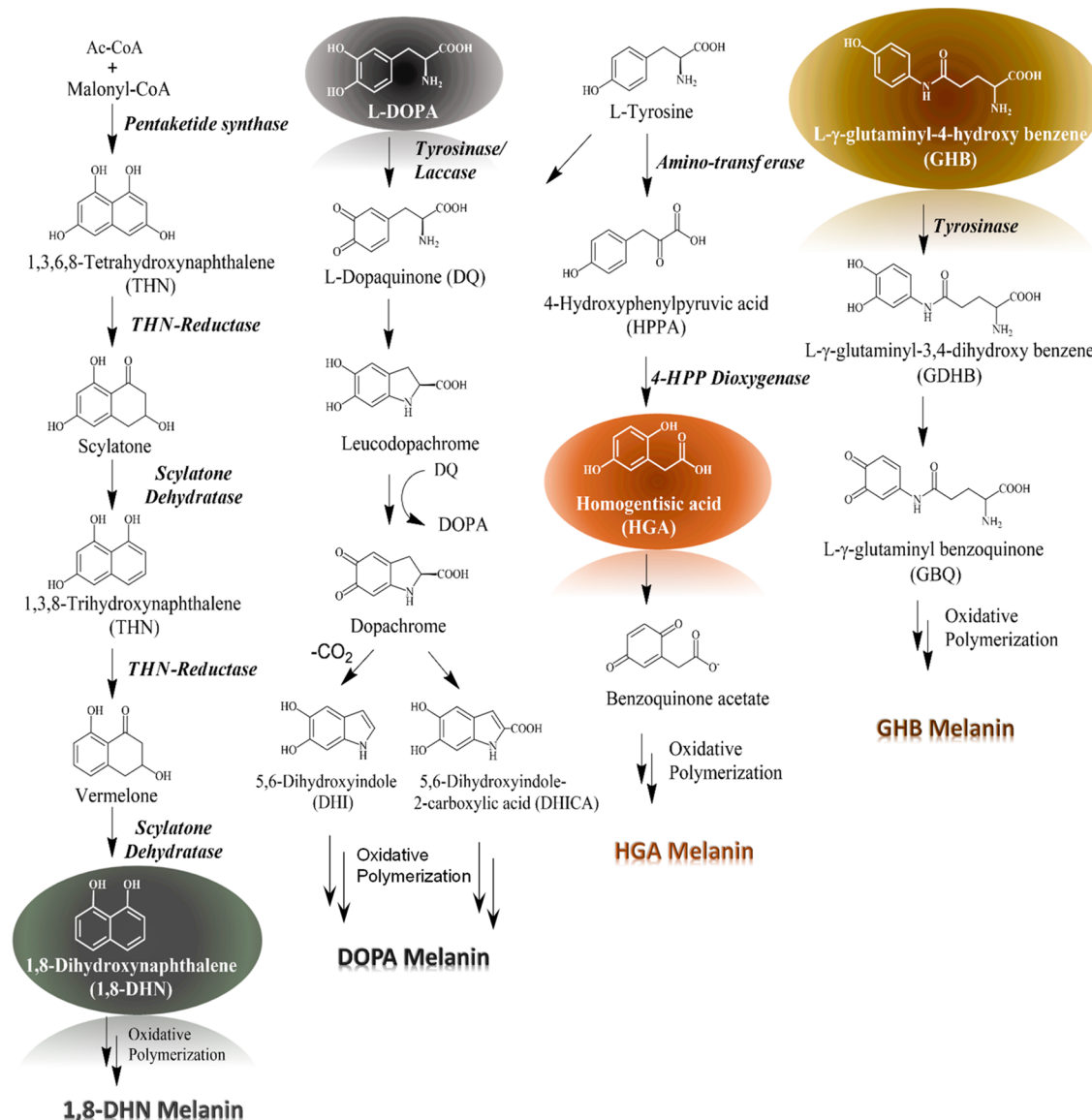


Figure 2. Biosynthetic pathways of fungal allomelanins.

chemical, structural, redox, and supramolecular disorder. For these reasons, unlike most of the natural pigments, the structure of melanins is not well defined and only a description of the main units, connections, and functional groups is available. Despite these challenging features making the investigation of the properties of melanins a difficult task, it has been possible to delineate, particularly in the case of eumelanins, a unique set of chemical–physical properties^{2,3} that includes (a) a broad-band visible-light absorption spanning throughout the entire UV–vis range and accounting for the dark coloration, (b) the intrinsic free radical character associated with a permanent electron paramagnetic resonance (EPR) signal, (c) a water-dependent hybrid ionic–electronic conductor behavior, and (d) a structure-dependent redox behavior. Thanks to these achievements, eumelanins have been successfully tested as active layers in different organic electronic devices (organic thin film transistor (O-TFT),⁴ organic photovoltaic (OPV),⁵ organic light-emitting diode (OLED),⁶ supercapacitors⁷), as a biocompatible interface for the adhesion, proliferation, and differentiation of different cell lines and stem cells,⁴ and as a functional biomaterial able to

sequester different kinds of metal ions and accumulate drugs within their structure.⁸

Recently, fungal allomelanins have received much attention with an emergent body of literature dealing with (a) isolation and tentative structural characterization of the natural pigment,⁹ (b) investigation of the chemical–physical properties, (c) design of synthetic procedures for the preparation of fungal allomelanin mimics,¹⁰ and (d) development of innovative biotechnological applications of both synthetic and natural pigments.¹¹

In this review, we propose an overview of the main properties of fungal allomelanins that have inspired the design and synthesis of 1,8-DHNmel mimics with tailored structural and functional properties for potential technological applications.

2. FUNGAL ALLOMELANINS: BIOSYNTHESIS AND BIOLOGICAL FUNCTION

Fungi can synthesize the melanin pigments following two main metabolic pathways: the 1,8-DHN pathway and the L-DOPA

pathway (Figure 2).¹² The L-DOPA pathway is quite similar to the one leading to the formation of mammalian eumelanins with the enzymes tyrosinase, operating on L-tyrosine, and laccase, operating on L-DOPA, triggering the first event generating dopaquinone. The latter undergoes intramolecular cyclization followed by a rearrangement step affording dihydroxyindole units (5,6-dihydroxyindole, DHI, and 5,6-dihydroxyindole-2-carboxylic acid, DHICA) whose polymerization generates the melanin pigment.

According to the nitrogen economy principle, many fungi pursue the 1,8-DHN metabolic pathway for the biosynthesis of melanins. In this process, the endogenously produced acetylCoA and malonylCoA are used for the buildup of the naphthalene skeleton, leading to the formation, under the action of a set of enzymes, of 1,3,6,8-tetrahydroxynaphthalene, scytalone, 1,3,8-trihydroxynaphthalene, vermeline, and 1,8-DHN. The latter enters the complex cascade of oxidative reactions leading to formation of the melanic polymer.

Other secondary pathways for the biosynthesis of melanins in fungi may involve the polymerization of dihydroxybenzene species via the formation of reactive *o*-quinones, as in the case of γ -glutaminy-3,4-dihydroxybenzene (GDHB), or *p*-quinones, as in the case of homogentisic acid (HGA) affording pyomelanins.

As is evident from the data reported in Table 1, there is no straight correlation between the type of fungal melanin and the

Table 1. Representative Fungi and the Allomelanins They Biosynthesize

species	phylum	melanin type ^a	ref
<i>Aspergillus nidulans</i>	Ascomycota	1,8-DHN/L-DOPA	13
<i>Aspergillus fumigatus</i>	Ascomycota	1,8-DHN/HGA	13
<i>Aspergillus niger</i>	Ascomycota	1,8-DHN/L-DOPA	15
<i>Alternaria alternata</i>	Ascomycota	1,8-DHN	18
<i>Cryptococcus albidus</i>	Basidiomycota	L-DOPA	19
<i>Cryptococcus neoformans</i>	Basidiomycota	L-DOPA	20
<i>Cryptococcus laurentii</i>	Basidiomycota	L-DOPA	19
<i>Cryptococcus curvatus</i>	Basidiomycota	L-DOPA	19
<i>Agaricus bisporus</i>	Basidiomycota	L-DOPA/GDHB	14

^aMonomer precursors have been listed to identify the melanin type.

phylum from which each fungus derives, even if 1,8-DHNmel seems to be commonly biosynthesized by fungi belonging to the Ascomycota phylum whereas basidiomycetes use the L-DOPA pathway or alternative pathways. In some cases, it has been reported that the same fungus can produce two different kinds of melanic pigments, as in the case of *Aspergillus fumigatus* that biosynthesizes mainly 1,8-DHN melanin and to a minor extent pyomelanin¹³ and *Agaricus bisporus* affording both DOPA and GHB melanins.¹⁴ In other cases, identification of the melanin type is still controversial as for *Aspergillus niger*,¹⁵ *Aspergillus nidulans*,¹³ *Histoplasma capsulatum*,¹⁶ and *Paracoccidioides brasiliensis* for which both the DOPA and the 1,8-DHN pathways have been proposed by independent studies.¹⁷

This high uncertainty can be traced back to the many difficulties found in isolating in high yields and in pure form the intact pigment due to its strong association with the polysaccharidic matrix of cell walls and in assessing its structure due to its high insolubility.⁹ For these reasons, most of the assignments of the fungal melanin type have been made by pursuing an indirect approach, specifically by identifying the

presence of the enzymes involved in the melanin biosynthetic pathways (i.e., pentaketide synthase, laccase, and tyrosinase) or by inducing the selective inhibition of specific melanin biosynthetic pathways, for example, via the administration to fungi of kojic acid or tropolone, inhibitors of the L-DOPA pathway, or tricyclazole or phthalide, inhibitors of the 1,8-DHN pathway.

Recently, an important step forward in the definition of the structure of fungal allomelanins has been made by Casadevall and co-workers, who demonstrated that the melanin pigment produced by *C. neoformans* during human infection derives from the polymerization of a mixture of monomer precursors, including L-DOPA and the catecholamines dopamine, norepinephrine, and epinephrine.²¹ This evidence suggests that fungi are able to biosynthesize polytypic melanin pigments whose composition reflects the availability of the monomer precursors in the infected tissues, opening for the first time to a new concept of structural complexity in the melanin pigments.

The main interest in fungi allomelanins relies on the benefits that each microorganism earns when producing the pigment and that are strictly related with its peculiar chemical–physical properties (Figure 3).

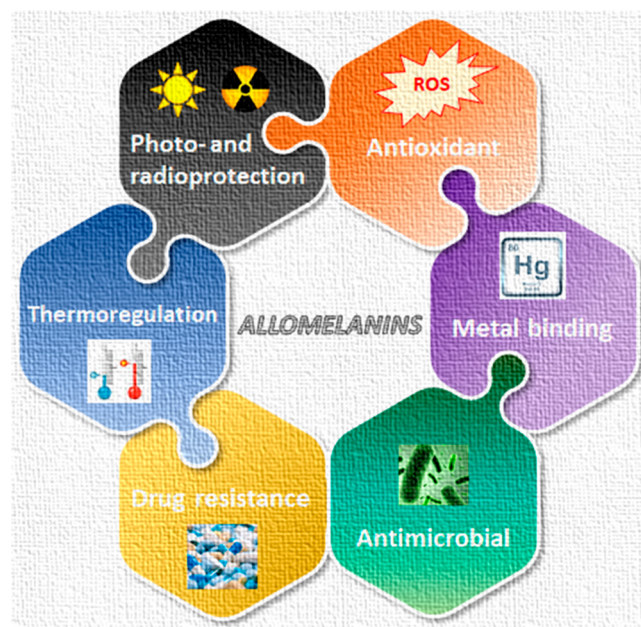


Figure 3. Main functions exerted by allomelanins in fungi.

Many studies have indicated that melanized fungi are more resistant under stress conditions with respect to nonmelanized ones, enabling the microorganisms to survive even under extreme environmental conditions.²² Particularly fascinating is the way fungal allomelanins can interact with electromagnetic radiation. The role of melanins in photoprotection has been widely discussed for the important implications in the processes related to human skin photodamage and strictly associated with the broad-band absorption of the pigment in the whole UV–vis portion of the electromagnetic spectrum. By a similar approach, allomelanins can protect some fungi against heat/cold stress probably thanks to the ability of melanins to adsorb solar radiation and dissipate it nonradiatively as heat. Valuable examples are the black meristematic microfungi that commonly grow on rocks in hot climates²³ and the halophilic ascomycetous black fungi *Hortaea werneckii*, *Phaeothea*

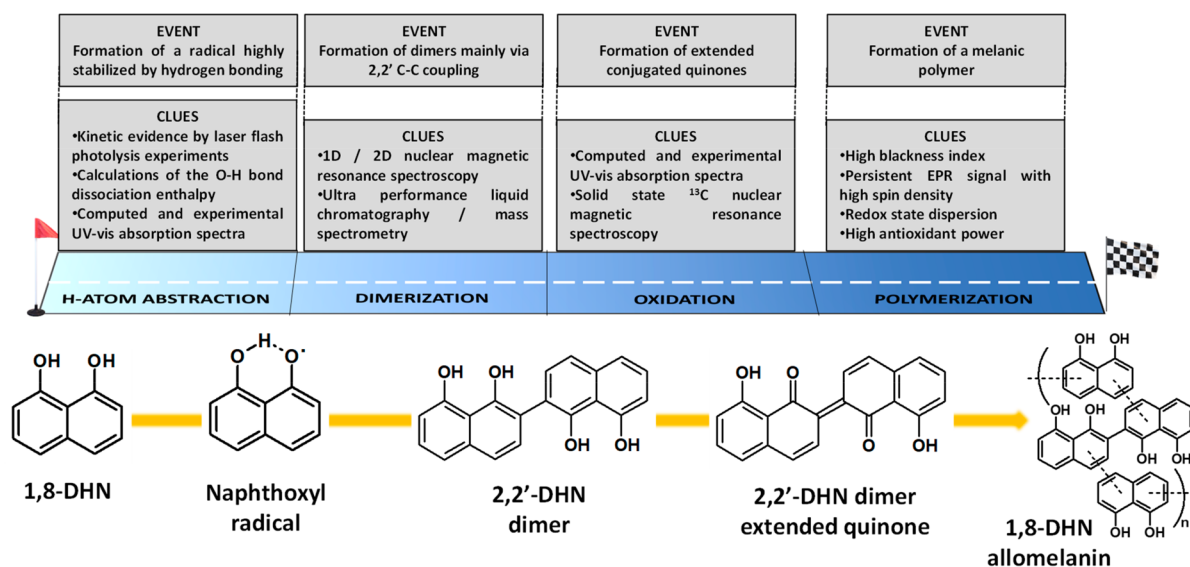


Figure 4. Key steps in the buildup of 1,8-DHN allomelanins.

triangularis, and *Trimmatostroma salinum* that can inhabit cold and hypersaline environments by producing 1,8-DHNmel.²⁴

Quite interesting is the interaction of allomelanins with high-energy radiation, as witnessed by the impressive case of the fungi populating the Chernobyl reactors after the tremendous accident in 1986. By actuating a sort of metabolic protection system, more than 37 species of fungi, including *Alternaria alternata* and *Cladosporium sphaerospermum*, spontaneously grew up despite the strong radioactive environment by activating the production of the melanin pigment inside the cell walls that acted as a real radiation shield.²⁵

The ability of melanins to protect fungi against the severe impact of high-energy radiation has been also confuted in the case of *Cryomyces antarcticus*, able not only to inhabit the McMurdo Dry Valleys in Antarctica²⁶ despite the cold temperature, dryness, and high UV radiation but also to survive external injuries such as in the case of gamma radiation²⁷ (Co⁶⁰, up to 55.61 kGy), densely (deuterons, ²H up to 1500 Gy) and sparsely (X-rays up to 300 Gy) ionizing radiation,²⁸ and accelerated helium ions²⁹ (150 MeV/nucleon) up to 1 kGy, still maintaining its metabolic activity with no detectable DNA and ultrastructural damage.

Activation of the chemical pathways leading to the polymerization of 1,8-DHN in response to photoirradiation has been also demonstrated in separate experiments carried out on the monomer precursor 1,8-DHN under different conditions (i.e., 170 MeV proton beam at 243 K under vacuum,³⁰ Hg–Xe lamp at 80 K under vacuum³¹).

Another important feature peculiar of fungal allomelanins is the chemical robustness that makes fungi highly resistant to a series of external agents. The structural diversity of allomelanins, featuring carboxylic acid, phenolic, and catecholic functional groups, accounts for the efficient interaction of the pigment with drugs, pesticides, proteins, and enzymes through the formation of stable ionic/covalent bonds and is responsible for the high binding affinity to metal cations. For this reason, melanized fungi can resist the action of degradative enzymes³² of structural polysaccharides (Chitinase or cellulase), heavy metals,³³ antimicrobial drugs (i.e., itraconazole and ketoconazole), and bactericidal and fungicidal proteins⁹ (i.e., defensins, magainins, or protegrins), exhibiting a potentiated virulence


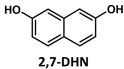
with respect to nonmelanized ones.³⁴ Allomelanins can contribute to fungal virulence also by means of their high antioxidant power. Thanks to the presence of unpaired electrons delocalized throughout the pigment backbone, fungal allomelanins can scavenge reactive oxygen and nitrogen species, such as hydrogen peroxide, hydroxyl and peroxide radicals, hypochlorite, nitric oxide, and peroxyxynitrite, that accumulate in consequence to the onset of pathological states.³⁵

3. 1,8-DHN ALLOMELANIN MIMICS: SYNTHESIS, PROPERTIES, AND APPLICATIONS

Taking inspiration from the amazing properties conferred to fungi by allomelanins, many efforts have been produced to make the pigments available on a large scale with the aim of engineering them for technological applications. Considering the many difficulties found in isolating the pigments from natural sources in high yields, a significant body of work has been focused on the development of synthetic procedures allowing for the preparation of allomelanin mimics. This approach would be desirable because it would bypass the biosafety problems associated with the industrial use of pathogenic fungi for isolation of the pigment. In this regard, most of the studies have dealt with the preparation and applications of melanins obtained from the oxidation of L-DOPA, being quite similar to the parent eumelanins. However, in the past decade, particular attention has been paid to the development of a synthetic approach to 1,8-DHNmel and on a comparative study of its structure–function relationship, disclosing for the first time the unique properties conferred by the 1,8-DHN unit to the melanin polymer.

By pursuing a biomimetic approach, 1,8-DHNmel mimics have been prepared by oxidizing the monomer precursor in phosphate buffer at pH 7.4 with the enzymatic system horseradish peroxidase/H₂O₂.³⁶ The mass analyses carried out on the dark polymer gave precious information on the dominant C–C mode of coupling of the naphthalene rings, also disclosing the high degree of polymerization from the detection of ion peaks up to the XXXIIImer species.³⁷ Supporting this evidence, ultra-performance liquid chromatography/mass analysis (UPLC/MS) of the oxidation reaction of

Table 2. Main Technological Application for Synthetic DHN Allomelanin Mimics^a

DHN monomer unit	Oxidation conditions	Synthetic melanin mimic	Application	Ref
 1,8-DHN	NaIO ₄	Spherical melanin nanoparticles	Gas and toxin sorbent	10
		Melanin-coated nylon-cotton fabrics	Protective dye for uniforms	10
	HRP/H ₂ O ₂	Quasi-spherical melanin nanoparticles	Antioxidant filler	36
 2,7-DHN	AISSP	Melanin thin films	Bio-interface for stem cells differentiation	43
	Laccase	Melanin thin films	Antimicrobial and antioxidant coating, pollutant sorbent	42

^aHRP = horseradish peroxidase; AISSP = ammonia induced solid state polymerization.

1,8-DHN carried out with the enzyme laccase from *Trametes versicolor* as a milder oxidizing agent led to the identification of a set of low molecular weight oligomers, mainly 1,8-DHN dimers and trimers.³⁷ Finally, isolation of the 1,8-DHN dimers as acetyl derivatives and their characterization by 1D and 2D NMR spectroscopy clearly indicated that the coupling between the naphthalene units occurred preferentially at the C-2 and C-4 positions.³⁸

A combined experimental and theoretical investigation suggested that the first stages of the oxidative polymerization were triggered by the initial H-atom abstraction from 1,8-DHN (Figure 4). This event, leading to the formation of a naphthoxyl radical, is strongly supported by the peculiar *peri* asset of the hydroxyl groups on the naphthalene ring stabilizing the radical via intramolecular H bonding. Evidence for this came from the following: (a) laser flash photolysis experiments indicating that the second-order rate constant (k_H) in the H-atom abstraction process mediated by phthalimide-*N*-oxyl radical and promoted by laser irradiation was higher for 1,8-DHN with respect to monohydroxynaphthalenes and other regioisomeric DHNs;³⁸ (b) theoretical calculations of the O–H bond dissociation enthalpies predicting the higher reactivity of 1,8-DHN in HAT processes;³⁹ (c) the analogies found between the simulated UV–vis absorption spectrum of the naphthoxyl radical and the time-resolved absorption spectra registered in the early phase of the oxidative polymerization process.³⁸ Moreover, through the comparative inspection of time-resolved spectra assisted by DFT calculations, it was possible to conclude that once the naphthoxyl radical has formed, the 2,2'-dimerization is the most favorable event, followed by a further oxidation step affording the corresponding extended quinones (Figure 4).³⁸

As shown by MALDI mass spectrometry and ATR-FTIR, solid-state ¹³C NMR, and EPR spectroscopies,^{36,40} the resulting synthetic polymer was characterized by a certain degree of redox complexity, in good agreement with what was reported in the case of eumelanins, exhibiting the typical features of the phenolic, semiquinone, and quinone moieties. In particular, the latter were clearly evident from the presence of both O–H and conjugated C=O stretching bands at 3100–3600 and 1621 and 1665 cm⁻¹, respectively, and from the ¹³C resonances at 190 and 160 ppm ascribable to conjugated ketones and aromatic C–OH carbon atoms.

The presence of unpaired electrons delocalized throughout the polymer backbone was revealed by EPR spectra showing the presence of a persistent signal centered at a *g* value of 2.0030, indicative of a more pronounced C-centered character of unpaired spins typical of natural 1,8-DHN allomelanins, associated with a quite high spin density value (10¹⁸ spin per g).

Finally, mass spectra revealed a consistent degree of cluster complexity in correspondence to high molecular weight oligomers, witnessing the coexistence of 1,8-DHN units at different redox states.

In a comparative study carried out on a set of fungi-inspired allomelanin mimics obtained from the oxidation of isomeric dihydroxynaphthalenes (namely, 1,6-, 1,8-, 2,7-, and 2,6-DHN), the clear-cut correlation between the redox state dispersion, the EPR spin density, the visible absorption properties, and the oligomer size has been shown.⁴⁰ This evidence suggested that the “blackness” in DHN allomelanins, defined as the property of the polymer to adsorb in the visible part of the spectrum, may be considered as a primary index of π -electron complexity.

As observed in the case of the natural pigment, also the synthetic 1,8-DHNmel tends to form supramolecular aggregates, dominated by π -stacking interactions, whose morphology can be tuned in a predictable manner by varying the oxidation conditions. In particular, the formation of quasi-spherical supramolecular aggregates connected in loose aggregates with approximate sizes in the range from 200 nm to 2 μ m was evident when using enzymatic reagents under biomimetic conditions,³⁶ whereas more regular aggregates featuring a spherical profile with diameters ranging between 100 and 300 nm were formed using chemical oxidants such as NaIO₄ or KMnO₄.¹⁰

Molecular dynamics simulations showed that hydrogen-bonding interactions are involved in the complex mechanisms driving the self-assembly of 1,8-DHN into nanoparticles in aqueous solutions. This effect is straightforward in the case of 1,8-DHN oligomers, such as in the case of 2,2'-, 2,4'-, and 4,4'-dimers, for which the relative position of the hydroxyl groups imposed by the different mode of connection of the two naphthalene rings can induce a different mode of aggregation.⁴¹ In particular, while the 2,2'- and 2,4'-dimers tend to form spherical aggregates with diameters ranging from 100 to 500 nm, the 4,4'-dimer can aggregate into planar sheets whose

morphology can be tuned into ellipsoidal platelets of different thickness by varying the amount of water incorporated within the aggregates. This behavior is due to the peculiar anisotropic packing of the 4,4'-dimer of 1,8-DHN driven by the π -stacking interaction between the naphthalene rings and the hydrogen bonding between the hydroxyl groups and the water molecules. The particle morphology proved to be retained even after the exposure to air-equilibrated ammonia vapors, allowing for the preparation of 1,8-DHNmel mimics with tailored supra-molecular assembly.

Given the many analogies between the natural pigment and the synthetic allomelanin mimic in terms of the structure–property relationship, the potential of the latter as a functional material has been tested for different applications (Table 2).

In the last 5 years a great amount of interest has focused on the applications of synthetic fungal allomelanins in functional coatings. Studies carried out on melanin thin films obtained from 2,7-DHN have shown the potential of this material to serve as a platform for the development of engineered surfaces with bactericidal and radical scavenging properties.⁴²

In this connection, recently Gianneschi and co-workers reported on the intrinsic microporosity of artificial allomelanin nanoparticles, opening up new intriguing possibilities for the use of this material for gas sorption¹⁰ (NH₃, CO₂) and toxin remediation (diazinon and paraoxon) processes, the latter experienced also in the case of melanin-coated nylon–cotton fabrics.

Allomelanin mimics from 1,8-DHN exhibited efficient radical scavenging properties in both *in vitro* and *in vivo* experiments with the antioxidant power being even higher than that registered in the case of DHICA–melanin and phenolic/catecholic plant allomelanins.³⁶

By taking inspiration from eumelanins, recently a processing protocol for the deposition of synthetic 1,8-DHNmel thin films has been reported based on an ammonia-induced solid-state polymerization (AISSP) approach.⁴³ This led to the formation of ultrasoft thin films characterized by a high robustness even under harsh conditions and good biocompatibility allowing for the adhesion, proliferation, and differentiation of stem cells, suggesting the potential use of this material as an active biointerface in (bio)optoelectronic devices.

Overall, this evidence represents a consistent starting point for future perspective in the technological applications of synthetic allomelanins as bioinspired multifunctional materials.

5. CONCLUSIONS

This mini-review offers a general overview on the recent findings on the structure and functions of fungal 1,8-DHNmel. Although the many difficulties found in isolating the pigment from natural sources have significantly limited the studies in this regard, the development of synthetic procedures for the preparation of 1,8-DHNmel mimics allows one to trace a profile of the peculiar set of chemical–physical properties of these pigments. What emerged is the high robustness and the redox complexity that, along with the tendency to form supramolecular aggregates and the ability to act as a sort of energy transducer contributed to delineate the enormous potential of 1,8-DHNmel as a multifunctional biomaterial for different kinds of technological applications.

Overall, these recent achievements make research on fungal allomelanins an emerging topic for multisectorial involvement and multidisciplinary research.

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Notes

The authors declare no competing financial interest.

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