Guillermo Gosset *Editor*

Melanins: Functions, biotechnological production, and applications

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Preface

Melanin is the common name for a unique class of natural polymeric pigments found in most organisms, including bacteria, fungi, plants, and animals. These pigments are generated as a result of the enzyme-catalysed oxidation and subsequent random polymerization of diverse precursor molecules. The most common precursors include L-tyrosine, cysteinyldopas, homogentisate, dihydroxynaphthalene, and phenolic compounds, yielding the eumelanin, pheomelanin, pyomelanin, and the allomelanins. These polymers play very diverse roles related to the survival of organisms. Functions provided by these pigments include photoprotection, thermoregulation, visual signalling, mechanical strengthening, and antioxidation. In several organisms, the production of melanins also influences immunity and virulence.

These complex polymers display unique chemical and physical properties including broadband optical absorption, structural stability, metal chelation, redox activity, paramagnetism, and semiconductivity. As a result of these properties, melanins are considered valuable functional materials for multiple applications in industry. Recent advances in the development of biotechnological processes to produce these pigments at large scale are enabling the generation of novel melanin-based products. The melanins have physical and chemical characteristics that have been found useful in the optical, electronic, material, agriculture, cosmetic, pharmaceutical, and medical industries. These polymers are also considered eco-friendly and biocompatible materials that can be produced sustainably.

The objective of this book is to provide an up-to-date overview of the biological functions of melanins, their biotechnological production, and selected applications. This book is expected to become a source of reference for researchers and students interested in this field. The chapters were written by leading experts that include up-to-date information and in-depth analysis of the current issues and challenges in this field. This work offers a better understanding of the biological roles of melanins and insights into the current and future industrial applications.

The topics in this book comprise the evolution of melanin in vertebrates as well as the properties, biogenesis, and evolution of melanosomes. Melanins are also synthesized by several species of microorganisms. The metabolic pathways involved in

the synthesis of several types of polymeric pigments in microorganisms as well as their role in survival are reviewed. A chapter is devoted to reviewing the role of melanin in virulence and pathogenicity. This pigment aids the fungus during infection and persistence in the host.

The large-scale production and purification of melanins to provide this product for industrial applications is a formidable challenge. Two chapters in this book present and discuss the state-of-the-art technologies used for the generation of microbial melanin-production strains as well as the processes for the biotechnological synthesis and purification of these pigments. Melanin is a polymer that can interact with metals, a chapter reviews this capacity in the context of technological applications, including electrochemical energy storage and metal recovery. The melanins are materials having favourable characteristics for photonic applications, including UV blocking and broadband absorption. The current advances in the application of these pigments in optically active materials are presented and discussed.

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Cuernavaca, Mexico Guillermo Gosset
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Melanin Synthesis in Bacteria: Who, How and Why

María Julia Pettinari, María Elisa Pavan, and Nancy I. López

1 Introduction

Melanins were described hundreds of years ago as the main component of the ink of the cuttlefish *Sepia* (Bizio 1825). These pigments that result from the oxidative polymerization of indolic or phenolic compounds have been found not only in animals but also in plants, fungi, and bacteria (Solano 2014; Toledo et al. 2017). In animals, melanins have been proposed not only to offer protection against UV radiation and free radicals but also to play a role in animal behavior and communication (Carletti et al. 2014). Fungi are known to produce many different types of melanin that have been shown to protect them against environmental factors and to have an important role in fungal pathogens (Belozerskaya et al. 2017). In plants, melanin production is mostly associated with the darkening of damaged tissue, and although it is normally present on the dark surface of many seeds, it remains poorly studied (Glagoleva et al. 2020). In the early twentieth century, Beijerink described dark pigments derived from tyrosine metabolism produced by some bacteria (Beijerinck 1900, 1911), and since then, many different bacteria have been reported to produce melanins using a variety of metabolic pathways (Pavan et al. 2020). The fact that melanin synthesis is observed in organisms with such a broad phylogenetic distribution suggests that these pigments are relevant to different aspects of their physiology.

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2 Melanin Biosynthesis Pathways in Bacteria

Melanins are heteropolymers of different types of quinones that rise from the oxidation of hydroxylated aromatic compounds. Most known melanins, including DOPA and homogentisate melanin, are related to tyrosine metabolism, and a few are derived from malonyl-CoA (Pavan et al. 2020) or other substrates.

2.1 Eumelanin (DOPA Melanin)

This kind of melanin is the best known and characterized, thus denominated eumelanin, and is synthesized by polyphenol oxidases with tyrosinase or laccase activities.

Tyrosinases (EC 1.14.18.1) are a type of copper-containing polyphenol oxidases that catalyze the orthohydroxylation of tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and its oxidation to dopaquinone. This compound suffers cyclization and oxidation and is finally converted into indole-5,6-quinone in another reaction catalyzed by the tyrosinase. The reactive indole-5,6-quinone, or its 2-carboxylated form (5,6-dihydroxyindole-2-carboxylic acid, DHICA), spontaneously oxidizes and polymerizes to form eumelanin or DOPA melanin. As bifunctional polyphenol oxidases, tyrosinases can catalyze both the monooxygenation of monophenols to o-diphenols (monophenolase /monophenol hydroxylase /cresolase activity) and the oxidation of o-diphenols to the corresponding o-quinone (catechol oxidase/catecholase/diphenolase activity) (Sanchez-Amat et al. 2010). Different tyrosinases can have different monophenol and diphenol substrate specificities (Shuster and Fishman 2009; Harir et al. 2018). Bacterial tyrosinases and their relevant applications have been the subject of several comprehensive reviews (Faccio et al. 2012; Zaidi et al. 2014).

Another type of polyphenol oxidases involved in melanin synthesis are laccases (EC 1.10.3.2), that lack monophenol hydroxylase activity, but oxidize a variety of diphenols (Reiss et al. 2011). Just like tyrosinases, laccases have copper ions bound to conserved histidine residues in the active site (Shuster and Fishman 2009). Laccases are frequent and well known in plants and fungi (Sharma et al. 2018) but have only recently been studied in depth in bacteria (Singh et al. 2011).

Bacterial tyrosinases related to melanin synthesis were initially described in *Streptomyces* species. In 1972 the tyrosinase of *S. glaucescens* was purified and its molecular properties were compared with those of tyrosinases from eukaryotic organisms (Lerch and Ettinger 1972). *Streptomyces* tyrosinases are encoded by a bicistronic operon that includes genes coding for the tyrosinase (MelC2) and a chaperone (MelC1) involved in the incorporation of copper in the active site of the tyrosinase and its secretion (Leu et al. 1992).

Tyrosinases were also related to melanin synthesis in *Bacillus* species, such as *Bacillus megaterium* (Shuster and Fishman 2009) and *Bacillus thuringiensis* (Liu

et al. 2004; Ruan et al. 2004). Many proteobacteria also synthesize DOPA melanin through the action of tyrosinases such as MepA in *Sinorhizobium meliloti* (Mercado-Blanco et al. 1993), and Mel *in Stenotrophomonas maltophilia* (Wang et al. 2000).

Other bacteria produce DOPA melanin through the action of a laccase. The laccase of *Azospirillum lipoferum* was purified and characterized as a multimeric enzyme consisting of one catalytic chain and one or two heavy chains whose role is to be elucidated (Diamantidis et al. 2000).

In some melanogenic bacteria, tyrosinases and laccases activities coexist. *Marinomonas mediterranea* MMB-1 possesses a cytoplasmic tyrosinase (PpoB1) (López-Serrano et al. 2004) and also a membrane-bound polyphenol oxidase (PpoA) with tyrosinase and laccase activities, but only PpoB1 is involved in melanin synthesis in this bacterium (Solano et al. 2000). A tyrosinase and a laccase have been purified and biochemically characterized from cellular extracts of *Pseudomonas putida* F6 (McMahon et al. 2007), a strain that produces reddish-brown melanin in liquid cultures supplemented with tyrosine (Nikodinovic-Runic et al. 2009), yet the exact pathway for melanin synthesis in this strain remains unknown. A gene encoding for a tyrosinase (VIBNI_B1404), with the common central domain of tyrosinases, is present in the genome of *Vibrio nigripulchritudo* SFn1, a strain that forms brown pigmented colonies. This tyrosinase, together with a laccase (VIBNI_B0280), would be involved in melanin production in this emerging pathogen of farmed shrimp, as the deletion of each gene decreased colony pigmentation (Goudenège et al. 2013).

2.2 *Pyomelanin (Homogentisate Melanin)*

Early studies of bacterial melanin assumed that their synthesis was always related to tyrosinases leading to DOPA-melanin, but due to results observed with several tyrosine-containing media Arai and Mikami (1972) suggested that diffusible dark brown melanins produced by *Streptomyces* could have a different chemical nature and that other enzymes distinct from the tyrosinase could be involved in pigment formation. Two decades later it was found that a 4-hydroxyphenylpyruvate dioxygenase directed the production of a pigment derived from homogentisate in *S. avermitilis* (Denoya et al. 1994). A pigment of this kind had already been described in *Pseudomonas aeruginosa* and denominated "pyomelanin" (Yabuuchi and Ohyama 1972). This pigment is also found in the urine of human patients with alkaptonuria. This hereditary disorder results from the absence of the homogentisate 1,2 dioxygenase, the enzyme responsible for the breakdown of homogentisate in the tyrosine degradation pathway (Fernández-Cañón et al. 1996).

In the so called homogentisate pathway, tyrosine is catabolized in five successive steps. First, tyrosine is converted into 4-hydroxyphenylpyruvate by aromatic amino acid aminotransferases. The 4-hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27) acts on this compound to generate homogentisate in what can be considered one of the most complex reactions catalyzed by a single enzyme, as it involves dioxygen

addition, decarboxylation and rearrangement/migration of the side chain on the aromatic ring (He and Moran 2009). If the homogentisate 1,2-dioxygenase enzyme is missing the final degradation of the homogentisate to fumarate and acetoacetate is precluded. The accumulation and spontaneous autooxidation and polymerization of homogentisate lead to the synthesis of the pigment.

The formation of this kind of melanin has been also related to the homogentisate 1,2-dioxygenase in bacteria. Some *Aeromonas* produce dark diffusible pyomelanins, and in all of them *hmgA,* the gene coding for this enzyme, is mutated (Pavan et al. 2015). For example, in *A. salmonicida* subsp. *salmonicida* A449 *hmgA* is a pseudogene due to a frameshift caused by a single base pair deletion while in *A. salmonicida* subsp. *pectinolytica* 34mel^T *hmgA* is interrupted by a transposase associated with IS21 family insertion sequences. The same gene is discontinued by a transposase belonging to the IS66 family in *A. media* WS and by a nonsense codon in the strain RM^T (Pavan et al. 2015; Wang et al. 2015). In contrast, *Vibrio cholera* HTX-3, *Shewanella colwelliana* D, and a strain of *Hyphomonas* accumulate homogentisate due to increased amounts of the 4-hydroxyphenylpyruvate dioxygenase and not to a mutation in the homogentisate 1,2-dioxygenase gene (Kotob et al. 1995). Additionally, homogentisate accumulation and melanin formation was observed in highly clonal strains of *V. cholerae* that display an identical 15-bp deletion in *hmgA* (Wang et al. 2011), and a single amino acid substitution (G378R) in the homogentisate 1,2-dioxygenase is responsible for pigmentation in *Burkholderia cepacia* isolates (Gonyar et al. 2015). Transposon insertions in *hmgA* (Rodríguez-Rojas et al. 2009) or large chromosomal deletions that include *hmgA* (Hocquet et al. 2016) result in the accumulation of homogentisate and production of pyomelanin in clinical isolates of *P. aeruginosa*. In the photosynthetic betaproteobacterium *Rubrivivax benzoatilyticus* JA2^T, which displays multiple anaerobic and aerobic L-phenylalanine catabolic pathways, the absence of the homogentisate dioxygenase leads to homogentisate accumulation in aerobic conditions and thereby to pyomelanin synthesis (Mekala et al. 2019).

2.3 Polyketide Derived Melanin (PK Melanin)

A unique way of melanin synthesis in bacteria is through polyketide synthases, thought to be exclusive to plants until the first bacterial one was found in *Streptomyces griseus* in 1999 (Funa et al. 1999). By sequential decarboxylative condensation of five molecules of malonyl-coenzyme A, the structurally simple homodimeric type III polyketide synthase RppA catalyzes the synthesis of a pentaketide that is then cyclized to 1,3,6,8-tetrahydroxynaphthalene (THN). A member of the cytochrome P450 family whose gene lies in an operon with *rppA* catalyzes the oxidative dimerization of two THN subunits to yield 1,4,6,7,9,12-hexahydroxyperylene-3,10-quinone (hexahydroxyperylenequinone, HPQ) (Funa et al. 2005). The air oxidation and autopolymerization of this unstable precursor lead to the formation of brownish melanin (Funa et al. 2005). Genome analysis of *Streptomyces avermitilis* has

described several polyketide synthase clusters, including a type II polyketide synthase proposed to catalyze the synthesis of polyketide-derived melanin from malonylCoA (Omura et al. 2001). The bacterial melanin biosynthetic pathway found in *Streptomyces* is different from that of some fungi in which THN, synthesized by the evolutionarily different large and complex type I polyketide synthases, is an intermediate in the synthesis of 1,8-dihydroxynaphthalene (DHN) that then polymerizes to form melanin.

2.4 Allomelanins

Finally, the oxidation of aromatic compounds such as phenol, catechol, protocatechualdehyde, and 1,8-dihydroxynaphthalene (1,8-DHN), can lead to the formation of pigments collectively known as allomelanins, commonly found in fungi and plants (Martínez et al. 2019), and also described in bacteria.

Some bacteria can synthesize these aromatic compounds as end products or as intermediary metabolites during the degradation of aromatic substrates, so their accumulation could provide the substrates for the formation of melanins through oxidation and polymerization.

Black melanin derived from catechol was described in *Azotobacter chroococcum* (Shivprasad and Page 1989). Although catechol is formed in low or high aeration conditions, melanization only occurs in the presence of oxygen. The formation of melanin during the encystment stage of this microorganism is related to a membrane-bound laccase (Herter et al. 2011).

Serratia marcescens can produce brown melanin by spontaneous oxidation and polymerization of homoprotocatechuate (3,4-dihydroxyphenylacetate), an isomer of homogentisate (2,5-dihydroxyphenylacetate) (Trias et al. 1989). Homoprotocatechuate is a metabolite produced during tyrosine degradation in a pathway present in many bacteria such as *Paraburkholderia xenovorans* that leads to the formation of Krebs cycle intermediates (Méndez et al. 2011). The low activity of the enzyme that catalyzes homoprotocatechuate degradation, the 3,4-dihydroxyphenylacetate 2,3-dioxygenase (EC 1.13.11.15) leads to the accumulation of this metabolite in *S. marcescens* (Trias et al. 1989). The metabolic process that leads to homoprotocatechuate accumulation is comparable to the one that leads to the formation of homogentisate melanin covered in Sect. 2.2, found in a great number of bacteria (Table 1). Although (i) the homoprotocatechuate pathway is present in many bacteria (Méndez et al. 2011), and (ii) mutations in the gene that codes for the 3,4-dihydroxyphenylacetate 2,3-dioxygenase would be expected to lead to homoprotocatechuate melanin synthesis in other bacteria, this kind of melanin has only been described in *S. marcescens*.

Table 1 Bacteria producing different kinds of melanin

Phylum	Class	Order	Family	Species	Melanin type	References
Proteobacteria	*Alpha*	*Rhodospirillales*	*Azospirillaceae*	*Azospirillum brasilense*	HMG?	Gowri and Srivastava 1996
				Azospirillum lipoferum	DOPA	Givaudan et al. 1993
		Caulobacterales	*Caulobacteraceae*	*Brevundimonas vitisensis*	HMG	Jiang et al. 2021
				Brevundimonas sp.	DOPA	Surwase et al. 2013
		Hyphomonadales	*Hyphomonadaceae*	*Hyphomonas* sp.	HMG	Kotob et al. 1995
			Rhizobiaceae	*Rhizobium etli*	DOPA	Piñero et al. 2007
				Rhizobium leguminosarum	DOPA	Cubo et al. 1988
				Rhizobium phaseoli	DOPA	Borthakur et al. 1987
				Rhizobium radiobacter	DOPA	Wu et al. 2022
				Sinorhizobium fredii	DOPA	Cubo et al. 1988
				Sinorhizobium meliloti	DOPA	Cubo et al. 1988
	Beta	*Burkholderiales*	*Alcaligenaceae*	*Alcaligenes faecalis*	HMG	Singh et al. 2018
				Bordetella parapertussis	HMG	Hiramatsu et al. 2021
			Burkholderiaceae	*Burkholderia cenocepacia*	HMG	Keith et al. 2007
				Burkholderia cepacia	HMG	Gonyar et al. 2015
				Ralstonia solanacearum	HMG	Ahmad et al. 2016
				Ralstonia pickettii	HMG	Seo and Choi 2020
			Comamonadaceae	*Rubrivivax benzoatilyticus*	HMG	Mekala et al. 2019
	Gamma	*Aeromonadales*	*Aeromonadaceae*	*Aeromonas media*	HMG	Wang et al. 2015
				Aeromonas salmonicida subsp. *pectinolytica*	HMG	Pavan et al. 2015
				Aeromonas salmonicida subsp. *salmonicida*	HMG	Pavan et al. 2015
		Alteromonadales	*Pseudoalteromonadaceae*	*Pseudoalteromonas aliena*	DOPA	Ivanova et al. 2004
				Pseudoalteromonas distincta	DOPA	Ivanova et al. 2004
				Pseudoalteromonas lipolytica	HMG	Zeng et al. 2017b
			Shewanellaceae	*Shewanella algae*	HMG	Turick et al. 2002
				Shewanella colwelliana	HMG	Kotob et al. 1995
				Shewanella oneidensis	HMG	Turick et al. 2009

		Enterobacterales	*Enterobacteriaceae*	*Klebsiella pneumoniae*	HMG	Singh et al. 2018
				Klebsiella sp.	DOPA	Sajjan et al. 2010
				Enterobacter sp.	HMG	Singh et al. 2018
			Morganellaceae	*Proteus mirabilis*	DOPA	Agodi et al. 1996
				Providencia rettgeri	ND	Laxmi et al. 2016
				Providencia sneebia	ND	Vijayan et al. 2017
				Providencia vermicola	ND	Vijayan et al. 2017
			Yersiniaceae	*Serratia marcescens*	HPC	Trias et al. 1989
		Legionellales	*Legionellaceae*	*Legionella pneumophila*	HMG	Flydal et al. 2012
		Oceanospirillales	*Oceanospirillaceae*	*Marinomonas mediterranea*	DOPA	Solano et al. 2000
		Pseudomonadales	*Moraxellaceae*	*Acinetobacter baumannii*	HMG	Coelho-Souza et al. 2014
			Pseudomonadaceae	*Pseudomonas aeruginosa*	HMG	Yabuuchi and Ohyama 1972
				Pseudomonas syringae pv. *porri*	DOPA	Samson et al. 1998
				Pseudomonas guinea	DOPA	Tarangini and Mishra 2013
				Pseudomonas koreensis	DOPA	Eskandari and Etemadifar 2021a
				Pseudomonas otitidis	DOPA	Seelam et al. 2021
				Pseudomonas putida	DOPA	McMahon et al. 2007
				Pseudomonas stutzeri	**HMG; DOPA**	**Kurian and Bhat 2018a; Ganesh Kumar et al. 2013**
				Azotobacter chroococcum	**DOPA; CAT**	**Banerjee et al. 2014; Shivprasad and Page 1989**
				Azotobacter salinestris	CAT	Page and Shivprasad 1991
		Cellvibrionales	*Cellvibrionaceae*	*Saccharophagus degradans*	DOPA	Kelley et al. 1990
		Xanthomonadales	*Xanthomonadaceae*	*Stenotrophomonas maltophilia*	DOPA	Wang et al. 2000
				Xanthomonas campestris pv. *phaseoli*	HMG	Goodwin and Sopher 1994
				Lysobacter oligotrophicus	DOPA	Kimura et al. 2015
		Vibrionales	*Vibrionaceae*	***Vibrio alginolyticus***	**DOPA + HMG**	**Kurian and Bhat 2018b**
				Vibrio antiquarius	ND	Vijayan et al. 2017
				Vibrio cholerae	HMG	Kotob et al. 1995
				Vibrio harveyi	ND	Vijayan et al. 2017
				Vibrio nigripulchritudo	DOPA	Goudenège et al. 2013
				Vibrio rotiferianus	ND	Vijayan et al. 2017
Firmicutes	*Bacilli*	*Caryophanales*	*Bacillaceae*	*Bacillus altitudinis*	ND	Vijayan et al. 2017
				Bacillus cereus	ND	Zhang et al. 2007

(continued)

Table 1 (continued)

				Bacillus haynesii	HMG	Marín-Sanhueza et al. 2022
				Bacillus licheniformis	DOPA	Gamal Shalaby et al. 2019
				Bacillus megaterium	DOPA	Shuster and Fishman 2009
				Bacillus safensis	DOPA	Tarangini and Mishra 2014
				Bacillus subtilis	DOPA	Ghadge et al. 2020
				Bacillus thuringiensis	**DOPA; HMG**	**Ruan et al. 2004; Cao et al. 2018**
				Bacillus weihenstephanensis	DOPA	Drewnowska et al. 2015
				Cytobacillus firmus	ND	Vijayan et al. 2017
			Staphylococcaceae	*Salinicoccus roseus*	ND	Vijayan et al. 2017
				Mammaliicoccus sciuri	ND	Vijayan et al. 2017
			Caryophanaceae	*Planococcus maritimus*	ND	Vijayan et al. 2017
Actinobacteria	*Actinobacteria*	*Pseudonocardiales*	*Pseudonocardiaceae*	*Actinoalloteichus* sp.	DOPA	Manivasagan et al. 2013
		Mycobacteriales	*Dietziaceae*	*Dietzia schimae*	DOPA	Eskandari and Etemadifar 2021b
		Frankiales	*Frankiaceae*	*Frankia sp.*	DOPA	Yuan et al. 2007
		Micrococcales	*Micrococcaceae*	*Glutamicibacter creatinolyticus*	ND	Vijayan et al. 2017
		Geodermatophilales	*Geodermatophilaceae*	*Modestobacter versicolor*	ND	Reddy et al. 2007
		Streptosporangiales	*Nocardiopsaceae*	*Nocardiopsis alba*	DOPA	Kiran et al. 2014
				Nocardiopsis dassonvillei	ND	Kamarudheen et al. 2019
		Streptomycetales	*Streptomycetaceae*	***Streptomyces avermitilis***	**HMG; HMG+ DOPA + PK**	**Denoya et al. 1994; Omura et al. 2001**
				Streptomyces castaneoglobisporus	DOPA	Ikeda et al. 1996
				Streptomyces cyaneofuscatus	DOPA	Al Khatib et al. 2018
				Streptomyces glaucescens	DOPA	El-Naggar and El-Ewasy 2017
				Streptomyces griseus	PK	Funa et al. 1999
				Streptomyces kathirae	DOPA	Guo et al. 2014
				Streptomyces lavendulae	DOPA	Mencher and Heim 1962
				Streptomyces michiganensis	DOPA	Held and Kutzner 1990
				Streptomyces parvus	DOPA	Bayram et al. 2020
Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	*Flavobacterium kingsejongi*	HMG	Lee et al. 2022

DOPA : Eumelanin, DOPA derived melanin; HMG: pyomelanin, homogentisate derived melanin; PK: polyketide derived melanin; CAT: allomelanin, catechol derived melanin; HPC: allomelanin, homoprotocatechuate derived melanin; ND: not determined. Species in which more than one kind of melanin have been described are highlighted in gray. Semicolons are used for cases in which different kinds of melanin have been described for a given species in separate studies.

3 Phylogenetic Distribution of Melanin Synthesis in Bacteria

3.1 Actinobacteria

The ability to synthesize melanin is a common property among *Streptomyces* (Table 1) and is used for the classification and identification of these filamentous soil-dwelling microorganisms (Arai and Mikami 1972). Although these bacteria mostly synthesize DOPA melanin through the action of tyrosinases (Manivasagan et al. 2013), several species are known to produce other kinds of melanin. The production of homogentisate melanin in bacteria was first related to 4-hydroxyphenylpyruvate dioxygenase in *S. avermitilis* (Denoya et al. 1994). Synthesis of melanins through type III polyketide synthases was also described in this genus and studied extensively in *S. griseus* (Funa et al. 2005). Synthesis of melanin in this microorganism shares regulatory control with spore formation and provides *S. griseus* enhanced protection from UV irradiation suggesting HPQ melanin would play a role in protection against environmental stress (Funa et al. 2005; Takano et al. 2007). Some bacteria from this genus, such as *S. avermitilis,* can synthesize three different kinds of melanin, two of them (DOPA melanin and homogentisate melanin) related to tyrosine, and a third (polyketide-derived melanin) from malonyl CoA. Analysis of its genome revealed that it contains two clusters coding for tyrosinases, a type II polyketide synthase (Omura et al. 2001), and genes leading to homogentisate synthesis (Denoya et al. 1994).

3.2 Firmicutes

Several species of *Bacillus* also synthesize melanin (Table 1). Some like *B. thuringiensis* and *B. megaterium* produce DOPA melanin through the action of tyrosinases (Shuster and Fishman 2009), while a recent study has described a thermophilic *B. haynesii* that produces homogentisate melanin (pyomelanin) when submitted to stress (Marín-Sanhueza et al. 2022). *B. thuringiensis* strains produce melanin when cultures are exposed at 42 °C (Liu et al. 2004; Ruan et al. 2004), and *B. cereus* isolates were also observed to produce melanin (Zhang et al. 2007). The melanin produced by these bacteria protects the spores and insecticidal crystals from UV exposure (Ruan et al. 2004; Zhang et al. 2007). A large screening study also identified psychrophilic melanin-producing *B. weihenstephanensis* (Drewnowska et al. 2015). However, this study that screened nearly a thousand isolates of the *B. cereus* group from different origins found that only six of them (all strains of *B. weihenstephanensis*) produced melanin, suggesting that the ability to produce this pigment is not widespread among this group (Drewnowska et al. 2015).

3.3 *Proteobacteria*

Many proteobacteria synthesize DOPA melanin (Table 1). Its synthesis has been studied in several of them and has been related to tyrosinase coding genes such as *mepA* in *S. meliloti* (Mercado-Blanco et al. 1993), and *mel* in *S. maltophilia* (Wang et al. 2000). Some proteobacteria only produce melanin in certain conditions, so it is possible that the capability to produce this pigment has been overlooked in many of them. For example, the plant pathogen *Ralstonia solanacearum*, that in standard growth conditions does not produce melanin, synthesizes DOPA melanin through the action of a tyrosinase when grown in the presence of tyrosine if deprived of some other nutrients (Hernández-Romero et al. 2005). DOPA melanin production has also been described in several species of *Pseudomonas*, including animal and plant pathogens, and environmental strains (Table 1).

In contrast to DOPA melanin which involves special enzymes, the synthesis of melanin derived from the oxidation and polymerization of homogentisate is due to deficiencies in the degradation of homogentisate. In species belonging to the genus *Pseudomonas*, the production of homogentisate melanin was detected 50 years ago in clinical isolates of *P. aeruginosa*, giving rise to the name pyomelanin (Yabuuchi and Ohyama 1972).

As discussed in the previous section, the synthesis of homogentisate-derived melanin, which has been extensively studied in *Aeromonas* (Pavan et al. 2015), is common among many groups of proteobacteria, including *Shewanella* (Kotob et al. 1995; Turick et al. 2009), *Burkholderia* (Keith et al. 2007), *Ralstonia* (Hernández-Romero et al. 2005; Seo and Choi 2020), *Brevundimonas* (Jiang et al. 2021), *Xanthomonas* (Goodwin and Sopher 1994), *Klebsiella*, *Alcaligenes*, and *Enterobacter* (Singh et al. 2018) among others (Table 1).

Homogentisate melanin was also described in *Vibrio cholerae* (Kotob et al. 1995), although other species of this genus like *V. nigripulchritudo* have been proposed to synthesize DOPA melanin based on the presence of a tyrosinase and a laccase (Goudenège et al. 2013). In addition, the simultaneous synthesis of both DOPA melanin and homogentisate-derived melanin has been reported in *V. alginolyticus* (Kurian and Bhat 2018a) and in *P. stutzeri* strains (Kurian and Bhat 2018b; Ganesh Kumar et al. 2013).

Although DOPA melanins and homogentisate-derived melanins are the most common types of melanins produced by proteobacteria, some cases of the production of melanins derived from other substrates have been described in this taxon. *A. chroococcum* produces black melanin derived from catechol proposed to fulfill a role in aeroadaptation by minimizing oxidative stress, allowing this soil-dwelling bacterium to fix nitrogen in an aerobic environment (Shivprasad and Page 1989), and *S. marcescens* produces a pigment derived from homoprotocatechuate (Trias et al. 1989), the only bacterium in which this kind of melanin has been described.

3.4 Other Bacterial Phyla and Archaea

Recent studies have characterized melanin produced by bacteria belonging to other phyla. For example, *Flavobacterium kingsejongi* was found to produce high levels of homogentisate-derived melanin (Lee et al. 2022).

An *in silico* search predicted that laccases are widespread in bacteria as putative laccases were identified in members of *Aquificales*, high G+C Gram-positive bacteria, and α-, γ- and ε-*Proteobacteria,* but their relationship to melanin synthesis in many of these microorganisms remains nearly unknown (Alexandre and Zhulin 2000).

Although melanin synthesis has never been observed in archaea up to our knowledge, it is noteworthy that applying *in silico* data mining putative laccases were identified in *Haloarcula marismortui*, *Halorubrum lacusprofundi,* and *Natronomonas pharaonis* (phylum *Euryarchaeota*), *Pyrobaculum aerophilum* (phylum *Crenarchaeota*), and *Nitrosopumilus maritimus* (phylum *Thaumarchaeota*) (Sharma and Kuhad 2009). Moreover, a psychrophilic tyrosinase from the marine *Candidatus Nitrosopumilus koreensis* was expressed in *Escherichia coli* and found to exhibit a high monophenolase/diphenolase activity ratio (Kim et al. 2016).

Taking into account the different metabolic pathways related to melanin production, there is no apparent association of melanin synthesis mechanisms with any particular phylogenetic groups of prokaryotes (Table 1). It can be stated that the production of this pigment appears in different groups of bacteria, both gram-positive and gram-negative. Likewise, the capability to synthesize melanin does not include all the members of a hierarchical phylogenetic group, but rather seems to have appeared sporadically.

4 Control of Melanin Synthesis in Bacteria

Despite the diverse melanin synthesizing pathways, in most bacteria melanin formation is stimulated in the presence of tyrosine and Cu. In the case of DOPA melanins, this effect can be obviously attributed to the fact that tyrosinases contain this metal in their structure. This explanation would not apply to cases in which melanin synthesis is not related to tyrosinases or other Cu-containing enzymes, so the mechanism through which Cu and other metals enhance melanin in these bacteria remains unknown (Pavan et al. 2020).

As discussed in previous sections, many bacteria synthesize melanin in specific growth conditions, or a certain growth phase, indicating that pigment formation is regulated, even in bacteria that produce the pigment through an apparently non-dedicated pathway (Pavan et al. 2020).

Pigmentation in rhizobacteria depends on several nutritional factors and is related to nitrogen fixation. *Rhizobium leguminosarum* bv. *phaseoli,* which carries melanin-related genes in the symbiosis plasmid, produces melanin in a rich medium but not in

a minimal medium even when both tyrosine and Cu are added (Borthakur et al. 1987). Transcription of the tyrosinase coding gene in this microorganism is activated by the regulator of nitrogen fixation, especially at low oxygen availability (Hawkins and Johnston 1988). Melanin formation has been linked to several kinds of stress or to the formation of resistance structures in different bacteria. *M. mediterranea* (Lucas-Elío et al. 2002) and *R. solanacearum* (Ahmad et al. 2016) synthesize melanin when they reach the stationary phase while in *Azospirillum* (Gowri and Srivastava 1996) and *Azotobacter* (Herter et al. 2011) melanin production is related to cyst formation. Other bacteria such as *B. thuringiensis* (Ruan et al. 2004) and *Pseudoalteromonas* (Zeng et al. 2017a) produce melanin at high temperatures. Both high temperatures and hyperosmotic stress increased pigment formation in *V. cholerae*, especially in nutrient-poor growth media (Coyne and Al-Harthi 1992). Melanin synthesis is also related to biofilm formation, as seen in *Pseudoalteromonas* (Zeng et al. 2017a) and in *Vibrio anguillarum*. In this last microorganism, both biofilm formation and pigment production are controlled by a Lux-R-like transcriptional regulator (Croxatto et al. 2002). Studies of melanin synthesis in *R. solanacearum* reveal that it is controlled by transcriptional regulators OxyR, RpoS, and HrpG, involved in the regulation of stress response and pathogenicity (Ahmad et al. 2016).

In several *Streptomyces* species, melanin formation is under the control of the transcriptional regulator AdpA, which controls morphological development in response to A factor levels. This regulator activates tyrosinase (Zhu et al. 2005) and also regulates the synthesis of the type III polyketide synthase RppA, responsible for polyketide-derived melanin formation, along with many genes required for both morphological development and secondary metabolism (Takano et al. 2007).

In contrast to the melanins synthesized by special dedicated biosynthetic pathways, melanin formation from degradation intermediates such as homogentisate is due to the accumulation of these compounds caused by alterations in catabolic pathways. In the homogentisate pathway, phenylalanine or tyrosine are degraded to 4-hydroxyphenylpyruvate that is converted to homogentisate by Hpd. In the complete pathway, homogentisate is further degraded by HmgA to acetoacetate and fumarate. The genes that code for the enzymes in this pathway are controlled by transcriptional regulators in response to the presence of phenylalanine or tyrosine (Herrera et al. 2009; Palmer et al. 2010).

Accumulation of homogentisate has been shown to be due to overproduction, e.g. due to an increase in the expression of Hpd that converts 4-hydroxyphenylpyruvate to homogentisate (Kotob et al. 1995), or to lack of degradation, linked to the absence (Pavan et al. 2015) or decreased activity (Sanchez-Amat et al. 1998) of HmgA, the enzyme that leads to homogentisate degradation. Apart from the enzymes involved in the synthesis and degradation of homogentisate, several transcriptional regulators and transport proteins can affect melanin formation in *P. aeruginosa, A. media,* and *R. solanacearum* (Hunter and Newman 2010; Wang et al. 2015; Ahmad et al. 2016). Additionally, the presence of glycerol was observed to inhibit melanin synthesis in *A. salmonicida* subsp.

pectinolytica, and attributed to a combination of metabolic and regulatory effects including a decrease in *hpd* transcription (Pavan et al. 2019).

Many factors, including nutrient availability, different kinds of stress, formation of resistance structures, and interaction with other organisms have been observed to affect melanin synthesis in bacteria. Pigment formation is controlled by transcriptional regulators both in bacteria that synthesize melanin through the action of specific enzymes or in those in which melanin synthesis is due to imbalances in aromatic amino acid degradation or other pathways. The growing evidence that indicates that melanin synthesis is subject to different levels of regulation in all these bacteria suggests that melanin production is highly relevant to their physiology.

5 Physiological Role of Bacterial Melanins

Melanins may have a wide variety of functions that confer adaptive advantages to enhance survival under many stress conditions. The role of melanin in bacterial fitness was verified in different lifestyles and ecological niches encompassing both free-living cells, biofilms, or associations with other microorganisms, plants, or animals, including both pathogenic and non-pathogenic interactions (Fig. 1).

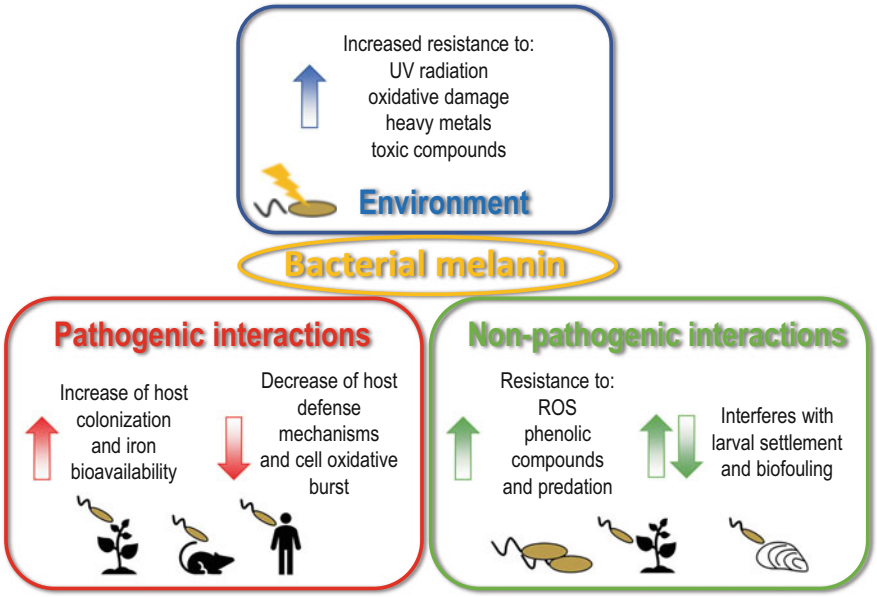

Fig. 1 The physiological role of melanins in bacteria. Melanin production increases bacterial fitness and provides resistance against several environmental stress factors. Melanin affects interactions among bacteria and both pathogenic and non-pathogenic interactions with animals and plants

5.1 Contribution of Melanin to Bacterial Fitness in Different Environments

Melanin production can enhance the capability of bacteria to respond to changing environmental conditions, increasing their survival in many environments (Fig. 1). *Shewanella* can use melanin as a terminal electron acceptor for anaerobic respiration and also as an electron shuttle during the respiration of Fe(III) minerals at low oxygen concentration helping this microorganism to live in anoxic or highly oxygenated environments (Turick et al. 2002, 2009).

Many melanin-producing bacteria have been discovered in extreme environments such as deserts and places with extreme hot or cold temperatures. *Streptomyces cyaneofuscatus* was isolated from the Sahara Desert (Harir et al. 2018), and *B. haynesii* from a hot spring in Chile showed melanin production under salinity stress (Marín-Sanhueza et al. 2022). The psychrotolerant melanin producer *B. weihenstephanensis* was isolated from cold environments in Poland (Drewnowska et al. 2015), and *Lysobacter oligotrophicus*, that was isolated in Antarctica, increased melanin production after UV exposure (Kimura et al. 2015). Nuclear waste, heavy metals, or oil spills can also generate extremely harsh environments where microorganisms have to cope with a variety of stress conditions. In *A. salmonicida* subsp. *pectinolytica*, a bacterium isolated from a heavily polluted river (Pavan et al. 2000), melanin production has been proposed to help this microorganism cope with the high concentrations of heavy metals, hydrocarbons, and other pollutants found in this environment (Pavan et al. 2015). Melanin-producing bacteria could thus be used for bioremediation applications as proposed for *A. chroococcum* which secretes melanin with high metal chelating activity (Rizvi et al. 2019).

In addition, one of the traditional roles assigned to melanin is the protection of cells from UV radiation (Fig. 1). Pigmented mutants of *Bacillus anthracis* (Han et al. 2015), and melanized spores of *S. griseus* are more resistant to UV irradiation than colorless mutants (Funa et al. 2005). Melanin has also been related to protection against oxidative stress, as melanogenic *P. aeruginosa* strains show higher tolerance to photodynamically induced oxidative stress (Orlandi et al. 2015).

5.2 Melanin in Pathogenic Interactions with Animals and Plants

Melanin production has been extensively associated with increased virulence in several bacterial pathogens (Nosanchuk and Casadevall 2003; Plonka and Grabacka 2006) (Fig. 1). This increase was attributed both to the reduction of the susceptibility of the pathogen to host defense mechanisms and to the alteration of the host immune response to infection (Nosanchuk and Casadevall 2006). Melanin production in

V. cholerae increases cholera toxin and pilus expression and enhances host colonization (Valeru et al. 2009).

In some melanin-producing epidemic strains of *Burkholderia cenocepacia* the pigment can diminish host cell oxidative burst due to its free radical scavenging potential, protecting this pathogen from oxidative stress (Keith et al. 2007). This was also observed for the plant pathogen *R. solanacearum*, in which protection against oxidative stress could help the bacteria cope with plant defense mechanisms (Ahmad et al. 2016) (Fig. 1). The ability of melanin to increase iron bioavailability through the reduction of ferric to ferrous form allows growth of *Legionella pneumophila* under iron-limited conditions such as those encountered in mammalian hosts and natural environments (Zheng et al. 2013). Melanin-producing *P. aeruginosa* emerge spontaneously during chronic infections in humans due to deletions in a genomic region containing *hmgA*. This generates mixed populations of pigmented and non-pigmented bacteria in an evolutionary process driven by continuous selective pressure due to intraspecific competition (Hocquet et al. 2016).

In the melanin producer *Bordetella parapertussis,* studies performed using a melanin-deficient mutant showed that melanin protects the bacteria from intracellular killing in macrophages and that the melanogenic strain was able to persist in the respiratory tract of mice more than the mutant strain (Hiramatsu et al. 2021). In line with this, it was observed that melanin from *Klebsiella pneumoniae* acts as a virulence factor suppressing the most important pro-inflammatory cytokines to counteract immune responses in mice (Saud and Alaubydi 2019).

Melanogenic bacteria having proteolytic, chitinolytic, and lipolytic activities were associated with tail fan necrosis in lobsters. It was proposed that the melanization of necrotized tail fans by lobsters was a sign of an effective immune response to eliminate pathogens (Perdomo-Morales et al. 2007). However, melanin-producing bacteria were isolated from the tail fans of the sick lobsters, suggesting that pigment formation in the characteristic melanized tissues associated with this disease could also be of bacterial origin (Zha et al. 2018). Melanin-producing bacteria can tolerate the presence of melanin from both bacterial or lobster origin. This fact may increase tail fan necrosis by protecting bacteria from lobster defenses, probably also enabling bacterial access to haemolymph and expanding infection (Zha et al. 2018). Melanosis is also recognized as one of the symptoms of fish diseases associated with some *A. salmonicida* melanin-producing strains (Janda and Abbott 2010).

5.3 Melanin in Non-pathogenic Interactions

Melanin is related to several interkingdom interactions involving non-pathogenic bacteria (Fig. 1). Tyrosinase activity, and consequently, melanin production is necessary during the first stages of nodulation during symbiosis of *Rhizobium etli*, a stage in which bacteria face reactive oxygen species (ROS) and phenolic compounds derived from plant defenses (Piñero et al. 2007). The melanin produced under metal exposure by the nitrogen-fixing *A. chroococcum* possesses a high metal

chelating activity, which makes this bacterium a good candidate to protect plants from toxicity in metal-stressed environments, also supplying N (Rizvi et al. 2019). Studies conducted on the melanin-producing endophytic *Bacillus subtilis* 4NP-BL isolated from the halophile plant *Salicornia brachiata* suggest that bacterial melanin could be involved in the protection of the host plant from environmental stresses and pathogens (Ghadge et al. 2020).

Induction of melanin production in *Pseudoalteromonas* sp. SM9913 that occurs at elevated temperatures or during biofilm formation might have a role in the adaptive responses of marine bacteria to environmental signals (Zeng et al. 2017a). In addition, melanin is related to interactions between bacterial biofilms and other organisms. Melanin secreted by biofilms of the marine bacterium *Pseudoalteromonas lipolytica* inhibits the larval settlement and metamorphosis of mussels, thus reducing the colonization of surfaces that leads to biofouling (Zeng et al. 2017b) (Fig. 1). *Shewanella* species establish symbioses with several metazoans (Lemaire et al. 2020), and in particular, in *S. colwelliana* melanin was reported to play a role in this process. A study mimicking the niche intertidal conditions performed using this bacterium showed higher biofilm formation and enhanced exopolysaccharides and melanin production that in turn increased the settlement of oyster larvae (Mitra et al. 2015). Contrasting results regarding biofouling activities suggest that melanin could act differently depending on the species involved or the environmental conditions, so further studies are necessary to elucidate the effect of melanin in complex communities in which bacteria interact with different organisms.

The photodamage protection conferred by bacterial melanin can be extended to other organisms living in close association. The dark pigmentation of sponges provided by melanogenic symbionts belonging to different bacterial genera such as *Vibrio*, *Bacillus*, *Providencia*, and *Shewanella,* also protects sponge cells from photodamage (Vijayan et al. 2017). The fact that several non-phylogenetically related bacteria sharing the same ecological niche produce melanins suggests that these pigments have an important role in this ecosystem (Fig. 1).

Melanin can also contribute to defenses against predation (Fig. 1). In *V. cholerae* biofilms, melanin production increases ROS production, protecting the bacteria from amoeba predation (Noorian et al. 2017).

When *Myxococcus xanthus* predates on *S. meliloti*, copper accumulation is observed in the region of contact between these bacteria. This induces copper detoxification mechanisms in the predator, and melanin production in the prey. Mutants of *S. meliloti* unable to produce melanin are more sensitive to predation (Contreras-Moreno et al. 2020).

6 Concluding Remarks

The broad distribution of melanin synthesis among many groups of bacteria and the diverse metabolic pathways that can lead to their synthesis suggest that these compounds have important roles in several aspects of bacterial physiology.

Furthermore, some bacteria can synthesize melanins with different chemical compositions suggesting that the benefits of synthesizing these pigments outweigh the metabolic burden involved in maintaining multiple melanin synthesizing pathways.

The diverse roles already described for melanins involve protection from environmental stress, especially in extreme environments, and affect the relationships between bacteria and those of bacteria with animals and plants. Phylogenetic analysis of melanin synthesis in bacteria reveals that this trait seems to have risen or been acquired independently many times, probably as a characteristic associated with fitness and survival. The growing body of evidence that links melanin formation to a great number of physiological processes suggests that the role of melanin synthesis is diverse and relevant to microorganisms with many different lifestyles.

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The Role of Melanin in Fungal Disease

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1 Fungal Melanin Biosynthesis

Members of the fungal kingdom make many types or classes of melanins. Most fungal species produce melanins of the allomelanin or eumelanin classes, canonically from the polymerization of 1,8-dihydroxynaphthalene (1,8-DHN) and derivatives of L-3,4-dihydroxyphenylalanine (L-DOPA) and other catecholamines, respectively (Eisenman and Casadevall 2012). Other fungal melanin subtypes have been reported, including pyomelanin production from homogentisate (HGA) by *Aspergillus fumigatus* (Schmaler-Ripcke et al. 2009) and GHB-melanin from the precursor glutaminyl-hydroxy-benzene (GHB) by *Agaricus biosporus* (Weijn et al. 2013). The production of a novel 5-deoxybostrycoidin-based melanin in *Fusarium* species (Frandsen et al. 2016), and an aspulvinone E-based melanin (Asp-melanin) by *Aspergillus terreus* (Geib et al. 2016) have been reported. However, further study of these novel compounds is necessary. Here, we describe the reported mechanisms of fungal biosynthesis for the three most prevalent melanin subtypes.

Aspergillus spp. as well as *W. dermatitidis* and *Sporothrix schenckii,* produce allomelanins, also known as DHN melanins (Gow et al. 2017). These melanins are black or brown, are typically attached to the inner side of fungal cell walls (Tran-Ly et al. 2020), and are produced from the polymerization of 1,8-dihydroxynaphthalene (DHN) (Britton 1983). As such, they do not contain nitrogen. DHN is produced from the polyketide pathway, which begins with either Acetyl-CoA or Malonyl-CoA.

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These precursors undergo decarboxylative condensation via polyketide synthase to form 1,3,6,8-tetrahydroxynaphthalene (THN) (Singh et al. 2021). The reduction of THN by THN reductase to form scytalone is followed by two rounds of dehydration to ultimately produce the monomer DHN (Singh et al. 2021). Fungal mutants with mutations of the gene encoding polyketide synthase (pksP or alb1 for "Albino 1") produce albino conidia in the absence of exogenous scytalone (Tanguay et al. 2006). In *Aspergillus fumigatus*, the genes for all six enzymes involved in DHN-melanin synthesis are encoded by a 19 kb gene cluster on the second chromosome (Tsai et al. 1999).

Other fungi, including *Cryptococcus neoformans, Candida auris,* and *Paracoccidioides brasiliensis*, produce nitrogen-containing eumelanin from catecholamine-based derivatives such as L-DOPA (Eisenman et al. 2007; Gómez et al. 2001). Eumelanin, a typically black-brown pigment, is formed by the polymerization of indoles. These indoles are canonically formed through a multi-step biosynthetic pathway, beginning with the oxidization of catecholamines into reactive quinones. These quinones can then cyclize via an intramolecular nucleophilic attack, after which their spontaneous oxidization and tautomerization form the necessary indoles (Eisenman and Casadevall 2012). While this pathway can occur spontaneously through autopolymerization, in most fungal species the first conversion to quinone is catalyzed by laccases (EC 1.10.3.2) or phenol oxidases (EC.1.14.18.1 and EC.1.10.3.1). Further, for fungal species that produce the phenol oxidase tyrosinase, the enzyme can catalyze both the oxidation of catecholamines to quinone as well as an additional precursor step converting L-tyrosine into the catecholamine L-DOPA, allowing for the production of eumelanin from an endogenous amino acid (Smith and Casadevall 2019).

In addition to the biosynthetic L-DOPA pathway originating from L-tyrosine, melanin can also be produced by the oxidation and subsequent polymerization of HGA, an intermediate in the degradation pathway of L-tyrosine. Mutation of the enzyme homogentisate dioxygenase that normally catalyzes the conversion of HGA to maleylacetoacetate in this pathway causes a rare human genetic disorder called alkaptonuria (La Du et al. 1958), characterized by a build-up of HGA and production of alkaptomelanin. Its microbial counterpart, pyomelanin, was first identified in the bacterium, *Pseudomonas aeruginosa* (Yabuuchi and Ohyama 1972), and has been discovered subsequently in several fungal species, such as *Apergillus fumigatus*, *Sporothrix* spp., and *Histoplasma capsulatum* (Schmaler-Ripcke et al. 2009; Almeida-Paes et al. 2012, 2018). For fungal species that produce melanin by multiple pathways, pyomelanin is identified by its unique susceptibility to sulcotrione, a specific inhibitor of 4-hydroxyphenylpyruvic acid dioxygenase (HppD), the enzyme that converts 4-hydroxyphenylpyruvic acid to HGA (Lorquin et al. 2022). At least one instance of direct competition between pathways has been noted wherein pyomelanin synthesis by *Alternaria alternata* supplants the more canonical DHN-melanin pathway through down-regulation of CmrA, the key transcriptional activator of DHN-melanin synthesis genes (Fernandes et al. 2021). The upregulation of HppD that occurs upon the transition from the filamentous to parasitic yeast form of several fungal species including *H. capsulatum,*

Paracoccidioides brasiliensis, and *Talaromyces marneffei* (Nunes et al. 2005; Boyce et al. 2015; Hwang et al. 2003) argues that pyomelanin may play a key role in fungal virulence.

2 Melanin Structure and Localization

The elucidation of melanin's exact chemical structure has been hampered due to the insoluble and heterogeneous character (e.g., variable starting monomers) of this biomaterial (Prota 1988). Any attempt at solubilization disrupts its structure and adds complexity to its structural analysis, the macroscale assembly of melanin is amorphous ("disordered") and thus not approachable with standard methods of structure determination such as X-ray crystallography. It involves supramolecular interactions within melanin and between melanin and other surrounding components. In the past three decades, the use of alternative and non-destructive spectroscopic methodologies (Casadevall et al. 2012; Chatterjee et al. 2012, 2014, 2015; Chrissian et al. 2020a–c; Baker et al. 2021; Camacho et al. 2017) along with high-resolution transmission electron microscopy (TEM) (Eisenman et al. 2005, 2009; Walker et al. 2010; Wolf et al. 2014; Franzen et al. 2008; Alviano et al. 1991; Almeida-Paes et al. 2017; Romero-Martinez et al. 2000; Freitas et al. 2019) and proteomics (Camacho et al. 2019; Almeida-Paes et al. 2020) have provided tremendous insights about the complex hierarchical assembly structure of fungal melanins.

In most fungal species, melanins are mainly deposited in layers within the cell wall displaying variations in their distribution (e.g., in inner or outer regions) (Eisenman et al. 2005; Walker et al. 2010; Franzen et al. 2008; Romero-Martinez et al. 2000; Nosanchuk and Casadevall 2003a; San-Blas et al. 1996; Bayry et al. 2014). However, given that melanin synthesis starts intracellularly, it is also detected in cytoplasmic deposits within membrane-enclosed compartments known as melanosomes as well as along the plasma membrane (Freitas et al. 2019; Camacho et al. 2019; San-Blas et al. 1996). The distribution and maintenance of melanin within the cell wall depend on covalent and non-covalent interactions with other cell wall components such as chitin, chitosan, glucan, and lipids.

The molecular organizational structure of melanins consists of locally-ordered oligomer sheets that form planar stacks with variable stacking distances due to differences in the chemical composition (Büngeler et al. 2017). In agreement with this model, studies using X-ray powder diffraction demonstrated that fungal melanins isolated from *C. neoformans, Wangiella dermatitidis*, *Aspergillus niger,* and *Coprinus comatus* conserved this basic stack sheet structure, with stacking distances between the melanin layers ranging from 3.46 to 4.39 Å, which may serve as a key parameter for further melanin categorization (Casadevall et al. 2012). While the supramolecular "disordered" structure is a consequence of the planar structures exhibiting diverse orientations to one another stabilized by hydrogen bonding, cation-π, and van der Wall interactions (Hong et al. 2018).

The most studied system of fungal melanin supramolecular architecture is that of *C. neoformans,* where complementary approaches have been used to elucidate its

cell wall building block unit (Camacho et al. 2019). That work investigated melanin hierarchical buildup from "melanin ghosts" (melanin carcasses from a hollow cell after acid exposure) and from structures released in the culture supernatant. Two main structures were identified: (1) Melanosomes; and (2) Melanin granules. The **melanosomes**, the structural unit of the cell-wall melanin that corresponds to ~30 nm in diameter smooth nanospheres. These are visualized by TEM within multivesicular bodies (MVBs) and vesicles in the cell cytoplasm or exposed in the cell wall after extended acid-hydrolysis of melanin ghosts. Similar melanosomes in the structure had been reported for other natural eumelanins (Xiao et al. 2018; Franzen et al. 2006). The **melanin granules** are aggregated melanosomes measuring from 40 to 200 nm in diameter, which result from the accumulation and crosslinking of melanosomes among each other and to surrounding non-pigmented components with different degrees of melanization. These are found intracellularly, within the cell wall, or in the extracellular media.

2.1 Cell-Wall Associated Melanin

Melanin granules can be arranged in layers within the cell wall (Chrissian et al. 2020a; Eisenman et al. 2005; Franzen et al. 2008; Romero-Martinez et al. 2000) or clustered on the cell wall surface (Walker et al. 2010; Romero-Martinez et al. 2000; Bayry et al. 2014). In *Cryptococcus* species and *Candida albicans* cell-wall chitin or its deacetylated form, chitosan, plays a key role in the melanin accumulation and distribution within the cell wall (Chrissian et al. 2020a; Camacho et al. 2017; Walker et al. 2010). Disruption of the chitin synthesis in *C. neoformans* (Tsirilakis et al. 2012) results in a leaky-melanin phenotype where melanin is not retained within the cell wall and is released to the extracellular medium. A similar leaky phenotype is also observed upon binding of cell wall dyes that interfere with melanin deposition (Perez-Dulzaides et al. 2018). Aliphatic groups identified as triglycerides (TGs) within fungal melanins are associated with their synthesis within vesicles (Eisenman et al. 2009; Zhong et al. 2008; Rodrigues et al. 2007) and cell-wall remodeling processes during budding (Nosanchuk and Casadevall 2003a). More recently, ss-NMR studies determined that melanized cells of *C. neoformans* were not only associated with TGs but also with sterol esters (SE) and polyisoprenoids. These lipids were also found in non-melanized cells but given that TGs and SEs are the typical cargo of lipid droplets, it may be possible that these organelles are involved in *C. neoformans* melanin synthesis (Chrissian et al. 2020c).

2.2 Secreted Melanin

During fungal growth and cell replication, melanized fungal cells have to remodel their cell wall during budding and morphological transitions such as making hyphae. To allow cellular budding, the local cell-wall remodeling might be driven by

secreted enzymes (peptidase, chitinases, and glucanases) (Geddes et al. 2015) that break melanin linkages to cell-wall components. In *C. neoformans*, using isopycnic gradient sedimentation, detached and secreted melanin granules in the culture supernatant were isolated and analyzed for proteins (Camacho et al. 2019). This study identified four proteins (Qsp1, Cig1, Blp1, and CNAG_05313) that may play important roles in the fungal melanogenesis and adaptation/survival of the fungus inside the host.

3 Role of Fungal Melanin in Human Disease

While mammalian endothermy, among other factors such as advanced immunity, protects against many fungal species, some species have evolved to be pathogenic in humans (Köhler, Hube, et al.). A concern particularly in those who are immunocompromised, the continued evolution of fungal virulence represents a growing threat to global health. While many fungal infections are superficial and mild, some may evolve into severe diseases, especially in the aforementioned immunocompromised hosts. For example, while *Candida albicans* is commonly a harmless colonizer of human mucous membranes, it can lead to fatal systemic candidemia in those with neutropenia. Meanwhile, infections with *Cryptococcus neoformans* or *C. gattii* can cause disseminated cryptococcosis in both healthy and immunocompromised adults, often leading to subacute meningoencephalitis (Köhler, Casadevall, et al.). To be pathogenic in humans, these fungi must evolve to be able to withstand the human febrile temperatures of 38–39 °C and resist the efforts of the immune system targeting fungal cells. Notably, melanin produced by the pathogens *Cryptococcus neoformans* and *Monilina fructicola* has been shown to confer thermotolerance, representing a potential role for fungal melanin in this evasion of mammalian endothermy (Cordero and Casadevall), especially when exogenous melanization substrates are available in the extracellular environment, such as in infection of the substantia nigra. Further work is needed to better elucidate the contributions of melanin's conferral of thermotolerance to the development of fungal pathogenesis; however, other roles for melanin in fungal virulence are well described.

3.1 Cell-Host Interaction

Among the innate immune receptors, host pattern recognition receptors (PRRs) can be divided into two groups: secreted receptors and transmembrane signal-transducing receptors (Mortaz et al. 2017; Brubaker et al. 2015; Latgé 2020). Pathogen-associated molecular patterns (PAMPs) are highly conserved molecular structures found in some pathogenic microorganisms and are known to be critical in

initiating innate immune responses and inducing and directing subsequent adaptive immunity (Latgé 2020; Kurup and Tarleton 2013).

Most host cells express at least one type of cellular PRRs. PRRs can be divided into five different types: Toll-like receptors (TLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), C-type lectin receptors (CLRs), and absent in melanoma-2 (AIM2)-like receptors (ALRs). Among them, TLRs and CLRs have been extensively studied in the context of fungal infection and also play central roles in antifungal immunity (Mortaz et al. 2017; Brubaker et al. 2015).

Several immunologically fungal ligands have been described as PRRs, including melanin 2,3. A C-type lectin receptor, called Melanin sensing C-type Lectin receptor (MelLec/CLEC1A), was shown to play an essential role in antifungal immunity through recognition of the naphthalene-diol unit of 1,8- dihydroxynaphthalene (DHN)-melanin. However, MelLec is not able to bind to DOPA-melanin, produced by other fungal pathogens, including Cryptococcus sp. (Smith and Casadevall 2019; Stappers et al. 2018). In humans, this receptor is expressed by endothelial cells and leukocytes, including monocytes, dendritic cells, and granulocytes, but not by lymphocytes (Sattler et al. 2012). In aspergillosis experimental infection, MelLec was required for early leukocyte recruitment in the lungs (Stappers et al. 2018). In summary, MelLec is a receptor recognizing an immunologically active component commonly found in fungi and plays an essential role in protective antifungal immunity in both mice and humans, showing the importance of fungal melanin as PAMPs and how it can be sensed and recognized by immune host cells, activating the development of the appropriate immune response (Stappers et al. 2018).

3.2 Mechanisms of Resistance to Human Host Immune Factors

3.2.1 Oxidative Stress

Two key features, namely a negative charge and a stable free radical population, are shared among the characteristics of melanins produced by fungi (Smith and Casadevall 2019). These properties confer upon fungal melanins the ability to reduce oxidizing free radicals, highly reactive molecular species with one or more unpaired electrons (Lobo et al. 2010). In the context of fungal infections, free radicals produced by host immune cells during oxidative bursts can be absorbed and neutralized by melanin in the fungal cell wall before they can enter the cell and elicit cytotoxic damage (Nosanchuk and Casadevall 2003b). For example, DOPA-derived eumelanin produced by *Cryptococcus neoformans* imparts a survival advantage to cells treated with oxidative reactants *in vitro* (Jacobson and Tinnell 1993; Wang and Casadevall 1994a) or during phagocytosis by macrophages (Wang et al. 1995). Enhanced survival of melanized cryptococcal cells inside macrophages is expected to contribute to virulence by promoting dissemination from the lungs to the brain

through a Trojan horse mechanism (Liu et al. 2012) and a recent study reporting a survival advantage for melanized compared to non-melanized *C. neoformans* cells in a mouse model of cryptococcal infection supports this hypothesis (Baker and Casadevall 2023). Other forms of melanin have been shown to protect fungal species from the types of free radicals released by host macrophages during phagocytosis, including DHN-melanin in *Sprorothrix schenkii* and *Fonsecaea pedrosoi* (Romero-Martinez et al. 2000; Cunha et al. 2010) and both DHN and pyomelanin in *Aspergillus fumigatus* (Schmaler-Ripcke et al. 2009; Jahn et al. 1997). Thus, melanization is a widespread adaptation that permits prolonged survival of infective fungal cells within host phagolysosomes thereby increasing their propensity to cause disease.

3.2.2 Melanin Interference with Antifungal Drug Activity

Melanin also contributes to fungal virulence through the sequestration of antifungal drugs. Melanization of *C. neoformans* and *H. capsulatum* reduces the potency of the polyene amphotericin B and the echinocandin caspofungin (van Duin et al. 2002). These compounds have also been observed to change the elemental composition of fungal melanin after incubation *in vitro*, suggesting a mechanism of direct binding and sequestration (Nosanchuk and Casadevall 2006). Melanized *P. brasiliensis* cells have also demonstrated reduced susceptibility to amphotericin B, and, in contrast to that observed in *C. neoformans*, also manifested reduced susceptibility to azoles like fluconazole, ketoconazole, itraconazole, and sulfamethoxazole (Gómez et al. 2001). Notably, the direct sequestration of azole drugs by melanin has yet to be demonstrated. However, fungal melanins have been shown to bind to a variety of drug types beyond just the antifungals, with binding efficacies approaching other known absorbers like medicinal activated charcoal (Bridelli et al. 2006).

Further, treatment with the DHN-melanin synthesis inhibitor tricyclazole increased the potency of terbinafine in *Sporothrix brasiliensis* and *Sporothrix schenckii* species (Almeida-Paes et al. 2016). And, interestingly, antifungal drug treatment has also been shown to increase rates of DHN-melanin synthesis in *A. infectoria*, affecting the compound's deposition in the cell wall (Fernandes et al. 2015). Electron micrographs of melanized *C. neoformans* cells have demonstrated melanin deposition in the cell wall, providing support for extracellular drug capture by melanins (Eisenman et al. 2005). Melanization makes the cell wall less porous to amphotericin-containing liposomes suggesting another mechanism by which this pigment can reduce fungal susceptibility to this antifungal drug (Walker et al. 2018). Taken together, the role of melanin in antifungal drug resistance and uptake has been robustly established.

3.2.3 Immune Evasion

The extracellular localization of fungal melanin also contributes to its role in the evasion of host immunosurveillance. Melanin in *Aspergillus fumigatus* has been

shown to mask pathogen-associated molecular patterns (PAMPs) like mannans and β-glucan from the immune recognition, significantly attenuating the observed cytokine response (Liu et al. 2021). Likewise, melanin from *A. nidulans* was shown to have an anti-inflammatory effect, decreasing the production of nitric oxide and TNF-α in stimulated macrophages (Gonçalves et al. 2013). In *C. neoformans,* phagocytosis of melanized cells was observed to be lessened compared to those unable to form melanin (Mednick et al. 2005). In addition, Rosas et al. showed that the injection of *C. neoformans* melanin isolated particles could induce granuloma formation in mice; interestingly, the granulomas and the latent infection commonly associated with them are observed in pathogens capable of melanization (Nosanchuk and Casadevall 2006; Rosas et al. 2002). In addition to the protection conferred by its immunomodulatory and ROS scavenging roles, melanin has also been shown to be protective against enzymatic degradation and secreted antimicrobial peptides like defensins, likely due to its negative charge and promiscuous binding affinity (Rosas and Casadevall 2001). Broadly, melanin knockout has been shown to decrease fungal virulence (McClelland et al. 2006), underscoring its importance in fungal pathogenesis and human disease.

4 Interactions Between Fungal Melanins and Insect Hosts

Insect hosts provide an interesting context for melanized fungi. Insects, like most arthropods, produce their own melanin as an important part of their immune response to microbes, including fungi (González-Santoyo and Córdoba-Aguilar 2012). Insect immune melanization is produced by the oxidation of catecholamines in the hemolymph by activated phenoloxidases, resulting in the formation of DOPA melanins. The melanization reaction is believed to kill the microbes through the oxidative and toxic intermediates produced by the melanization reaction (Zhao et al. 2011). The interactions between fungal melanins and insect melanins have not been extensively studied, but current evidence in the literature indicates that fungal melanins are not advantageous for fungal survival and limit infection within insects. In contrast to mammalian and plant fungal pathogens, many entomopathogenic fungi—or fungi that infect insects—do not produce melanin pigment, including *Metarhizium anisopliae* and *Beauveria bassiana* (Lu et al. 2021; Fang et al. 2010). Since insect melanins are used for immunity and wound healing, fungal melanins may act as damage-associated molecular patterns (DAMPs), which in turn would activate more immune and wound-healing responses as seen in *in vitro* studies investigating melanization of insect hemolymph (Smith et al. 2022).

During infections of *Galleria mellonella* wax moth larvae, pigmentation mutants of *Aspergillus fumigatus* are more virulent than their wild-type melanized counterparts (Jackson et al. 2009). Conversely, melanin-deficient *A. fumigatus* mutants (*alb1Δ)* were less virulent during oral and topical infection of *Drosophila melanogaster* fruit flies deficient in *Toll,* an immune gene responsible for recognizing microbes (Lionakis et al. 2005). While these findings appear contradictory, if the

fungal melanin activates an effective immune response via *Toll* signaling, then the *Toll*-deficient *D. melanogaster* mutants would not reveal an enhanced virulence phenotype of albino fungal mutants. Non-melanin-producing mutants of *Fonseca monophora* are more virulent than the melanized counterparts in *G. mellonella* larvae (Liu et al. 2019). Lastly, cultures from wildtype non-melanized *Cryptococcus neoformans* are also more virulent than the wildtype melanized cultures in *G. mellonella* (Eisenman et al. 2014). The melanized cells induced larger inflammatory nodules, indicating that melanin can activate inflammation and immune reactions in the larvae (Eisenman et al. 2014). These nodules are often sites of the insect's immune melanization reaction and are key in controlling infection (Dubovskiy et al. 2016). Additional evidence shows that the melanin-producing enzyme laccase from *C. neoformans* can activate the insect's melanization response (Smith et al. 2022), although the laccase-null *lac1Δ* mutant is hypovirulent in *G. mellonella* infections, possibly due to other non-fungal melanin related roles (Lu et al. 2021; Mylonakis et al. 2005). On the other hand, a strain of the entomopathogenic fungus *M. anisopliae* that was genetically modified to produce melanin resulted in mutants that had enhanced virulence and enhanced stress tolerance overall in a wide variety of insect pests (Tseng et al. 2011, 2014).

In studies investigating the correlation between virulence factors, fungal properties, and survival of insect hosts, the role of fungal melanin is less clear. Survival of *D. melanogaster* positively correlated to the degree of melanization of *Cryptococcus spp.* isolates, indicating that degree of fungal melanization is maladaptive in the case of infections of insect hosts (Thompson et al. 2014), while fungal melanization from *Cryptococcus gattii* isolates was correlated with increased virulence in *G. mellonella* (Firacative et al. 2014). These studies only provide correlations between virulence and melanization, which may be influenced by many other virulence factors and conditions, including capsule size and fungal growth rate. Additional experiments studying the nuanced and mechanistic interactions between fungal melanin and the insect immune response need to be done.

5 Role of Fungal Melanin in Plant Infections

The melanin in melanized fungi can also play a surprisingly crucial role beyond human and insect hosts. Black fungal pathogens have a significant impact on agriculture globally. One example is the species *Colletotrichum.* This species predominantly causes anthracnose disease, red rot, crown rot, and brown blotch (Cannon et al. 2012). The fungi are so expansive that it affects: papaya, citrus, strawberry, tomato, corn, alfalfa, pepper, legumes, radish, coffee, and sorghum plants to name a few (Dean et al. 2012). The melanin in melanized fungi can also play a surprisingly crucial role beyond human and insect hosts. More specifically, melanized fungi use melanin to create and maintain high turgor pressures in the appressorium while inserting themselves into the plant hosts, absorbing essential

minerals which function as a reservoir for the fungi, and preventing loss of glucose (Nosanchuk and Casadevall 2003b; Butler et al. 2001).

The melanin produced by fungal plant pathogens plays a significant role in the colonization of the plant host. To colonize a plant host, fungi produce appressoria, or tiny hyphal cell formations containing glycerol, which help create enough turgor pressure to penetrate the epidermal cells of plants. Specifically, melanized appressoria are comparatively advantageous to non-melanized appressoria in terms of generating and maintaining sufficient turgor pressure to invade the plant (de Jong et al. 1997). Melanized fungi more effectively prevent diffusion of glycerol which retains a higher turgor pressure necessary for the degradation of the cuticle (de Jong et al. 1997). A notable example of this phenomenon is the fungus *Magnaporthe grisea* which utilizes melanin to invade rice plants and result in rice blast disease (Howard and Valent 1996). When the same *M. grisea* is treated with a tricyclazole, a reagent that prevents the synthesis of melanin, or an albino mutant of *M. grisea* is used, the appressoria are unable to generate sufficient turgor pressure (Howard and Valent 1996).

The role of melanin in melanized fungi is not limited to just host-invasion processes; it can also play a defensive role. Melanin can aid the survival of melanized fungi even while in a dormant state (Butler et al. 2001). Fungi which produce melanized sclerotia, a bundle of hyphae, are far more resistant to chemical attacks (Butler et al. 2001); this is incredibly important for resistance against fungicides as well. For fungi that do not produce sclerotia, melanin still plays a role in protecting the fungi (Butler et al. 2001). This is evident, for example, in mutant versions of fungi *G. Graminis* which are more susceptible to ultraviolet radiation, lytic enzymes, and some antimicrobial agents (Frederick et al. 1999).

Melanotic fungi pose clear offensive and defensive advantages over their non-melanized counterparts. More specifically, melanized fungi are more effectively able to invade plants, maintain nutrients, and protect themselves against an array of chemical, radioactive, and other physical threats.

6 Fungal Melanin as a Target for Antimicrobial Therapies

Fungal melanin is of particular consideration in the development of antimicrobial therapies due to its roles both in fungal virulence and antimicrobial resistance. Melanized fungi can exhibit decreased susceptibility and enhanced resistance to antifungal medications. For example, while amphotericin B (AmB) is often effective against non-melanized *C. neoformans* in vitro (Wang and Casadevall 1994b), it likely acts by causing an increase in ROS (Sangalli-Leite et al. 2011). Due to melanin's antioxidant properties, *C. neoformans* grown with L-Dopa exhibited significantly enhanced survival against AmB at concentrations up to 0.3 μg/ml (Wang and Casadevall 1994b). In vivo melanization of *C. neoformans* may hinder amphotericin B's fungicidal action in clinical settings (Nosanchuk and Casadevall 2006).

Past research has suggested that inhibition of melanization can be an effective infection control strategy. When Alviano et al. collected sera from patients with chromoblastomycosis, purified melanin-binding antibodies were shown to opsonize melanotic *Fonsecaea pedrosoi* conidia in vitro. In addition, treating mice with monoclonal antibodies (mAbs) to melanin significantly improved survival against *C. neoformans* infection (Rosas et al. 2001). In addition, mice administered mAbs experienced significantly less *Cryptococcal* growth both in the lungs and the brain (Rosas et al. 2001).

Glyphosate, a glycine analog, and a component of the herbicide Roundup interfere with the shikimate pathway, which is used by many organisms for the synthesis of aromatic amino acids. Because melanin is synthesized from phenolic compounds, glyphosate can also interfere with melanin synthesis in fungal pathogens such as *C. neoformans*. Nosanchuk et al. demonstrated that mice infected with *C. neoformans* and administered glyphosate displayed prolonged survival and decreased *C. neoformans* melanization (Nosanchuk et al. 2001). On the other hand, in organisms that rely on melanin production for the immune defense such as insects (See Sect. 4), glyphosate increases host susceptibility to microbial infection (Smith et al. 2021). In total, the body of existing research points to a need for further studies into the use of inhibiting melanin synthesis and/or activity for antimicrobial purposes.

7 Concluding Remarks

Melanin is a multifunctional polymer that has varied roles in fungal pathogenesis ranging from interfering with the immune system in animals to promoting turgor pressure for plant-invasive fungi to protecting fungal cells from antifungal drugs. Melanin functions in virulence stand apart from the other mechanisms by which this pigment promotes fungal survival in the environment (Cordero and Casadevall 2017) such as conferring protection against amoeba predators (Steenbergen et al. 2001), ultraviolet light (Wang and Casadevall 1994c) and cellular mechanical strength (Mattoon et al. 2023) and promoting the capture of electromagnetic energy for growth (Dadachova et al. 2007) and thermal regulation (Cordero et al. 2018). Interference with melanization is a potential therapeutic strategy that is currently unexploited in drug development. Despite all we know about this enigmatic polymer there are major unresolved problems in the biology of melanin ranging from uncertainty in its structure to the mechanisms by which it is assembled in the cell wall and rearranged during budding and cellular morphological transitions. The study of melanization and its effects on virulence are exciting and productive frontiers in fungal pathogenesis.

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Melanosome Origins, Diversity and Functional Relevance Across Animals

Liliana D'Alba

1 Melanosome Origins and Development

Melanins characterize a defense system as well as a resistance mechanism to stress in all organisms. Dopaquinone, a highly reactive o-quinone plays an essential role in melanogenesis, the production of both eumelanin and pheomelanin (Ito and Wakamatsu 2008). While some of the reactions involved in melanogenesis are favorable to the organisms, most of the reactions of o-quinones, for example, those including binding to macromolecules like proteins and DNA, are strongly detrimental, including increases in cytotoxicity and carcinogenesis (Ito et al. 2020). Therefore, melanin production commonly occurs in the confined space of membrane-bound organelles called melanosomes, or it is externalized, forming physical barriers or encrustations on targeted surfaces. For example, bacteria produce diffuse melanin in extracellular spaces (Polacheck and Kwon-Chung 1988); fungi produce melanin granules at the surface of the cell wall (Butler and Day 1998) and deposit these granules in the area close to pathogens or intruding bodies. Invertebrate animals also deposit diffuse melanin pigments on their scales (e.g. Papilionid butterflies; Shawkey et al. 2009), exoskeleton (*Aedes aegypti*; Christensen and Forton 1986) and mantle in hydrobiid snails (Kabat and Hershler 1993). Vertebrates compartmentalize the process of melanin synthesis into melanosomes but these have not been found in insects or most other invertebrates. So, where is melanin produced and how is it localized in invertebrates?

The bulk of melanin production in invertebrates is due to the activity of pro-phenoloxidases (PPOs) systems that can be combined with a cellular response involved mostly in immunity, and to a lesser degree, in pigment production. In a few invertebrates including cephalopods (Schraermeyer 1994), some bivalves (e.g. Han

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et al. 2022) and some cnidarian (e.g. *Gornonia ventalina*; Mydlarz et al. 2008), melanogenesis is confined in cells where the pigment is concentrated in membrane-bound melanosome like granules. Of particular interest in the context of the origins of the melanosome are ascidians, because they are the sister taxa of vertebrates (Delsuc et al. 2006). They present an immune system based on circulating immunocytes (phagocytes, cytotoxic granular cells) that produce an amyloid-protein scaffolding to package melanin into granules to fill their cytoplasm (Franchi and Ballarin 2017; Franchi et al. 2019). Subsequently, these melanin-filled cells degranulate and localize melanin where encapsulation is needed. It is important to highlight that across Animalia, the production of melanin seems to be consistently supported and intrinsically linked to the formation of amyloid fibrils (Grimaldi et al. 2012; Franchi et al. 2019).

Insects have been heavily researched in the context of melanogenesis because they have evolved alternative ways to avoid the deleterious effects of toxic by-products from melanogenesis, by strictly limiting melanin distribution and targeting deposition only where it is needed. How the localization of melanization is controlled in those tissues is not fully known but several important discoveries, which deserve further mention, have been made in recent years.

Invertebrates rely exclusively on innate immunity to combat infections (Janeway and Medzhitov 2002; Ratcliffe and Whitten 2004). Thus, their immune defense is mainly based on cytotoxic effector responses, including phagocytosis, antimicrobial peptides and the synthesis of extracellular matrix and reactive intermediates of oxygen (ROI) and nitrogen (RNI), (Beutler 2004). More specifically, the production of melanin in insects contributes to the formation of cytotoxic molecules that interact with ROI and RNI to provide an efficient immune response (Nappi and Christensen 2005). Consequently, the typical demonstration of defensive responses against pathogens in insects is melanization.

Production and localization of melanin in insects involves the recruitment of phenoloxidases (POs) to initiate the biogenesis of melanin. Hemocytes, the blood cells of insects, are essential for melanogenesis and act as the main source and carriers of PPOs. The hemocytes containing PPOs break to deposit them at specific locations, for example, around wounds in the cuticle or infected areas. POs in the hemolymph enzymatically convert tyrosine into DOPAquinone (via hydroxylation followed by oxidation). DOPAquinone autocatalyse to form DOPAchrome and finally, an isomerase converts it into DHI, which polymerizes to form eumelanin (Whitten and Coates 2017).

Perhaps through a highly conserved mechanism (Grimaldi et al. 2012), both systems of melanin containment in vertebrates and invertebrates involve the production of amyloid proteins (protein fibrils configured into cross-β sheet quaternary structures), which act as scaffolds to direct and enhance the rate of melanin accumulation (Fig. 1). In vertebrates, PMEL17, a pre-melanosomal protein specific to pigment cells, forms amyloid fibrils in the luminal space of melanosomes. In insects, amyloid proteins were first discovered in 2012 (Falabella et al. 2012), forming bundles of fibrils surrounding the nuclei of hemocytes. Other studies since then have shown that, indeed, amyloids in insects provide an anchoring mechanism for

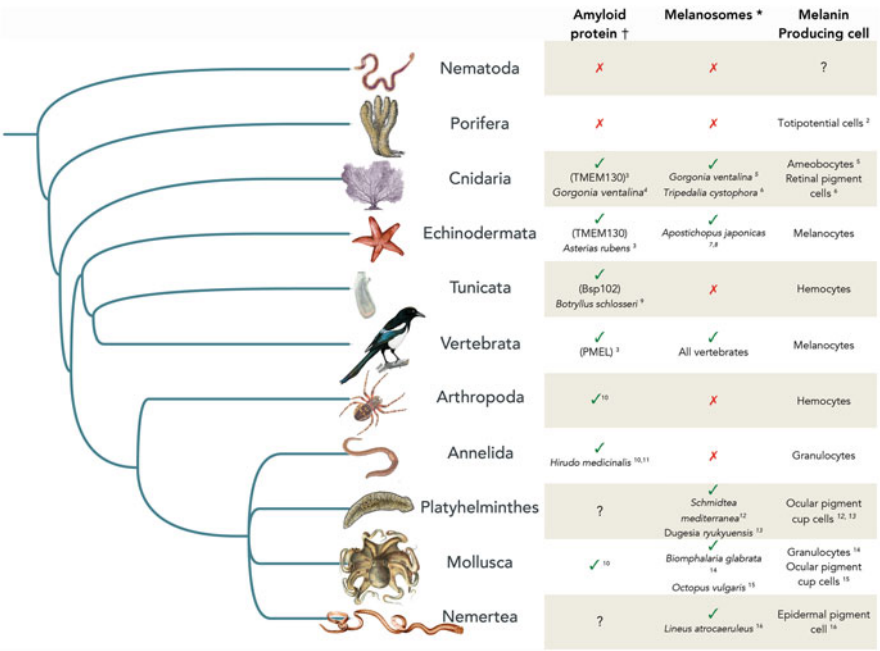

Fig. 1 Simplified phylogenetic tree of Animalia showing, for each major phyla, the correspondence between the production of amyloid proteins (when associated with melanin production) and the occurrence of melanosomes (reported in the literature as membrane-bound granules). References cited are summarized: (1) Freitas et al. (2019); (2) Araujo et al. (2012); (3) Chrystal et al. (2021); (4) Petes et al. (2003); (5) Mydlarz et al. (2008); (6) Kozmik et al. (2008); (7) Zhao et al. (2012); (8) Xing et al. (2017); (9) Ballarin et al. (2018); (10) Grimaldi et al. (2012); (11) Girardello et al. (2015); (12) Lambrus et al. (2015); (13) Hase et al. (2006); (14) Matricon-Gondran and Letocart (1999); (15) Rogers et al. (2019); (16) Moretto et al. (1988); (17) Pascale et al. (2014)

melanin and allow attachment of hemocytes to encapsulate pathogens (Pascale et al. 2014; Di Lelio et al. 2014).

In vertebrate skin, melanin is synthesized in specialized cells called melanophores (or melanocytes in humans; Ratcliffe and Gagen 1977), specifically inside melanosomes, which are present in skin, eyes and internal tissues. Melanophores are the most common type of chromatophores, responsible for the black, brown and brownish red coloration of integuments and their derivatives. Melanosomes are derived from early endosomal membranes and have become specialized to synthesize and store melanin. As a generality, melanin is composed of 30–50 nm nanoparticles, in which melanin monomers are cross-linked together and form stacking structures by Π-Π interactions (noncovalent interactions between the Π-bonds of aromatic rings. Melanosomes within melanophores often vary in size, shape and composition depending on the types of melanin they contain. Eumelanosomes (containing predominantly eumelanin, but see below) tend to be ellipsoidal and larger compared to pheomelanosomes, which are generally spherical but also more irregularly shaped

(Liu et al. 2005). It was hypothesized that the irregular shape of pheomelanosomes is due to the absence of amyloid protein scaffolds (PMEL17; Moyer 1966; Jimbow et al. 1983), which anchor the deposition of melanin in eumelanosomes. However, a few recent discoveries call the notion of a total absence of amyloid proteins in pheomelanosomes into question. First, it has been shown that PMEL17 protein is readily available in membranes and cytosol of epithelial cells, even in unpigmented cells, and that the high reactivity of the thiol group of cysteine residues in the PMEL17 molecule promotes bonding with melanin precursors (Ito et al. 2020). Second, across Animalia, from cnidarians, annelids, mollusks and insects, amyloid protein fibrils template and accelerate the production of melanins (Fig. 1), bind to reactive melanin precursors to prevent their harmful diffusion into the cytoplasm of hemocytes and are crucial in driving melanin close to intruders and pathogens in a process that seems to be evolutionarily conserved (Grimaldi et al. 2012). Third, using solid-state NMR spectroscopy, Thureau et al. (2012) showed that, as in eumelanosomes, a proteinaceous matrix is also present as a substrate for melanin polymerization within pheomelanosomes (from red human hair) but this matrix is irregular and defined by the configuration of the intraluminal globular vesicles of the premelanosome (see below; Fig. 2).

1.1 Non-integumentary Melanosomes

Besides their important roles in the integument, melanosomes also occur in animal eyes. Melanins and ommochromes protect the photoreceptor cells of the eye from prooxidant molecules both by neutralizing free radical products and by screening or filtering unwanted light. All vertebrates studied so far use eumelanin as their screening pigment, which is always contained in melanosomes (Ostrovsky et al. 2018) of diverse morphologies (Liu et al. 2005) and organized into distinct layers within the retinal pigment epithelium, basal to the rod and cone photoreceptors. In invertebrates, however, there seem to be mixed reports about the presence of granules containing melanin. Most invertebrates are assumed to use ommochromes, and to some extent, pterins and carotenoids to shield their photoreceptors and filter light (Vopalensky and Kozmik 2009). Notoriously, melanosomes are found in the inverse cup eye cells of platyhelminthes (*Schmidtea mediterranea*; Lambrus et al. 2015, *Dugesia ryukyurnsis;* Hase et al. 2006), photoreceptor cells in cnidarians (Tripedalia cystophora; Kozmik et al. 2008), retinal pigment epithelium, iridial and scleral cells in cephalopods (Rogers et al. 2019), shell eyes of polyplacophora (*Acanthopleura granulata*; Speiser et al. 2014) and the compound eyes of some arthropods, including fossil specimens (e.g. crane-fly; *Diptera*; Lindgren et al. 2019). In contrast to the large variation of melanosome shape in vertebrate eyes, invertebrate ocular melanosomes seem restricted to small ovoid morphologies (Clements et al. 2016). Importantly, the precise chemistry of invertebrate melanin granules remains poorly studied.

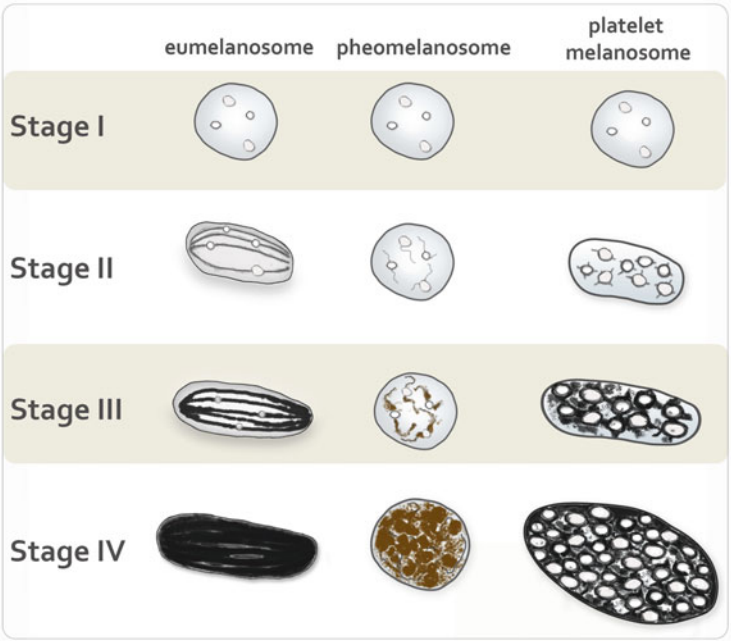

Fig. 2 Stages of development of eumelanosomes, pheomelanosomes and bird melanosome platelets. All melanosome types are thought to originate from multivesicular endosomes, which contain numerous ILVs. Stage I and II precede the production and deposition of melanin and are characterized by the formation of protein scaffolds, which guide de deposition of melanin. In pheomelanosomes and platelets the protein scaffold does not seem to form regular sheets as in eumelanosomes. In bird platelets the protein fibrils are hypothesized to form around the ILVs. Stage III is evidenced by the production of melanin. Mature melanosomes increase in size and asopt different shapes towards Stage IV. Complete melanization happens in Stage IV and in most cases also the occlusion of the ILVs

Melanosomes are also present in internal organs of vertebrates, including the heart, lungs liver, kidneys and spleen. These melanosomes contain mixed concentrations of eu- and pheomelanin and show large variations in shape and size, but this morphological diversity is not associated with their chemical composition (Rossi et al. 2019). The function of melanin within internal organs is hypothesized to include the detoxification, antioxidation and immune defense of cells in these tissues (Rossi et al. 2019). The concentrations of free radicals (e.g. O_2^-) inside cells are constantly maintained at low levels by the antioxidant action of catalytic enzymes that require metals like Cu, Zn and Mn to function (Geremia et al. 1989). Across vertebrates, there seems to be a negative correlation between melanin content in the liver and the activity of these enzymes, particularly that of superoxide dismutase (SOD), suggesting that melanin might be the main scavenger of singlet oxygen ($^1O_2$) in the melanized organs.

1.2 Melanosome Biogenesis

Melanosomes containing exclusively eumelanin or pheomelanin are rare, exceptions being the melanosomes of the RPE (Peles et al. 2010). It even has been argued that pure pheomelanin does not occur in nature (Simon and Peles 2010). In most cases, melanosomes contain co-polymers and mixed amounts of the two melanin types (Ito and Wakamatsu 2003; Liu et al. 2005) in a configuration that follows a "casing model" (pheomelanin core encased by eumelanin; Agrup et al. 1981). Nevertheless, in an initial stage, all melanosomes contain tyrosinase, the enzyme that converts L-tyrosine to L-dopaquinone (DQ). After this first step, two different processes can occur. In eumelanogenesis, dopachrome, a result of the oxidization of cyclodopa, accumulates preferentially and is converted to DHI and DHICA and subsequently oxidates to form eumelanin. Only if a sufficient concentration of L-cysteine (Cys) is present within melanosomes, a redox exchange with DQ occurs to eventually produce pheomelanin (Ito et al. 2020). Only in eumelanosomes, two membrane-bound enzymes, tyrosinase-related protein-1 and -2 (TRP-1 and TRP-2), form a multi-enzyme complex and help stabilize tyrosinase (Kobayashi et al. 1998). The production of eumelanin or pheomelanin is then modulated by the opposing action of alpha-melanocyte stimulating hormone (MSH) and agouti protein. A shift between eumelanin and pheomelanin production can occur rapidly through changes in the pH inside melanosomes, where eumelanin is produced around neutral pHs but the production of pheomelanin proceeds faster and even suppresses eumelanogenesis at acidic pHs (Wakamatsu et al. 2017).

Based on observations of mammalian melanocytes, melanosomes are formed through a series of well-defined stages starting with the fusion between an endosome and intraluminal vesicles (ILVs) from the transcisternae of the Golgi apparatus to form a multivesicular body (MVB) (Hurbain et al. 2008) (Fig. 2). The first stage of melanosomes (I) begins with an MVB that lacks pigment and is characterized by incipient proteinaceous fibrils present in the organelle's lumen. In melanosomes containing predominantly eumelanin, fibrils are completely formed in stage II and the melanosome adopts an ellipsoidal shape. The protein PMEL17 (also known as gp100) is exclusively synthesized by melanocytes and represents the main structural constituent of fibrils (Raposo and Marks 2007). PMEL17 expression is strongly downregulated in cells synthesizing pheomelanin by agouti signaling (Furumura et al. 1998; Kobayashi et al. 1994) but a proteinaceous matrix is still found to be associated with the synthesis of melanin in pheomelanosomes (Thureau et al. 2012) (Fig. 2). PMEL17 has been used as a tracker of transferred melanin into keratinocytes (Singh et al. 2008).

In stages III and IV electron-dense melanin is synthesized and progressively deposited on the fibrils until the internal structure of the melanosome is completely obscured at the end of stage IV. Human pheomelanosomes seem to always be spherical throughout development and contain only granular melanin and amorphous proteinaceous material that does not form sheets (Jimbow et al. 1983). In derived melanosomes in birds (e.g. the hollow platelets in hummingbird feathers),

the ILVs seem to be retained and even increase in number throughout melanosome maturation, and do not become occluded by melanin deposition, giving the melanosome their distinctive porous appearance (D'Alba et al. 2021). Noticeably, a clear fibrillar proteinaceous structure was not observed in the early stages. Instead, melanin seems to be heavily deposited around the vesicles until all the intraluminal space of the melanosomes becomes filled, suggesting that, if present, the scaffolding protein could be seeded around the ILVs. The platelet melanosomes in hummingbird feathers dramatically increase in size, first elongating but then widening until they adopt their characteristic disc shape (D'Alba et al. 2021) (Fig. 2). The precise biogenetic path resulting in the formation of melanosomes is still a topic of active research and much information is needed particularly about pheomelanosomes and non-mammalian systems.

From epidermal melanocytes, stage IV melanosomes are then transported to keratinocytes through four different hypothesized processes (reviewed in D'Alba and Shawkey 2019). The vast majority of research in the field of melanin transfer has been done in mammals with a few exceptions on amphibians (Aspengren et al. 2006) and birds (Durrer and Villiger 1967). The most supported model of melanin transfer to keratinocytes in mammal skin is the exocytosis model, where melanin units, free of their melanosomal membranes are released into the extracellular space between cells and then internalized by keratinocytes through phagocytosis (Wu and Hammer 2014). Alternatively, the vesicle transfer model (Wu et al. 2012) has been supported by studies of melanogenesis in developing feathers (Durrer and Villiger 1967; Shawkey et al. 2015).

2 Melanosome Diversity Across Animal Taxa

2.1 Evolution of Melanosome Shape

In general, vertebrate melanosomes tend to have a globular or ovoid shape, about 600 nm in diameter, and seem to be very conserved across vertebrates (Li et al. 2014). It is only in mammals and birds where a much larger diversity of melanosome morphologies has been consistently observed (Li et al. 2014; Eliason and Clarke 2018). In these two groups, melanosomes that mostly contain eumelanin are oblong, with a length of about a micrometer, while melanosomes containing mostly pheomelanin are spheroidal and about 500 nm in diameter (Fig. 3).

However, the precise mechanism behind the correlation between melanin chemistry and melanosome morphology still needs to be clarified. For example, very little is known about the distribution of pheomelanin across other metazoans and whether melanosome morphology conveys information about eumelanin/phomelanin content. Phaeomelanin has so far been chemically identified in insects (Galván et al. 2015; García et al. 2016), chiton molluscs (Speiser et al. 2014), frogs (Wolnicka-Glubisz et al. 2012), lizards (Megía-Palma et al. 2018) and tortoises (Roulin et al. 2013); none of which display large morphological diversity in melanin packaging.

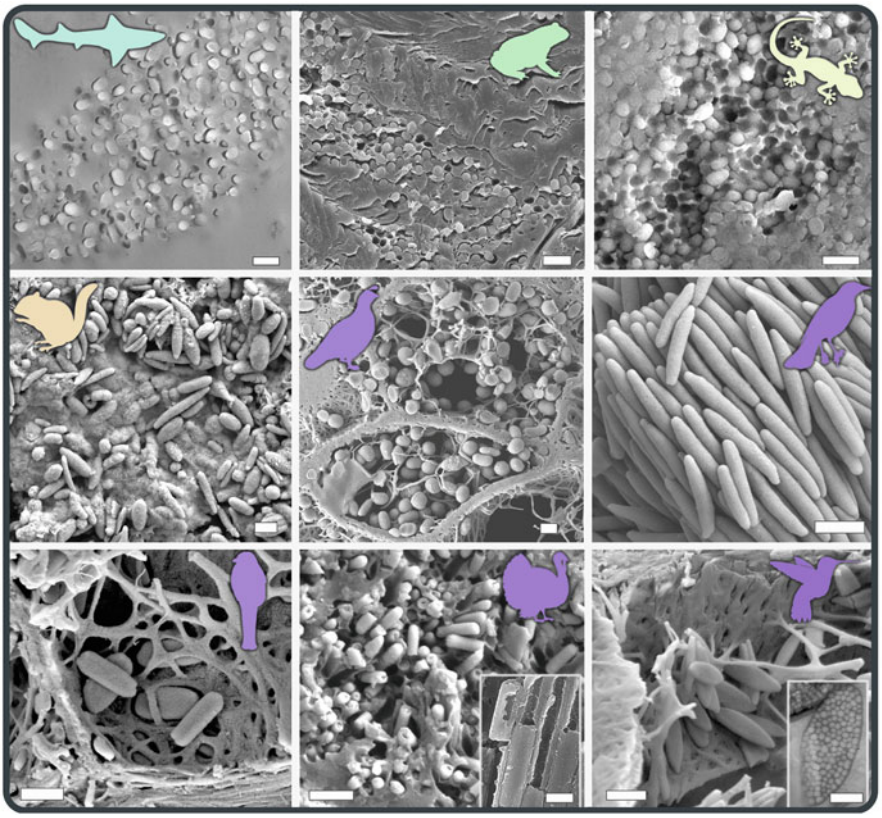

Fig. 3 Diversity of melanosome shapes in vertebrates. Melanosomes are globular and lack morphological diversity in fish, amphibians and reptiles. In mammals and birds (purple silhouettes) melanosomes display a greater diversity that ranges from spherical, to rod-like. The most derived melanosomes are found in birds, where cylinders, platelets and disc-shape melanosomes, solid and hollow, can be observed. (**a**) Fish: *Squalus acanthias*; (**b**) frog: *Bufo bufo*; (**c**) reptile: *Gekko gecko*; (**d**)mammal: *Tamias striatus*; (**e**) *Callipepla californica*; (**f**): *Agelaius phoeniceus*; (**g**) *Priotelus temnurus*; (**h**) *Meleagris gallopavo*; (**i**) *Melanotrochillus fuscus*. Scale bars: 1 μm, insets in (**h**) and (**i**): 500 nm

The presence of pheomelanin outside vertebrates also suggests a more extensive and earlier origin than previously anticipated.

A unique set of melanosome shapes that includes spheres, hollow cylinders, platelets and porous platelets has been discovered exclusively in bird feathers, with no equivalents anywhere else in metazoans (Fig. 3); these derived melanosomes are closely related to color production mechanisms in feathers. How melanosomes develop these geometries is as yet unclear. Some evidence suggests that melanosomes become hollow after their deposition in the developing feather cells (e.g. in *Meleagris gallopavo* Shawkey et al. 2015). The cores of melanosomes within the melanocyte seem to degenerate gradually and become hollow while in transit to the

developing barbule cell, perhaps after the pheomelanin core is lost during transport (Shawkey et al. 2015). This hypothesized process would agree with the eumelanin casing model of melanosomes (Agrup et al. 1981). However, the reason for the stability of melanosome cores in most (solid) melanosomes but not in a few types of feathers is unclear, and only using advanced techniques to verify the internal chemistry of developing hollow melanosomes would allow us to move towards clarification. Alternatively, holes within melanosomes could stem from the intraluminal vesicles already present in premelanosomes (see above), which could increase in size or fuse together during development as was suggested for hollow platelet melanosomes (D'Alba et al. 2021).

Recently, the presence of diverse melanosome geometries was reported in a tapejarid pterosaur (Cincotta et al. 2022). Melanosome shape was specific to the tissue in which melanosomes were embedded and interestingly elongate and ovoid melanosomes were present in the filamentous and branched feathers (integumentary appendages) while spheroidal melanosomes were found in the skin. The discovery of cylindrical melanosomes in pterosaurs and their similarity with melanosome morphology in birds and other theropod dinosaurs could indicate that either, the genes controlling melanosome formation and their phenotypes are ancestral to avemetatarsalians (i.e. clade that includes all dinosaurs and pterosaurs) or that they evolved separately (Cincotta et al. 2022). Parsimony suggests the former scenario is more likely.

So, what drove the evolution of animal melanosomes? Given that melanogenesis is a crucial component of immunity in invertebrates (Grimaldi et al. 2012) and in vertebrates melanin and immunity are tightly bound through the melanocortin pathway (Ducrest et al. 2008), it is reasonable to hypothesize that simple melanosomes could be present in organisms since the origin of the immune system. Later in animal evolution, the need for specialized pigmentation could have resulted in novel melanosome morphologies in two vertebrate lineages, bird and mammals. However, our estimation of the time of melanosome origins is limited to their availability in the fossil record. At present, the oldest known fossil-bearing melanosomes is the bilaterian *Tullimonstrum*, which only dates back to the Carboniferous (307 Ma; Clements et al. 2016). Older specimens may reveal an earlier origin of melanosomes.

3 Functional Relevance of Melanosome Diversity

3.1 General Properties of Melanin and Melanosomes

A general function of melanin across organisms is the protection against diverse forms of stress imposed on cells and tissues. Melanin interacts with environmental radiation and pollution causing structural changes to its molecules, which are not fully understood yet but that determine the ability to protect biological tissues. Melanins absorb light in the UV, visible and near-infrared regions (to at least 1300 nm; Wolbarsht et al. 1981; Medenhall et al. 2015) of the electromagnetic

spectrum and protect their embedding tissues by scattering or dissipating light by converting it into heat (Wolbarsht et al. 1981; Zonios et al. 2008; Bustamante et al. 1993; Hennessy et al. 2005). Melanin, behaving as a semiconductor (Prota 1988) is capable of transferring almost 90% of UV radiation to heat within a nanosecond (Mostert et al. 2012). Thus, exposure to UV radiation promotes melanogenesis in vertebrate integument, leading to the production of more melanin to protect the skin from UV damage.

Ionizing radiation, however, induces a transformation of melanin to an excited state. Excited melanin molecules transfer energy to molecular oxygen ($^3O_2$) forming singlet oxygen ($^1O_2$). UVB and UVA also produce an array of radical species including hydroxyl and hydrogen radicals, which can induce DNA damage. Ionized pheomelanin generates reactive oxygen species making it a potentially damaging molecule (Chiarelli-Neto et al. 2011).

Another very important chemical property of natural melanin is its ability to extract metals from the environment and chelate them to coordination sites within its polymeric structure. Melanin is negatively charged and readily binds to diverse metal ions through the formation of ionic and charge transfer complexes; specifically, metal ions bind to the o-semiquinone radical centers within melanin polymers (Felix et al. 1978). Thus, melanin's affinity for metals leads to the accumulation of several metal ions in melanized tissues including the internal organs of many vertebrates (Rossi et al. 2019). Melanosomes sequester bio-relevant metals (Na, Ca, Cu, Fe, Zn) as well as more toxic ones (Hg, Pb, Cr, Mn). Sepia melanin granules contain Mg, Ca, Na and K (Liu et al. 2014) and can store up to 8% Fe^{3+} by weight (Simon et al. 2006). Melanosomes have been reported to play an important role in calcium homeostasis in vertebrate cells (Bush and Simon 2007; Hoogduijn et al. 2003) and have been proposed as a mechanism of metal detoxification in fish (Cooper and Midling 2007), birds (Chatelain et al. 2014) reptiles (Martin et al. 2022) and mammals (Nicolaus 2005).

Nevertheless, the mechanism of this protective role is still poorly understood. For instance, it is not known how the metal ion transport and storage inside the melanosome take place. It has been shown that the metal ions first need to bind to the melanosome surface but they are not stored there (Simon et al. 2008). Similarly, how does the integrity of a melanosome change upon metal chelation? Or, are some metals more prone to degrading the polycatechol structure than others? Techniques that retrieve *in situ* magnetic and spectroscopic information are currently showing promising opportunities to track changes in melanosome structure, metal coordination and oxidation state under radical stress.

The cross-linked network of melanin inside melanosomes and proteins, as well as their binding to metals, is also behind the enhancement of the mechanical strength of numerous types of tissues. In birds, melanin strengthens feathers (Bonser 1995; Burtt 1986) and increases resistance to bacterial degradation (Goldstein et al. 2004). Melanized tissue help resists mechanical damage in insect exoskeleton, and *Glycera* marine worms use melanin to strengthen their jaws (Moses et al. 2006).

Melanosome properties have been interpreted almost exclusively in terms of the properties of the melanin pigment they contain. Nevertheless, melanosomes contain

many types of proteins, enzymes and lipids which most certainly influence their behavior, the interactions between these organelles and other molecules, and ultimately their function. For example, proteomic analyses of melanosomes have revealed that nearly 1500 proteins are involved in the formation of different types of melanosomes (Chi et al. 2006). Similarly, an analysis of different types of ocular melanosomes showed that the content and composition of lipids vary greatly with melanosome type (Simon et al. 2006). Accordingly, the surface properties of melanosomes will vary with their composition and given that it is on the surface where melanin interacts with molecules inside cells, the presence of proteins and lipids is likely an important determinant of melanosomes' ability to chelate metals or absorb or reflect radiation.

These studies show that melanosomes show great complexity of molecular composition and that a complete understanding of the structure and biological functions of melanosomes can only be achieved by considering this mixed chemistry into account.

3.2 Optical Properties and Color Production by Melanosomes

Melanosomes are responsible for important variations in animal coloration, which play many essential roles in camouflage and communication. Due to its dense, cross-linked polymeric structure melanin possesses two unique optical properties, high refractive index (RI) and broadband absorption (Chen et al. 2014) which form the basis for the production of structural coloration, photoprotection, and thermal absorption in many biological systems. However, direct measurements of RI assume translucence of the material being measured, therefore, researchers have primarily used indirect methods to measure RI of melanin. The RI of melanin from damselfly wings and bird feathers was first estimated using polarizing interference microscopy and optical modeling (Stavenga et al. 2012, 2015). In those studies, the RI was calculated to be ~1.7–1.8. More recently, Xiao et al. (2015) measured the RI of aqueous synthetic melanin and corroborated these values. Nevertheless, optical modeling assuming an RI of 2.0 has produced an excellent agreement between theoretical and empirical reflectance spectra (e.g. Eliason and Shawkey 2012; Xiao et al. 2014), therefore more direct measurements of diverse natural melanins are further needed.

Melanosomes can attenuate the incident light through absorption and scattering. Specific melanosome morphologies likely determine the extent to which they absorb or reflect light. Researchers have been able to determine the optical properties of single melanosomes using both indirect (pulsed laser disruption; Jacques et al. 1996) and direct methods (photoemission electron microscopy; Peles et al. 2010; Peles and Simon 2011). These studies have provided valuable insight into the photoabsorption behavior of melanosomes. For example, we now know that melanosomes from different types of tissues (e.g. skin, choroid, RPE, internal organs) and embryonic origin show different absorption coefficients, most likely explained by differences in

their melanin content (Jacques et al. 1996), chemical composition and/or structural configuration (Peles et al. 2010).

Eumelanin is built from varying amounts of two building monomers, 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) while pheomelanin is derived from isomers of cysteinyl dopa. Correspondingly, the photoabsorption coefficient of melanosomes increases with larger DHICA content relative to DHI (Peles and Simon 2011) and decreases with larger ratios of pheomelanin to eumelanin (Peles and Simon 2010). Melanosomes are mostly comprised of melanins but they also contain ions, proteins, and lipids. From these components, some amino acids significantly absorb in the UV portion and therefore they could contribute to the absorption properties of melanosomes, nevertheless, this still awaits further examination.

One limitation of the aforementioned studies is that the optical properties have been measured mostly within the UV range (240–400 nm) and in some visible wavelengths (500–600 nm). The main focus so far has been to reveal functional differences in photoprotection provided by melanosomes in the context of melanoma and skin cancer. Thus, we still do not know much about the variation in optical properties of different types of melanosomes from the perspective of color production or thermoregulation.

Even though melanin is a dark pigment and it is responsible for colors ranging from black to greys and browns, some of the brightest colors in nature are produced by nanoscale arrangements of melanosomes (Maia et al. 2013; Eliason et al. 2013). Further, melanosomes vary greatly in shape and size, and this diversity is particularly large in birds (see above; Fig. 3) where we also see some of the best examples of brilliant iridescence. This is due to its strong absorption and high RI that can sharply contrast with those of other materials with lower RI such as feather keratins (~1.56) and air (1.0). Consequently, when melanosomes are spatially organized with keratins or air (as in hollow melanosomes), they can produce bright iridescent colors. Without spatial organization, these melanosomes produce typical dark melanin-based colors. However, the precise mechanism of color production by melanosomes is still not fully understood. Some studies have shown that melanin enhances the color saturation and or purity by absorbing incoherently scattered light (Shawkey and Hill 2006; Xiao et al. 2017). Experimental work on synthetic melanin particles demonstrated that coloration can be adjusted either by varying the spacing between the melanosomes (Xiao et al. 2017) or the degree of layering and separation between melanin and keratin.

In recent years a basic separation of the types of iridescent colors produced by melanosomes has been proposed. "Thick films" have a single layer of melanin granules below a single superficial keratin layer (Brink and van der Berg 2004; Doucet 2006); here, the layer of melanosomes apparently serves primarily to define the thickness of the superficial keratin layer (Brink and van der Berg 2004). An example of this type of coloration is found in the coppery-purple iridescence of the dark plumage of the hadeda ibis, *Bosthrychia hagedash* (Brink and van der Berg 2004), which is produced primarily by coherent scattering from a thick (~0.8 μm) and uniform keratin cortex. Conversely, "Thin films" are formed by multiple layers

of melanin granules and keratin (Durrer 1986; Prum 2006). In some cases, a single continuous layer of melanosomes under a thin keratin cortex is enough to produce intense reflectance peaks and iridescent effects (e.g. European and Cape starlings; Freyer et al. 2021). In other cases, brilliantly-colored iridescent feathers are produced by coherent light scattering from multiple (~20), alternating layers of keratin and air-filled, disk-shaped melanin granules (e.g. hummingbirds; Family Trochilidae, Greenewalt et al. 1960; Eliason et al. 2020).

Melanosomes may also be arranged two-dimensionally, for example, in the hexagonal close-packed configuration that confers diverse iridescent colors to dabbling duck wing patches (Eliason and Shawkey 2012). The authors in that study showed that small changes in melanosome diameter and spacing can cause strong shifts in hue, but these parameters and thus the resulting colors are limited relative to what could be theoretically possible, in other words, structural colors in birds have not occupied the full extent of the potential avian colorspace. This could perhaps be due to energetic constraints during development, like nutritional or environmental stress. Optical nanostructures like the ones formed by melanosomes enable the production of colors otherwise hard to produce, for example, green and blue colors that require rare pigments (Durrer 1986). Accordingly, the functions facilitated by these colors such as camouflage may have enabled the colonization of new niches or the production of novel signals for sexual selection (Maia et al. 2013).

Hollow melanosomes introduce an additional low refractive index material (air) into organized nanostructures and thereby produce even brighter colors (Eliason et al. 2013). These types of melanosomes have independently evolved numerous times in some lineages with bright colors (e.g. turkeys, hummingbirds, African starlings) and have recently been shown to accelerate both the rate of color evolution and speciation in lineages that have them (Maia et al. 2013; Beltrán et al. 2021). The ability of melanosome arrays to selectively interact with wavelengths of light with high precision, and to be "tuned" to specific wavelengths through slight changes in dimensionality makes them ideal models for bioinspired optical devices (Parker and Townley 2007).

In some animals like cephalopods and reptiles, melanosomes are also involved in producing highly dynamic and fast-changing colorations. Controlling melanosome density and distribution in their integument can switch from dull or obscure colors to vibrant and intense hues (Sköld et al. 2013). In other organisms, specific arrangements of melanosomes can lead to enhanced antireflection. For example, deep-sea fishes have evolved an efficient antireflective skin, which they use to improve camouflage and being undetected in dark deep waters. These fish show a simple arrangement of close-packed, small melanosomes with a size and shape optimized to produce the lowest reflectance. These packed melanosomes scatter light and increase the optical path length and absorption by melanin (Davis et al. 2020).

3.3 Thermal Properties of Melanized Tissue

Melanin-based coloration can determine the thermal properties of the vertebrate integument. Coloration strongly depends on the concentration of pigments deposited in animal tissues and pigments selectively absorb particular wavelengths of visible light. Variation in reflectance and absorption of light affect the heating of the skin surface (Wolf and Walsberg 2000; Rogalla et al. 2021). When a body surface is heated by the sun under cold conditions, the temperature gradient between the surface and the interior is reduced, resulting in a decrease in heat loss to the environment (Lustick et al. 1970; Lustick 1969; Heppner 1970; Cowles 1967). However, when ambient temperatures are high and skin surface temperature exceeds body temperature due to solar heating, animals gain excessive heat.

Structurally iridescent feathers are less reflective than pigmentary-colored feathers and heat up more under exposure to solar radiation (Rogalla et al. 2021). Iridescent feathers contain large concentrations of melanosomes, often higher than black feathers, and these melanosomes are aligned forming multiple layers inside feather barbules. The organized arrangement and large concentration of melanosomes in iridescent feathers (Maia et al. 2013) may lower the reflectance in the NIR compared to non-iridescent feathers, (Shawkey et al. 2017). As a result, these arrangements could enhance photothermal absorption and result in higher surface temperatures. Both, experimental and theoretical studies on the thermal effects of melanosome properties in feathers have shown that in addition to melanin concentration, melanosome shape can have substantial effects on photothermal absorption within tissues. Optical simulations have estimated that melanosomes with greater aspect rations (i.e. flat and long platelets) could have higher photothermal absorption compared to oblong or spherical melanosomes, regardless of the arrangement in which they exist (organized or random arrangements of melanosomes). Therefore, tissues containing these platelet melanosomes are expected to heat up more than those containing rods or spherical melanosomes (Rogalla et al. 2021). Similarly, experiments using synthetic particles have demonstrated that hollow melanosomes exhibit enhanced absorption as hollow structures allow the transmission of light inside the air gaps (Wang and Guo 2017). This enhanced absorption suggests a potential enhanced photothermal efficiency of tissues with hollow melanosomes but future investigations using natural melanosomes will be needed in order to test this hypothesis.

4 Novel Techniques in the Study of Melanosomes

Identifying patterns in melanin composition of differently-colored tissues has been the goal of much research in recent decades and could give great insights into the relationship between melanin chemistry and color, as well as inform about the links between melanosome chemistry and the material's biomechanical properties.

Unfortunately, our knowledge of the chemistry of melanosomes remains limited and is primarily based on samples from human hair and squid ink sacs.

Different techniques can reveal different aspects of melanin chemistry, and a coordinated multi-pronged approach most likely will succeed where individual techniques have not. Some of the techniques so far used include nuclear magnetic resonance (NMR; solution and solid-state), Raman (molecule level) and MALDI (polymer level) spectroscopy, and ToF-SIMS to identify the fingerprint of the melanin at different levels of biological scale. X-ray photoelectron spectroscopy (XPS) analyzes the surface composition of melanosomes and provides high-resolution elemental data of the top ~100 nm of a sample, which would be perfect to determine the composition of the membrane that envelops melanosomes.

Pheomelanin has still not been well defined, either regarding its spectral or other physical characteristics. Among the techniques that have been applied to the structural and functional characterization of pheomelanin are gas chromatography-mass spectrometry (GC-MS) and ultrafast absorption spectroscopy. In the last decade, solid-state NMR spectroscopy has shown great potential to determine the structure of many biomaterials, but it has only been started to be used for the characterization of melanins, (Tian et al. 2003; Chatterjee et al. 2014; Thureau et al. 2012). 1D and 2D solid-state NMR techniques have been utilized to investigate the structure and dynamics of eumelanin and pheomelanin extracted from human black and red hair (Thureau et al. 2012) this procedure does not alter the pigment structure, thus, it has the advantage of retaining the morphology of intact melanosomes.

The surface features of individual melanosomes can be analyzed using atomic force microscopy (AFM), which, with an appropriately functionalized tip, can be applied to force probe surfaces to single-molecule resolution. Recent research using AFM has provided novel insights into the structure near the surface of melanosomes, for example, that melanosomes, regardless of their shape, have a substantially rough surface (Simon et al. 2008).

Very recently, synchrotron infrared nano-spectroscopy (SINS), a combination of atomic force microscopy (AFM) and infrared (IR) spectroscopy, emerged as a powerful tool for nanoscale chemical characterization of materials as it takes advantage of the low-noise, broadband, high spectral irradiance, and coherence of synchrotron infrared radiation for infrared measurements with nanometer spatial resolution. This powerful combination provides a qualitatively new form of broadband spatio-spectral analysis of nanoscale, mesoscale, and surface phenomena that were previously difficult to study with IR techniques and makes it ideal to analyze the chemistry of individual melanosomes. Stanic et al. (2018) used SINS for *in situ* nanoscale characterization of black and white sheep hair. The authors revealed a strong orientation of keratin fibrils along the hair fiber axis. More interestingly, a comparison of the white and black types of hair allowed them to isolate the IR fingerprint hair melanosomes, enabling them to achieve the unprecedented assignment of the vibrational modes of pheomelanin and eumelanin.

Melanosomes constitute a key component of animal pigmentary and defense systems but for a long time have resisted complete characterization due, to a great extent, to melanin's inherent physicochemical properties. However, only a better

understanding of melanosome properties and the relevance of their diverse forms across animals will lead to a full knowledge of their functions.

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Biotechnological Production of Melanins with Recombinant Microorganisms

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1 Introduction

Melanins are a family of polymeric pigments that are abundantly present in nature (d'Ischia et al. 2015). These polymers are the end products of the enzymatic oxidation of phenolic or indolic substrates. Additionally, it is thought that some of the oldest pigments found in nature are melanins, since this type of compound has been found in dinosaur and bird fossils (Zhang et al. 2010). Furthermore, intact melanin was also found in Jurassic-era squid ink sacs (Glass et al. 2012). Therefore, melanin has been proposed as a biomarker in the study of evolution (Wogelius et al. 2011). The four main types of melanin are eumelanin, pheomelanin, allomelanins, and pyomelanin. Eumelanin is produced by the oxidation of the amino acid L-tyrosine and/or L-dihydroxyphenylalanine (L-DOPA), resulting in a brown or black polymer. Alternatively, pheomelanin is formed when L-tyrosine and/or L-DOPA are oxidized in the presence of L-cysteine, resulting in a red-yellow pigment. Moreover, the oxidation of 4-hydroxyphenylacetic acid, catechols, dihydroxynaphthalene (DHN), -glutaminyl-4-hydroxybenzene, protocatechualdehyde, or tetrahydroxynaphthalene results in the formation of allomelanins. Finally, a particular kind of melanin called pyomelanin is produced when homogentisic acid is oxidized (HGA) (Fig. 1) (Lindgren et al. 2015).

Due to their chemical makeup, melanins exhibit unique physicochemical features, allowing them to function as cation exchangers, amorphous semiconductors,

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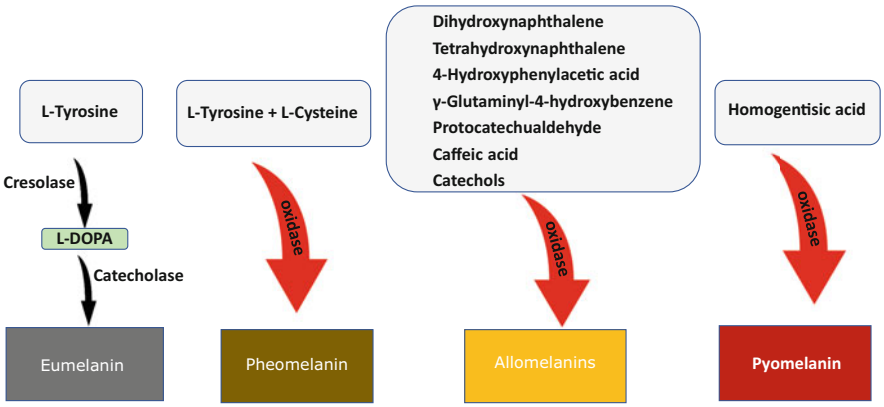

Fig. 1 Biochemical reactions that lead to the synthesis of eumelanin, pheomelanin, allomelanins and pyomelanin

X-ray, γ-ray, and ultraviolet light absorbers (della-Cioppa et al. 1990; Krol and Liebler 1998; Różanowska et al. 1999; Sarna et al. 1976; Ambrico et al. 2014). Additionally, it has been demonstrated that melanins contain antioxidant and antiviral properties (Nofsinger et al. 2002; Montefiori and Zhou 1991). Therefore, the ability to obtain these polymers in large quantities and at an affordable cost is necessary for the creation of a variety of valuable products and applications. Melanins can be chemically produced or isolated from animal and plant tissues. Nevertheless, these procedures can be rather expensive and are occasionally not sustainable (Saini and Melo 2015). An innovative and viable alternative to obtaining melanins is based on the development of biotechnological processes that use melanogenic microorganisms. Two advantages of this approach are its scalability and good yields for melanin production. Furthermore, this strategy can be strengthened by using genetic engineering techniques to boost the inherent melanogenic potential of some organisms or by creating new melanin-producing strains. In this context, the expression of genes encoding the enzymes involved in the oxidation of melanin precursors is the most typical genetic modification used to improve or generate a production strain (Martínez et al. 2019).

2 Enzymes Involved in Melanin Formation: Classification and Evolution

The initial process leading to the production of melanins is the enzyme-dependent oxidation of phenolic or indolic chemicals. Tyrosinases are the most common type of enzyme associated with melanogenesis. Tyrosinases are copper enzymes that catalyze both the ortho-hydroxylation of monophenols (cresolase activity) and the oxidation of catechols (catecholase activity), generating ortho-quinone products

(Garcia-Molina et al. 2007) (Fig. 1). More specifically, the enzyme tyrosinase catalyzes the hydroxylation of L-tyrosine to L-DOPA using molecular oxygen and then oxidizes this compound to dopachrome, which in turn nonenzymatically polymerizes to yield melanin (Ito 2003).

Copper proteins are classified into three categories according to their spectroscopic and structural properties. These are (1) mononuclear type-1 or blue copper proteins, which are mostly involved in electron transfer; (2) type-2 or non-blue copper proteins, typically found in enzymes that activate molecular oxygen and; (3) type-3 or binuclear copper proteins, which are part of the Di-copper superfamily or clan (CL0205) which includes phenoloxidases (i. e. tyrosinases and catechol oxidases) and hemocyanins (Jaenicke and Decker 2004; Aguilera et al. 2013; Kaintz et al. 2014). Phenoloxidases are enzymes involved in the oxidation of phenolic compounds. On the one hand, catechol oxidases have diphenolase activity, while on the other hand, tyrosinases are bifunctional enzymes with monophenolase and diphenolase activities (Aguilera et al. 2013; Kaintz et al. 2014). Hemocyanins are oxygen carriers found in arthropods and mollusks, although some may also have weak phenoloxidase activity (van Holde et al. 2001).

Type-3 copper proteins bind oxygen through two copper atoms (CuA and CuB) whose oxidation state changes from Cu^{+} (reduced) to $Cu2^{+}$ (oxidized) upon oxygen binding. Each Cu atom is coordinated by three histidine residues provided by two pairs of α helices, forming a four α helix bundle motif (Jaenicke and Decker 2004). The CuA binding site is typically characterized by an H1(n)-H2(8)-H3 motif and the CuB binding site by an H1(3)-H2(n)-H3 motif, where n is a variable number of residues between histidines (Aguilera et al. 2013). Sequence differences in the copper binding sites and domain architecture have been used as criteria for the classification of these proteins into three subclasses: (1) α-subclass with N-terminal signal peptide, suggesting secretion or vesicle localization (includes tyrosinases in the three domains of life, plant catechol oxidases and molluscan and urochordate hemocyanins); (2) β-subclass without a signal peptide and, therefore, presumably localized in the cytosol (includes arthropod tyrosinases and hemocyanins) and; (3) γ-subclass with a signal peptide, which is a cysteine-rich region upstream of the binuclear copper center and a transmembrane domain downstream of the binuclear copper center (includes various metazoan tyrosinases such as human ones) (Aguilera et al. 2013). Among these, the β-subclass appears as the most evolutionary divergent group, presenting a motif variation in the CuA binding site as H1(2)-H2(n)-H3 (Aguilera et al. 2013) (Fig. 2).

The presence of type-3 copper proteins in the three domains of life suggests an early origin probably dating back to the times of the last universal common ancestor to all cellular life (LUCA) (Jaenicke and Decker 2004; Aguilera et al. 2013) (Fig. 2), where a common ancestral protein with a mononuclear copper center (probably CuB binding site) duplicated resulting in a protein with a binuclear copper center (CuB + CuA binding sites) from which all type-3 copper proteins evolved. This ancestral protein may have protected primitive organisms from increasing levels of oxygen, which began to accumulate 3.5 billion years ago as a byproduct of photosynthesis (van Holde et al. 2001; Jaenicke and Decker 2004).

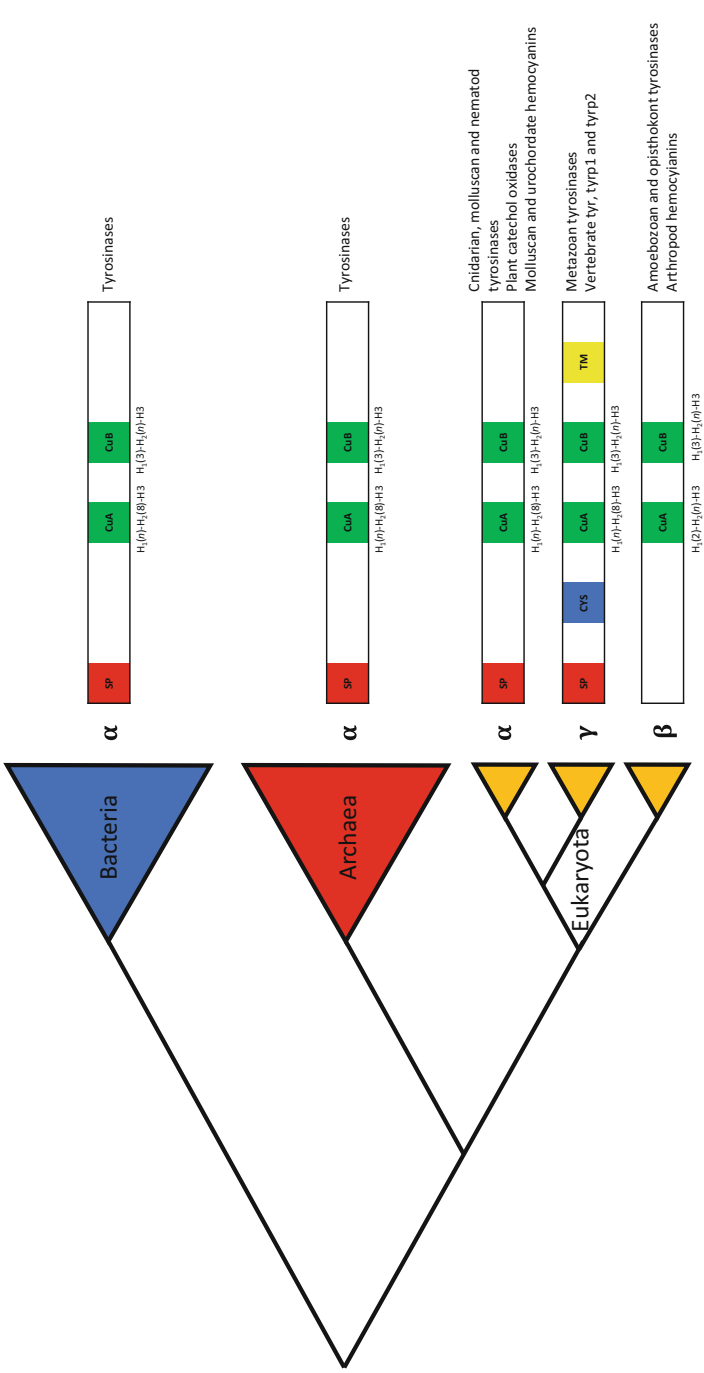

Fig. 2 Schematic representation of the taxonomic distribution of type-3 copper proteins. Domain architectures and sequence motifs characteristic of each subclass are represented to the right, as well as the type of enzymes present in each of them. SP signal peptide, CYS cysteine-rich region, CuA copper binding site A, CuB copper binding site B, TM transmembrane domain. The Eukaryota branch represents the branching order of α, β, and γ type-3 copper proteins. See the text for further detail on the taxonomic distribution and evolutionary processes related to type-3 copper proteins

Phylogenetic analyses have shown that type-3 copper proteins have a complex evolutionary history including multiple lineage-specific gene expansions and losses. Given the wide distribution of α-subclass proteins, it has been hypothesized that the first type-3 copper proteins belonged to this group. β and γ-subclasses appear to have originated from gene duplication within the Eukaryota domain. Among these, the most ancient one is the β-subclass, which seems to have originated before the divergence of amoebozoan and opisthokont lineages (present in amoebae, fungi, and some animals) where they lost the signal peptide and their sequences began to diverge at the CuA binding site. More recently, γ-subclass appears to have originated within the metazoan lineage (only found in animals), where they acquired the cysteine-rich region and the transmembrane domain (Aguilera et al. 2013) (Fig. 2).

Metazoan tyrosinases are mainly members of the γ-subclass (where α and β-subclass tyrosinases were lost); although α-subclass tyrosinases are maintained in Cnidaria, Nematoda and Mollusca; whereas β-subclass tyrosinases are found in Porifera, Urochordata, and Arthropoda. Regarding hemocyanins, α-subclass hemocyanins were probably duplicated before the divergence of bilaterians but were lost in most lineages except for urochordates and molluscans, whereas β-subclass hemocyanins were duplicated more recently within the Arthropoda lineage (van Holde et al. 2001; Aguilera et al. 2013). Although metazoans are the only kingdom that possesses proteins from the α, β, and γ-subclasses, gene loss has occurred to such an extent that *Ciona intestinalis* is the only organism possessing the three types in its genome (α-subclass hemocyanin and, β and γ-subclass tyrosinases) (Aguilera et al. 2013).

In vertebrates, in addition to tyrosinase, two tyrosinase-related proteins are also involved in different steps of melanin biosynthesis: tyrosinase-related protein-1 (tyrp1) and tyrosinase-related protein-2 (tyrp2) also known as DOPAchrome tautomerase (dct). These two enzymes influence the quantity and quality of melanins produced and are involved in the stabilization of the tyrosinase structure (Esposito et al. 2012). Phylogenetic analyses suggest a duplication event before the divergence of urochordates and vertebrates resulting in tyrosinases and a tyrosinase-related protein, and a second duplication event before the divergence of teleost fishes gave rise to the two tyrosinase-related proteins tyrp1 and tyrp2. Interestingly, it may be possible that these enzymes do not coordinate Cu atoms at the metal binding sites, as tyrp2 is known to coordinate two zinc atoms instead (Esposito et al. 2012).

To the best of our knowledge, the evolution of bacterial and archaeal tyrosinases has not been extensively explored. Thus, for the time being, a tentative scheme proposes at least three unambiguous types of bacterial tyrosinases based on their domain architecture. For example, the first type includes the well-studied tyrosinases from *Streptomyces* sp. which are produced as a heterodimer where tyrosinase (*melC2* gene product) is bound to a caddy protein (*melC1* gene product) involved in heterodimer secretion and delivery of copper atoms to the active site of tyrosinase. A second type is not associated with a caddy protein for copper incorporation and includes tyrosinases such as that of *Bacillus megaterium*. Finally, a third type includes tyrosinases produced as zymogens that, similarly to plant and fungal tyrosinases/catechol oxidases, require proteolytic removal of a C-terminal domain

for activation (Fairhead and Thöny-Meyer 2012; Pretzler and Rompel 2018). Interestingly, a preliminary analysis performed in our group suggests that tyrosinases from plant-associated bacteria (e. g. those from *Rhizobium etli* or *Ralstonia solanacearum*) are more closely related to plant catechol oxidases, which implies horizontal gene transfers that would further increase the complexity of the already intricated evolutionary history of this protein family. Additionally, a cold-adapted tyrosinase from the archaeon *Candidatus Nitrosopumilus koreensis* is distinct from other known bacterial tyrosinases (Kim et al. 2016).

Despite efforts to classify type-3 copper proteins based on their domain architecture, conserved sequence motifs, and tertiary structure elements, it is still unclear what changes have driven the functional differentiation of these proteins throughout evolution. However, besides the six conserved histidines, mutagenesis studies have shed some light on some amino acids of great catalytic importance that could explain the functional differentiation between catechol oxidases and tyrosinases. These include a variable gatekeeper residue which may support or inhibit substrate entry; a waterkeeper residue (Glu for tyrosinase activity) required to maintain a stable water network around the active center; variable residues (Asn and Asp for tyrosinase activity) one position ahead of the first and second histidines in CuB copper binding site, which are involved in increased basicity of adjacent histidines required for substrate deprotonation; a thioether bond (absent in tyrosinases) between a cysteine residue and the second histidine in the CuA copper binding site, which upon breaking allows the histidine to move more freely within the active site where it can also intervene in substrate deprotonation and; a seventh histidine one position before the third histidine in the CuB copper binding site and disulfide bonds that may stabilize the enzyme's catalytic activity (Kampatsikas and Rompel 2021).

Lacasses are another group of enzymes involved in melanogenesis. These enzymes which have been found in bacteria, fungi, and plants, are not related to tyrosinases but are also copper-dependent oxidoreductases (Valderrama et al. 2003). For example, the enzyme 4-hydroxyphenylacetic acid (4-HPA) hydroxylase is involved in the catabolism of 4-HPA in bacteria. This enzyme is a two-component flavin adenine dinucleotide (FAD)-dependent monooxygenase (Gibello et al. 1995). This group of enzymes displays a broad substrate range, they can hydroxylate various monohydric and dihydric phenols (Prieto et al. 1993).

3 Production of Melanins with Genetically Engineered Microorganisms

3.1 Eumelanin Production

The ability to obtain these pigments from plentiful and affordable sources is a prerequisite for the current and future uses of melanin. By using relatively simple procedures, these products can be recovered from natural sources such as the tissues

of animals or plants. However, the mixture of various melanin types and related compounds found in these sources frequently makes purifying processes difficult and could result in a product with varied compositions. Furthermore, these polymers can be produced chemically or enzymatically by oxidizing phenolic or indolic substrates (Saini and Melo 2015). These processes can produce melanins that are highly pure, but they come at a high price (d'Ischia et al. 2015). Alternatively, culturing melanin-producing microorganisms or microbes that have been genetically modified to produce melanin is another method for obtaining these polymers. This last method allows for the production of melanins at a high yield and relatively low cost.

The experimental procedures collectively referred to as "genetic engineering " allow altering the genetic make-up of bacteria to increase or give them the capacity to generate chemicals. An increasing number of microbes may be genetically engineered for producing melanin, and the identification of individual genes and melanin-producing pathways has been possible through the application of DNA sequencing technologies in conjunction with biochemical investigations. This knowledge and technologies are the basis for creating recombinant microbes with improved melanin production and the capacity to transfer this capability to non-melanogenic bacteria.

The latest developments in the design of recombinant microbial strains and the production techniques used for the synthesis of melanins are reviewed and analyzed in the following sections. The bacterium *Escherichia coli* was the first documented recombinant melanogenic microbe. Genes from the actinomycete *Streptomyces antibioticus* were altered to be expressed in *E. coli*. The two genes in question, *mel* and ORF438 at the *mel* locus in *S. antibioticus* are necessary for the synthesis of melanin. In agar plates and liquid cultures, the recombinant *E. coli* strain was capable of producing eumelanin from L-tyrosine. Furthermore, it has been demonstrated that the *S. antibioticus* tyrosinase enzyme could use synthetic, non-natural amino acids such N-acetyl-L-tyrosine and L-tyrosine ethyl ester, as substrates, to produce synthetic melanins (Della-Cioppa et al. 1990). In a different report, a recombinant *E. coli* strain derived from JM109 was created using the *mel* locus from *S. antibioticus*. In this case, the phage T5 promoter and two *lac* operators were used to control the expression of the *mel* gene. The resulting recombinant strain was cultivated in LB medium, producing 0.4 g/L of eumelanin (Table 1). Eumelanin was removed from the culture medium by precipitation at a pH of 3.0, followed by dissolution at a pH of 8.0 in distilled water. Afterward, liquid chromatography on Sephadex LH-20 was performed. To investigate how the presence of this polymer affected the antibacterial activity of several antibiotics, pure eumelanin was used. It was found that eumelanin dose-dependently decreased the antibacterial effect of ampicillin, kanamycin, polymyxin B, and tetracycline against *E. coli* (Lin et al. 2005). In addition to the potential therapeutic significance of such data, the observed results could be used to select for greater melanin-producing recombinant strains based on antibiotic resistance.

Another early study demonstrated that the *Bacillus thuringiensis* strain 4D11 was able to produce melanin when cultivated with L-tyrosine at 42 °C (Ruan et al. 2004).

Table 1 Engineered microbial strains used in the production of melanin

Promoter	Inducer	Expressed gene(s)	Expression vectors	Genes source	Production microorganism	Melanin precursor	Carbon source	T (°C)	Q_M (mg/L/h)	Titer (g/L)	Reference
Lac	Not reported	*Mel*	pGEM-7Zf	*Bacillus thuringiensis 4D11*	*Escherichia coli*	Casein	Casein	NR	155.5	5.6	Ruan et al. 2005
T5	IPTG 0.36 mM	*Mel*	pQE32	*Streptomyces antibioticus*	*Escherichia coli*	L-tyrosine	LB medium	37	8.3	0.4	Lin et al. 2005
Trc	IPTG 0.1 mM	*MutmelA*	pTrc99A	*Rhizobium etli*	*Escherichia coli*	L-tyrosine	Glucose	30	75	6	Lagunas-Muñoz et al. 2006
None	None	None	None	*Pseudomonas putida* strain F6	*Pseudomonas putida* strain F6-HDO	L-tyrosine	Citrate	30	17.5	0.35	Nikodinovic-Runic et al. 2009
P_{skmel}	Constitutive	*melC*	pIJ86	*Streptomyces kathirae*	*Streptomyces kathirae*	L-tyrosine	Amylodextrine, yeast extract	28	225	28.8	Guo et al. 2015
None	None	Not identified	None	*Escherichia coli*	*Escherichia coli*	Caffeic acid	Glucose	30	16.7	0.15	Jang et al. 2018
T7	IPTG 1 mM	*Fcs*	pRSF duet-1 pET duet-1	*Burkholderia glumae* BGR1	*Escherichia coli*	Caffeic acid	Glucose	30	NR	0.20	Jang et al. 2018
T7	IPTG 1 mM	*Ech*	pRSF duet-1 pET duet-1	*Burkholderia glumae* BGR1	*Escherichia coli*	*					Jang et al. 2018
pBAD	Arabinose 0.1%	*Hpd*	pVO	*Pseudomonas auruginosa* PAO1	*Escherichia coli*	CAA L-tyrosine	Glucose	37	NR	0.213	Bolognese et al. 2019
T7	IPTG 0.25 mM	*Hppd*	pET-28a(+)	*Ralstonia pickettii*	*Escherichia coli*	L-tyrosine	Glucose	30	13.1	0.315	Seo and Choi 2020
T7	IPTG 1 mM	*Hpd*	pET-21a(+)	*Flavobacterium kingsejongi*	*Escherichia coli*	L-tyrosine	NR	30	40	3.76	Lee et al. 2022
pTEF	None	*4HPPD*	Chromosome	*Yarrowia lipolytica*	*Yarrowia lipolytica*	None	Glucose	28	NR	4.5	Larroude et al. 2021

T7	IPTG 0.25 mM	*melC*	pET-24a(+)	*Bacillus megaterium*	*Escherichia coli*	L-tyrosine and L-tryptophan	NR	30	100	3.5	Park et al. 2020
T7	IPTG 0.25 mM	*cyp102G4*	pETduet-1	*Streptomyces cattleya*	*Escherichia coli*	L-tyrosine and L-tryptophan	NR	30			Park et al. 2020
T7	IPTG 0.1 mM	*TAL*	pRSFduet-1	*Rhodotorula glutinis*	*Escherichia coli*	Caffeic acid, L-tyrosine and L-lysine	NR	30	NR		Ahn et al. 2021
T7	IPTG 0.1 mM	*C3H*	pRSFDuet-1	*Saccharothrix espanaensis*	*Escherichia coli*	*					Ahn et al. 2021
T7	IPTG 0.1 mM	*FCS*	pETDuet-1	*Burkholderia glumae*	*Escherichia coli*	*					Ahn et al. 2021
T7	IPTG 0.1 mM	*ECH*	pETDuet-1	*Burkholderia glumae*	*Escherichia coli*	*					Ahn et al. 2021
T7	IPTG 0.1 mM	*melC*	pACYCDuet-1	*Bacillus megaterium*	*Escherichia coli*	*					Ahn et al. 2021
T7	IPTG 0.1 mM	*CadA*	pACYCDuet-1	*Klebsiella pneumoniae*	*Escherichia coli*	*					Ahn et al. 2021
Lac	IPTG 0.1 mM	$aroG^{fbr}$	pTrc99A	*Escherichia coli*	*Escherichia coli*	None	Glucose	30	26.8	3.2	Chávez-Béjar et al. 2013
Trc	IPTG 0.1 mM	*tyrC*	pTrc99A	*Zymomonas mobilis*	*Escherichia coli*	*					Chávez-Béjar et al. 2013
Trc	IPTG 0.1 mM	$pheA_{CM}$	pTrc99A	*Escherichia coli*	*Escherichia coli*	*					Chávez-Béjar et al. 2013
Trc	IPTG 0.1 mM	*MutmelA*	pTrc99A	*Rhizobium etli*	*Escherichia coli*	*					Chávez-Béjar et al. 2013
Lac	IPTG 0.1 mM	$aroG^{fbr}$	pTrc99A	*Escherichia coli*	*Escherichia coli*	None	Glycerol	30	16.8	1.21	Mejía-Caballero et al. 2016
PtktA	None	*tktA*	pTrc99A	*Escherichia coli*	*Escherichia coli*	*					Mejía-Caballero et al. 2016

(continued)

Table 1 (continued)

Promoter	Inducer	Expressed gene(s)	Expression vectors	Genes source	Production microorganism	Melanin precursor	Carbon source	T (°C)	Q_M (mg/L/h)	Titer (g/L)	Reference
Trc	IPTG 0.1 mM	*antABC*	pTrc99A	*Pseudomonas aeruginosa PAO1*	*Escherichia coli*	*					Mejía-Caballero et al. 2016
Trc	IPTG 0.1 mM	*MutmelA*	pTrc99A	*Rhizobium etli*	*Escherichia coli*	*					Mejía-Caballero et al. 2016

Q_M Volumetric productivity, NR not reported

*Only the first line of each example provides production data, the rest of the lines indicate the types of genetic modifications performed in the production strain

These findings suggested that in the genome of this organism there should be a gene encoding for tyrosinase. Considering that the genome sequence of *B. thuringiensis* 4D11 was unknown, a cloning technique was then developed based on the predicted sequence similarities with a tyrosinase gene from *Bacillus cereus* 10987. Based on the tyrosinase gene sequence from *B. cereus* 10987, a set of PCR primers was created and used to amplify an 1179 bp DNA fragment from *B. thuringiensis* 4D11 DNA. This DNA fragment shared 99% of its encoded amino acid sequence with the tyrosinase from *B. cereus* 10987. The *lac* promoter was used to express the PCR product in plasmid pGEM-7zf. This plasmid was then used to transform *E. coli* DH5. The resulting recombinant strain produced eumelanin at a titer of 5.6 g/L when cultured in a casein liquid medium (Table 1). Intriguingly, it was found that in trials involving exposure to UV radiation, this recombinant strain outperformed DH5 in terms of survival rates (Ruan et al. 2005). These findings demonstrate how, in addition to enabling the biotechnological production of melanin, the heterologous expression of a gene encoding a tyrosinase might improve the host's UV radiation resistance, a characteristic that might be advantageous in the case of microorganisms used in the field, such as *B. thuringiensis*. Moreover, microorganisms that can endure intense UV exposure could be of benefit in future long-term space missions and planet-colonization initiatives, as microbes are thought to be crucial to help maintain human existence by producing food, valuable compounds, and recycling trash (Horneck et al. 2010; https://blogs.scientificamerican.com/observations/microbes-might-be-key-to-a-mars-mission/). Furthermore, melanin can absorb X and γ rays, which may help engineered bacteria survive in conditions outside of our planet.

Rhizobium etli, a soil bacterium, is particularly significant for agriculture, as it can fix nitrogen by forming nodules in the roots of *Phaseolus vulgaris* plants. This bacterium can also synthesize melanin in the symbiotic nodules through a symbiotic plasmid that contains a gene encoding tyrosinase (*melA*) (González et al. 2003; Piñero et al. 2007). The expression vector pTrc99A was used to clone the *melA* gene under the strong *trc* promoter, resulting in pTrcmelA, which was used to transform *E. coli*. When L-tyrosine was used as a substrate, the recombinant *E. coli* strain was able to produce eumelanin at 30 °C, yet, increasing the temperature to 37 °C significantly lowered melanin yields (Cabrera-Valladares et al. 2006). Moreover, it was also discovered that melanin production occurred only in the stationary culture phase and, when compared to other colonies, a recombinant *E. coli* colony on media containing L-tyrosine showed a deeper color. Following DNA sequencing of this clone's *melA* gene, it was discovered that it had undergone a spontaneous mutation, changing the Asp535 residue in the MelA tyrosinase enzyme to a Gly residue. Therefore, MutMelA was the name given to this mutant version of MelA. When compared to a strain that expresses the wild-type enzyme, it was observed that eumelanin synthesis in liquid cultures begins sooner in cultures of *E. coli* expressing *MutmelA*. A study was performed to find the best conditions for pigment synthesis in liquid cultures utilizing a recombinant *E. coli* strain expressing *MutmelA*. The influence of culture temperature, pH, isopropyl-d-thio-galactopyranoside (IPTG) as a gene inducer, antibiotic concentration for plasmid selection pressure, and eumelanin concentration was assessed. It was found that the ideal bioreactor

conditions were: 0.1 mmol/L of IPTG, a culture temperature of 30 °C, and a change in medium pH from 7.0 to 7.5 at the beginning of the eumelanin production phase. Additionally, L-tyrosine was added to the culture medium at 6 g/L as a eumelanin precursor. With a final titer of 6 g/L under these conditions, a 100% conversion yield of L-tyrosine to eumelanin was observed (Table 1) (Lagunas-Muñoz et al. 2006). These findings emphasize the significance of optimizing culture conditions as a factor in achieving the highest yield and productivity while using a recombinant melanogenic strain.

As part of a bioprospecting investigation, microorganisms capable of producing melanin were identified from soil samples in China. Among these isolates, *Streptomyces kathirae* SC-1 had the best potential for melanin synthesis among all isolates. Employing this organism and a surface response approach to enhance the medium and growth conditions, 13.7 g/L of melanin was produced (Guo et al. 2014). It is significant to note that yeast extract, which provides a variety of melanin precursors, was a component of the culture medium used in this investigation. Therefore, to identify the type of melanin generated, the resultant polymer needs to be analyzed to ascertain its chemical makeup. To provide further light on this organism's melanogenesis process, a tyrosinase was homogeneously purified, which is a 30-kDa enzyme (called tyrosinase MelC) that has a Km of 0.42 mM for L-DOPA and 0.25 mM for L-tyrosine. The primers used to amplify the *melC* gene and its promoter region were designed using the partial amino acid sequence of this tyrosinase. Two potential promoters, Pskmel and P135, were discovered by sequence analysis. In the replicative plasmid pIJ86, the gene *melC* was cloned under the transcriptional control of either the putative promoter or the constitutive promoter PermE*. The resultant constructs were then transformed into *S. lividans* and *S. kathirae*. After characterizing the recombinant strains of *S. lividans*, it was demonstrated that Pskmel is the functional promoter for *melC*. *S. kathirae* recombinant strains that were grown in melanin-producing conditions. It was also found that the amounts of melanin generated by strains expressing *melC* from PermE* or Pskmel were 24.9 and 28.8 g/L, respectively (Table 1) (Guo et al. 2015). It should be emphasized that these are the highest reported melanin titers, showing the possibility of using genetic engineering methods to significantly increase a melanogenic organism's production capability (Table 1). This manufacturing method can be further optimized, particularly in terms of the culture medium composition, as yeast extract, at the concentration used in the referenced work (37 g/L), becomes a costly ingredient. Moreover, some of the components of yeast extract can interact with melanin precursors to produce a polymer that isn't completely derived from L-tyrosine. To enhance the existing manufacturing method, future research should aim at finding a growth medium that only contains salts and a simple carbon source.

3.2 Pyomelanin Production

When cultured on a medium containing L-tyrosine, the soil bacterium *Pseudomonas putida* strain F6 exhibits the ability to produce a dark pigment. Thus, transposon mutagenesis was done to learn more about the function of the genes involved in the production of this pigment. Two mutants with improved synthesis were obtained by this approach. When compared to *P. putida* F6, one of these mutants (F6-HDO) generated 0.35 g/L of the pigment, which was identified as a type of melanin (Table 1). It is interesting to note that, when compared to the wild-type strain, this mutant showed greater resistance to UV radiation and H_20_2. Genetic testing revealed that a gene encoding HGA 1,2-dioxygenase (HGO) was disrupted by transposon mutagenesis. This enzyme participates in the degradation process that turns HGA into 4-maleylacetoacetate. As a result, it is expected that this mutation will cause HGO to use less HGA. This finding suggests that in this mutant strain, HGA is the pyomelanin precursor (Fig. 3) (Nikodinovic-Runic et al. 2009). Precursors for HGA synthesis originate from the L-tyrosine biosynthetic pathway. Furthermore, the enzyme hydroxyphenylpyruvate dehydrogenase (HPPD) converts the intermediate 4-hydroxyphenylpyruvate (HPP) into HGA (Fig. 3).

A recombinant strain of *E. coli* for pyomelanin production was developed by cloning the chromosomal *hpd* gene that encodes the 4 hydroxyphenylpyruvate dioxygenase from *Pseudomonas auruginosa* PAO1, and then placing it in the pVO vector under transcriptional control of the arabinose promoter pBAD. A biotransformation protocol for pyomelanin synthesis with the recombinant *E. coli* strain was developed by defining the parameters influencing growth and production. The optimal growth conditions were determined to be as follows: use of mineral medium with glucose as carbon and energy source, arabinose 0.1% to induce gene *hpd*, casamino acids at 0.2%, and tyrosine 1 mM. Under these conditions, after 6 days of culture, 213 mg/L of pyomelanin was produced (Bolognese et al. 2019).

In another report, a strain of *Ralstonia pickettii* that produces pyomelanin was isolated from soil samples. From this strain, gene *hppD* encoding 4-hydroxyphenyl pyruvate dioxygenase was amplified and cloned in plasmid pET-28a(+). This plasmid was then transformed into *E. coli* that in turn was cultured to produce pyomelanin. In these experiments, a dependency on metal ions was observed. In this case, the highest pyomelanin production was achieved while supplementing Cu^{2+}, resulting in a titer of 315 mg/L (Seo and Choi 2020).

A new strain of *Flavobacterium kingsejongi* could produce a dark pigment when L-tyrosine was present in the culture medium. The chemical characterization of such pigment showed properties consistent with melanin. However, it was not clear if this pigment was eumelanin, pyomelanin or a mixture of both polymers. Regardless, the *hpd* gene encoding 4-hydroxyphenyl pyruvate dioxygenase from *F. kingsejongi* was amplified and cloned in expression vector pET-21a (+). The resulting plasmid was then transformed into *E. coli*. Afterward, this recombinant strain was cultured in a 5-L bioreactor containing TB medium supplemented with 10 g/L L-tyrosine and

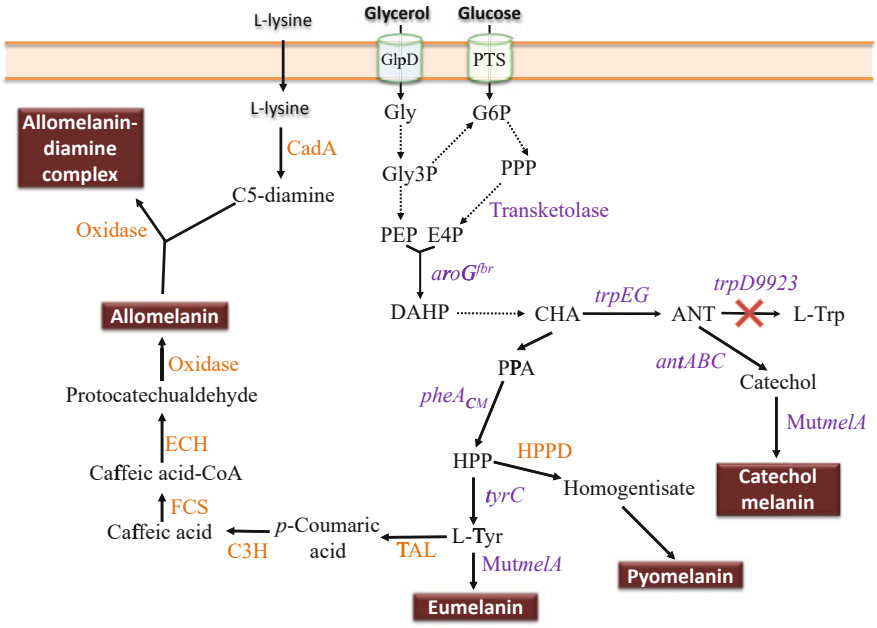

Fig. 3 Metabolic pathways and expressed genes related to the synthesis of melanins with engineered microorganisms. Dashed arrows indicate two or more enzyme reactions. Abbreviations: PTS phosphotransferase system glucose transport protein, Gly glycerol, Gly3P glycerol-3-phosphate, G6P glucose-6-phosphate, E4P D-erythrose 4-phosphate, PEP phosphoenolpyruvate, DAHP 3-deoxy-D-arabino-heptulosonate 7-phosphate, HPP 4-hydroxyphenylpyruvate, CHA chorismate, ANT anthranilate, PPA phenylpyruvate, HPPD hydroxyphenylpyruvate dehydrogenase, L-Tyr L-tyrosine, L-Trp L-tryptophan, *aroG*^{fbr} feedback inhibition resistant DAHP synthase, CadA L-lysine decarboxylase, *trpEG* anthranilate synthase component I, *trpD9923* mutant version of TrpD causing the loss of anthranilate phosphoribosyl transferase activity and retaining anthranilate synthase activity, *tyrC* cyclohexadienyl dehydrogenase, C3H p-coumarate 3-hydroxylase, TAL tyrosine ammonia-lyase, FCS feruloyl-CoA synthetase, ECH enoyl-CoA hydratase/aldolase, AntABC terminal oxygenase and the reductase components of anthranilate 1,2-dioxygenase, *pheA*$_{CM}$ chorismate mutase domain from chorismate mutase-prephenate dehydratase, Mut*melA* mutant version of tyrosinase (Martínez et al. 2019)

1 mM IPTG. Under these conditions, 3.76 g/L of melanin was produced (Lee et al. 2022).

Random mutagenesis is a reasonably simple approach for strain enhancement; however, it can only be used with organisms that naturally produce melanin. A drawback of this approach is that the site and type of mutation are not easily determined. This restricts the application of genetic engineering methodologies for strain enhancement. Additionally, the strain may return to a low producer phenotype since the genetic alterations caused by random mutagenesis might be unstable. The enhanced strain's genome may be sequenced to learn more about the type of

mutation that caused it as well as the genes and pathways that contributed to the observed phenotype. Knowing this, the melanogenic organism can be "reverse-engineered" by reintroducing the detected mutations through genetic engineering techniques. Furthermore, by using this method, it is possible to distinguish between genetic alterations that are connected to an enhanced phenotype from those that might be harmful to production purposes. In the preceding examples, recombinant strains and procedures for converting various aromatic chemicals into melanins were detailed. Tyrosinases may use different precursors as substrates to produce melanins with different colors (Table 1). Despite these benefits, several disadvantages might be thought. One of them is the use of expensive of using pure melanin precursors. Therefore, to decrease expenses, less pure melanin precursors such as protein hydrolysates or yeast extract are used. Yet, using these more affordable precursors causes new issues. For example, these culture media may contain a variety of substances that can act as substrates of tyrosinases or that can react with melanin precursor molecules, therefore, their usage can lead to diversity in the composition of the generated melanins. Additionally, techniques for purifying melanin become more complicated and costly when non-defined media are used.

4 Production of Allomelanin and Novel Types of Melanin

In the chemical and food industries, chemicals known as phenolic aldehydes are commonly used. Thus, a project was launched that aimed to synthesize this class of compounds through engineered *E. coli* strains that have been altered to produce caffeic acid from L-tyrosine. To do so, the expression of the enzyme tyrosine ammonia-lyase (TAL) converted L-tyrosine to p-coumaric acid and p-coumarate 3-hydroxylase (C3H) converted p-coumaric acid to caffeic acid (Fig. 3). The resulting product was a black pigment with melanin-like properties, which was probably created by some of the oxidases encoded in the *E. coli* genome that can oxidize the catechol moiety of various molecules. Additionally, it was shown that by culturing *E. coli* with protocatechualdehyde in the media, a brown melanin pigment was produced, whereas when caffeic acid was added, a black pigment was generated. Additionally, the genes from *Burkholderia glumae* BGR1's encoding for feruloyl-CoA synthetase (FCS) and enoyl-CoA hydratase/aldolase (ECH) were expressed in *E. coli* (Fig. 3), resulting in a recombinant strain with the capacity to transform caffeic acid into protocatechualdehyde. Furthermore, this study found that wild-type *E. coli* BL21(DE3) generated 0.15 g/L of melanin when exposed to 5 mM caffeic acid. (Table 1). The production of melanin increased when the same quantity of caffeic acid was given to a culture of a recombinant strain expressing *fcs* and *ech*, reaching a titer of 0.2 g/L (Jang et al. 2018). Although the produced melanin chemical makeup has not been determined, it is most likely a polymer made up of a combination of protocatechualdehyde and caffeic acid moieties. These findings show that recombinant *E. coli* can produce caffeic acid and protocatechualdehyde melanins. It can also be expected that this strain's FCS and ECH activities affect how

much melanin and/or melanin precursors are produced, but the exact processes behind these effects are not yet entirely known. Therefore, the synthesized melanin's detailed characterization should shed further light on the chemical precursors involved in its synthesis. Additionally, finding the native *E. coli* enzyme responsible for the oxidation of protocatechualdehyde and caffeic acid, which results in their polymerization into melanin, should also be of interest as the development of melanin-producing strains should be possible by the cloning and overexpression of the gene encoding this yet undiscovered oxidase. In a subsequent publication, it was shown that soft contact lenses may be dyed using protocatechualdehyde-based melanin (Ahn et al. 2019), providing an advantage when compared to chemically produced dyes, as the antibacterial and antioxidant properties of melanins could benefit these products.

The synthesis of a novel melanin polymer was explored in *E. coli* by the co-expression of tyrosinase (MelC) and cytochrome P450 monooxygenase (CYP102G4). The tyrosinase MelC synthesizes eumelanin in the presence of L-tyrosine as substrate, whereas CYP102G4 is involved in the synthesis of indigo dye from L-tryptophan-derived indole. The gene *melC* was obtained from *Bacillus megaterium*, and cloned in the pET-24a(+) vector. Gene *cyp102G4* was amplified from *Streptomyces cattleya* and cloned in pETduet-1. Single plasmids or both were transformed into the *E. coli* BL21 (DE3) strain. Cultures with a strain expressing *melC* in the presence of L-tyrosine and L-tryptophan resulted in the production of 1 g/L of melanin. In contrast, under the same conditions, a strain expressing both *melC* and *cyp102G4* produced 3.5 g/L of melanin. The characterization of this novel melanin demonstrated it had noticeable dyeing capabilities on cellulose paper. In addition, it displayed electrical conductivity, rendering it a potential material for organic electrodes (Park et al. 2020).

In another study, the production of novel synthetic melanin was explored by employing C5-diamine to increase the crosslinking of melanin units. *E. coli* strains expressing combinations of the following enzymes were evaluated: Tyrosine ammonia lyase, p-coumarate 3-hydroxylase, feruloyl-CoA synthetase, enoyl-CoA hydratase, lysine decarboxylase, and tyrosinase. Some of these strains produced melanin derived from caffeic acid, protocatechualdehyde, or L-DOPA to a maximum level of 400 mg/L.

A strain expressing all the previous enzymes showed the capacity to produce a melanin-diamine complex. Using this strain in biotransformation experiments, 20 mg/L of a melanin-diamine complex was formed while supplying 5 mM caffeic acid, L-tyrosine, and L-lysine (Ahn et al. 2021). This relatively low production level is attributed to toxicity from the C5-diamine, which is formed by lysine decarboxylase. Therefore, to improve the production of melanin-diamine, further studies must be done to contend with C5-diamine toxicity.

5 Metabolic Engineering Applied for De Novo Synthesis of Melanins

Developing microbial strains for the complete synthesis of melanins from simple carbon sources is one possible solution for the challenges stated previously. Based on metabolic engineering techniques, it is possible to increase flux into the shikimate pathway, which is responsible for producing the precursors of aromatic amino acids. For instance, L-tyrosine, a precursor of eumelanin, was produced from glucose by an *E. coli* strain modified through metabolic engineering techniques (Chávez-Béjar et al. 2008). To do so, carbon flow to the L-tyrosine biosynthetic pathway was increased by overexpressing the genes for cyclohexadienyl dehydrogenase (TyrC) from *Zymomonas mobilis*, the chorismate mutase domain from the original enzyme chorismate mutase-prephenate dehydratase, and a feedback-insensitive version of the enzyme 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase ($aroG^{fbr}$). Additionally, this strain expressed the tyrosinase MutMelA (Fig. 3). The resulting strain was able to produce eumelanin from glucose. But it was found that the L-tyrosine pool was decreased by MutMelA activity, which led to a defect in cell growth. It was then found that Cu is required as a cofactor for the correct functioning of the enzyme tyrosinase. Thus, to prevent L-tyrosine depletion by MutMelA, Cu was left out of the medium during the first half of the culture. Then, tyrosinase was activated by the addition of $CuSO_4$ to the medium, which began the eumelanin synthesis phase. This method was used in bioreactor cultures where the only carbon source was glucose. In this culture, 3.2 g/L of eumelanin was produced in 120 h (Table 1) (Chávez-Béjar et al. 2013). These results were the first instance of the application of metabolic engineering to create a strain for the complete synthesis of eumelanin from glucose. This work shed light on the possible detrimental effects on cell physiology brought on by tyrosinase expression at high levels. Yet, by using a delayed activation of the heterologous enzyme, this issue was solved. A different approach might be based on the precise regulation of gene induction during a particular stage of the production culture.

During the characterization of the enzyme MutMelA, it was found that in addition to L-tyrosine, catechol may be used as a substrate. Therefore, this enzyme might be used to produce catechol melanin. To put this theory to the test, a bioconversion process was created using an *E. coli* strain that expresses MutMelA and grows on a medium that contains catechol (0.85 g/L) as a tyrosinase substrate, and glycerol 40 g/L as a carbon source. In this experiment, 0.29 g/L of catechol melanin was generated after 54 h. To further enhance this process, metabolic engineering was evaluated to create a strain capable of producing catechol melanin from a simple carbon source. The method used was based on employing an engineered strain of *E. coli* capable of generating catechol from a simple carbon source (Balderas-Hernández et al. 2014). The chosen mutant strain was *E. coli* W3110 *trpD9923*, which overproduces the intermediate anthranilate in the L-tryptophan biosynthesis pathway (Yanofsky et al. 1971). This strain was altered to enhance carbon flow to anthranilate by overexpressing the $aroG^{fbr}$ and *tktA* genes that encode for the feedback-insensitive

form of the enzyme DAHP synthase and transketolase, respectively (Fig. 3). These modifications resulted in a twofold increase of the anthranilate titer in flask cultures (Balderas-Hernández et al. 2009). This strain was further modified through the expression of the genes *antABC* which encodes anthranilate 1,2-dioxygenase from *P. aeruginosa* PAO1, granting it the capacity to convert anthranilate to catechol (Fig. 3). Lastly, the strain was further modified by integrating the gene Mut*melA* into the chromosome at the location of the *lacZ* gene. The resultant strain was assessed in 1-liter bioreactor cultures. The carbon source in the culture medium was glycerol, 40 g/L. This carbon source was chosen instead of glucose because it does not utilize the aromatics precursor PEP during its internalization and phosphorylation. Furthermore, glycerol is a very affordable, plentiful, and renewable carbon source, as it is a byproduct of biodiesel and soap manufacturing (Tan et al. 2013). Additionally, 2 g/L of yeast extract was added to the culture medium because the employed strain is an L-tryptophan auxotroph. Under these conditions, the modified strain showed growth for 17 h before entering the stationary phase, which lasted 72 h. At 18 h, just at the beginning of the stationary phase, catechol melanin started to accumulate. A total of 1.21 g/L of catechol melanin were recovered from the culture medium, while 0.73 g/L of catechol was accumulated at the end of the culture (Table 1) (Mejía-Caballero et al. 2016). This finding suggests that the rate of synthesis of this precursor is greater than the capacity of MutMelA to metabolize it. Therefore, tyrosinase activity should be increased as a goal to improve strain performance.

The yeast *Yarrowia lipolytica* displays a natural capacity to produce pyomelanin. To improve the de novo pyomelanin synthesis capacity in this yeast, a strategy was followed based on the utilization of *Y. lipolytica* strain JMY8032 which was engineered for aromatic amino acid production. In this strain, flow into the L-tyrosine and L-phenylalanine pathways was increased by expressing unregulated mutant versions of enzymes DAHP synthase, chorismate mutase, and aromatic aminotransferase I. Meanwhile, gene *4HPPD* encoding 4-hydroxyphenyl pyruvate dioxygenase was disrupted. The resulting mutant strain did not produce pyomelanin, confirming that this gene is involved in HGA synthesis. Thus, to generate a strain that overexpresses *4HPPD*, this gene was cloned under the control of the strong constitutive pTEF promoter. The Golden Gate cloning procedure was employed to generate strain JMY8208 with *4HPPD* integrated into the chromosome (Larroude et al. 2021). Genetic characterization of this strain showed that it contained three copies of the *4HPPD* overexpression cassette. Then, this strain was grown in YNB medium supplemented with glucose at 28 °C in shake flasks, and, after 5 days of culture, 4.5 g/L of pyomelanin was synthesized (Larroude et al. 2021).

6 Conclusions and Perspectives

Melanins can be considered functional polymers with multiple potential industrial applications. Some significant technological problems related to melanins are achieving their large-scale synthesis while obtaining specific chemical composition

and maintaining affordable production costs. These challenges can be overcome by isolating and employing naturally occurring melanogenic bacteria. This proposal presents various benefits, such as the potential to rapidly establish a production process. However, using naturally occurring melanogenic organisms might have certain downsides, such as the need to use complicated conditions to activate the synthesis of melanin. Utilizing complex media makes purifying processes more difficult and increases the risk of producing melanin with undesirable chemical components. In turn, these issues can be solved by using genetic engineering techniques to create new melanogenic strains or to enhance the expression of naturally occurring genes involved in melanogenesis. Furthermore, it is increasingly easier to do so thanks to the growing body of information available regarding the biochemistry and genetics of melanin synthesis in many species. Through these innovative techniques, new melanogenic strains have been developed that can produce melanin from simple carbon sources. These efforts have resulted in strains and processes capable of producing melanin polymers at the gram scale (Table 1).

Tyrosinase gene overexpression is the primary genetic modification used to create or enhance melanogenic organisms. This process generally involves cloning the tyrosinase gene in a replicative plasmid vector controlled by an inducible promoter. By adding inducers, this method allows exact control of the amount and timing of gene expression, enabling the optimization of the production process. Furthermore, antibiotics must be added as a selective pressure when employing expression plasmids, for example, in cases when it is necessary to prevent the development of plasmid-less cells. The requirement for a chemical inducer to be present in the culture medium is another issue. Antibiotics and inducers drive up manufacturing costs and make purifying processes more challenging. Hence, alternative antibiotic-free plasmid selection methods, as well as non-chemically based gene induction techniques, need to be developed, including process optimization, to produce melanins at grams per liter level to simplify purification processes and to reduce production costs. (Vidal et al. 2008).

When comparing the strains that transform melanin precursors present in the culture medium, it can be observed that in several of the reports reviewed so far, the melanin titers and volumetric productivities are lower in processes where the production strain used was modified by metabolic engineering where the objective was challenging to convert the carbon sources into melanins (Table 1). In these cases, the reported titers and productivities for eumelanin are lower than those that have been seen when L-tyrosine was used as a precursor (Santos et al. 2012). This fact demonstrates that there may still be room for improvement in the production process and strain engineering.

To improve the currently available melanin production strains, the use of synthetic biology, mutagenesis techniques, and adaptive laboratory evolution (ALE), should be assessed (Bassalo et al. 2016). Specifically, the engineering of complicated phenotypes may be possible with the application of ALE. In one study, ALE was paired with a synthetic biosensor module that reacts to the intracellular concentration of aromatic amino acids, which resulted in an improved strain of *S. cerevisiae* able to produce muconic acid (Leavitt et al. 2017). This last strain displayed an

increased flux in the common aromatic amino acid pathway; thus, it could be further modified to enhance L-tyrosine synthesis by following established methods. The *S. cerevisiae* strain created in the such study could also serve as a platform for eumelanin production if such modifications are made. In a different publication, an *E. coli* strain expressing the MelA tyrosinase from *R. etli* was used to create a high-throughput screen for the manufacture of L-tyrosine by linking the synthesis of this amino acid to the production of melanin (Santos and Stephanopoulos 2008). This technique was used to find *E. coli* strains that have increased L-tyrosine synthesis capacity. In said work, *E. coli* was genetically modified using metabolic engineering techniques which resulted in high-level L-tyrosine synthesis. Global transcription machinery engineering (gTME) was applied to this strain to increase its capacity to synthesize L-tyrosine (Alper et al. 2006). This technique was performed by producing two different gTME libraries of the RNA polymerase *rpoA* and *rpoD* subunits in the modified strain of *E. coli*. Based on the melanin color intensity of colonies from these libraries, improved L-tyrosine producers from these two libraries were identified on agar plates. When compared to the modified parent strain, three mutant isolates showed a twofold increase in L-tyrosine titer (Santos et al. 2012). Additionally, it should be highlighted that these strains may be used for eumelanin production from glucose.

Tyrosinase activity is one of the factors limiting production in strains engineered to synthesize melanin from a simple carbon source (Chávez-Béjar et al. 2013; Mejía-Caballero et al. 2016), and it could also be limiting the synthesis of melanin in other modified strains. Thus, it is crucial to evaluate tyrosinase enzymes from various biological sources to identify those with the required characteristics for biotechnological application. The extensive genome and metagenome data that is currently available should provide many genes encoding tyrosinases that can be evaluated experimentally for melanin production. Meanwhile, protein engineering could be a practical way to enhance this family of enzymes. Furthermore, an advantage of working with tyrosinases is the simple activity assay based on visual detection of melanin production, which allows for high-throughput selection methods (Santos and Stephanopoulos 2008).

Despite the technological advancements in the design of strains and methods for the synthesis of melanin, there are still many fundamental concerns that need to be resolved. For instance, the kinetics of melanin polymerization is a significant issue, as it is believed that after being produced in the cytosol, melanin precursors leave the cell and begin to polymerize in the culture media. Consequently, as the polymer increases in size, a wide variety of melanin molecules are produced. These macromolecules should have unique physical characteristics. For example, distinct eumelanin isolates from different manufacturing cultures exhibit a range of hues, from yellow to black (Chávez-Béjar et al. 2013). Therefore, it is crucial to research the kinetics of melanin polymerization in production cultures and the features of polymers of specific sizes as this information could help isolate products with specific attributes.

As observed in the publications examined in this chapter, most of the published research on microbial melanin production is focused on eumelanin. This makes

sense, as it is a polymer that has undergone considerable characterization since it is the type of melanin found in humans. Therefore, the produced eumelanin may be applied in the medical and cosmetic fields. Additionally, it should be emphasized that in terms of their chemical composition, melanins are a diverse set of polymers. In consequence, only a small portion of this chemical diversity has been studied thus far. Yet, processes used for the production of catechol, caffeic acid, and protocatechualdehyde melanins have been reported, in addition to eumelanin. Therefore, it stands to reason that different forms of melanin would operate differently depending on the application. For example, a recent study found that protocatechualdehyde-based melanin performed better than eumelanin or caffeic acid melanin when used as a pigment in soft contact lenses (Ahn et al. 2019). Additionally, synthetic non-natural amino acids and other substances that tyrosinases can use as substrates can be used to create non-natural melanins (Della-Cioppa et al. 1990). As a result, a wide variety of this class of polymers is expected to be produced soon. Finally, it is expected that the range of applications for these aromatic polymers should significantly increase when new strains and production techniques for novel natural and synthetic melanins are developed.

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Extraction, Purification, and Characterization of Microbial Melanin Pigments

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1 Introduction

'Melanins' are natural polymeric pigments present in all forms of life, having a heterogeneous origin with an extensive diversity extending from structure, and functions to different colored pigments (Gosset 2017; Stepien et al. 2013). The melanin word was derived from 'melanos' meaning dark. However, a Swedish scientist proved the appearance of melanin in the 1840s after isolating the pigment from the iris of the eye. The initial procedure for the formation of polymeric pigments is via the oxidation of phenolic or indolic monomeric substrates involving enzyme catalysis. Melanin has slowly diversified from three different types into five different classes based on the monomeric unit involved in their formation. Those five types are eumelanin, pheomelanin, allomelanin, pyomelanin, and neuromelanin. Eumelanin and allomelanin contribute dark brown to black coloration to the cells

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unbiased for any specific kingdom. Whereas pheomelanin is mainly found in the animal kingdom and provides yellow to red pigmentation to the cells (Nicolaus 1968; Pralea et al. 2019). The precursor units of these melanins play an important role in understanding the synthesis, structure, and function of these pigments. Precursor units for the polymer eumelanin consist primarily of indole-type monomeric units that are formed as a result of L-tyrosine or L-DOPA (L-3,4-dihydroxyphenylalanine) oxidation reactions. Similarly, pheomelanins are also the products of tyrosine as eumelanins, however, cysteine moieties are incorporated in their structures (Singh et al. 2021). The synthesis of allomelanins is quite different and is derived by the oxidation of nitrogen-free diphenols such as catechol, 1,8-dihydroxynaphtalene, and γ-glutaminyl-3,4-dihydroxybenzene. Pyomelanins are polymers of 2,5-dihydroxyphenylacetic acid, a byproduct of tyrosine metabolism, and display better photo- and thermo-stability. Whereas neuromelanin is synthesized from catechol and quinones in the human substantia nigra (Haining and Achat-Mendes 2017; Pralea et al. 2019). Knowledge of the structure and biosynthetic pathways that lead to the various melanins found in nature can act as inspiration for the development of new artificial pigments and their materials. Melanins are well-known for providing pigmentation to cells, however, they play many major roles in different niches of life. Melanins provide shielding from harmful radiations, perform oxidation of reactive oxygen species, are responsible for a range of functions across kingdoms, help with defense mechanisms in arthropods, molluscs, and microbes, and enhance pathogenicity in various fungi and bacteria (Singh et al. 2021). Further, due to the intramolecular electronic interactions, melanins have applications in semiconductors (Bothma et al. 2008), as metal chelators, as optical imagers (Abbas et al. 2009), extending to cosmeceuticals and pharmaceuticals, MRI probes, soil bioremediations, etc. (Martinez et al. 2019). The presence of melanin plays an irreplaceable role in human lives, wherein the absence of melanin can lead to diseases like cancer, vitiligo, Waardenburg syndrome, etc. Despite such promising and diverse attributes, the complete potential of melanin is not harvested because of its heterogeneous nature, resulting in a lack of specific genetic makeup responsible for melanin biosynthesis and sequential metabolic pathways. Further, the capabilities of microbes to employ multiple precursors like tyrosine and DOPA for melanin synthesis ultimately leads to a complex process of biosynthesis (Cao et al. 2021). The isolation and complete characterization of melanin is very difficult because of its insolubility in organic solvents. The narrow-spectrum solubility of melanins makes the extraction process costly and hence reduces its industrial production (Borovansky and Riley 2011; Sun et al. 2016). As a result of such physical problems, the isolation of melanin from eukaryotic sources is hindered; in such conditions, microbial melanin can pave the way (Pavan et al. 2020; Sun et al. 2016). Culturing aspects of microbial melanin makes them feasible for easier upscaling for commercial production and efficient utilization in various sectors.

2 Extraction of Melanin

Due to the amorphous nature and structural diversity, extraction of melanin employs different methods which highly rely on factors like melanin source (fungal, bacterial, human hair) and its cellular localization (intracellular/extracellular) (Aghajanyan et al. 2005, 2017; Gómez-Marín and Sánchez 2010; Tarangini and Mishra 2014). For example, extracellular melanin extraction involves acid precipitation (Choi 2021), whereas intracellular melanin extraction employs alkali extraction, acid precipitation, ultrasonic-assisted extraction (Hu et al. 2015; Zou et al. 2010), and microwave-assisted extraction (Lu et al. 2014). The most conventional method for melanin extraction is alkali extraction and acid precipitation as melanins normally solubilize in alkaline solutions and precipitate as sediments in acidic solutions (Lu et al. 2014; Sajjan et al. 2010; Sun et al. 2016). Many alkali solutions such as sodium hydroxide, potassium hydroxide, ammonium hydroxide, calcium hydroxide, etc. are reported to be used at different concentrations of 0.1, 0.5, 1, 1.5, and 2 N for melanin extraction, whereas for precipitation purposes, double strength acid like HCl is used under slow magnetic stirring conditions. Many research groups have employed this conventional method to extract melanin from different microbial sources as summarized in review articles (Singh et al. 2021; Choi 2021). Apart from alkali extraction assisted with ultrasonication/microwave, an enzymatic method is also used for extraction of intracellular melanin due to the ability of enzymes to increase hydrolysis as well as degradation of impermeable cell walls. This method mainly utilizes specific cell wall lysing enzymes such as guanidine thiocyanate for protein denaturation and a serine proteinase for cleavage (Dadachova et al. 2007; Youngchim et al. 2004). This traditional method is economically feasible but has low extraction efficiency and is time-consuming; so nowadays there is a trend for advanced cavitation-based extraction methods which minimize the use of toxic solvents, improve extraction yield, shorten extraction duration, and use green solvents that are recycled and reusable (Ghadge et al. 2022; Panda and Manickam 2019; Zou et al. 2010). For example, melanin is extracted from *Streptomyces hyderabadensis* 7VPT5-5R using tetrabutylammonium hydroxide (40% w/w TBAOH in water) solvent which led to a 66% increase in the yield of melanin in comparison to the conventional method of extraction (Ghadge et al. 2022).

Cavitation is a phenomenon where a small low-pressure vapor-filled cavity is formed due to a rapid change in pressure under the liquid medium. Based on this phenomenon, various cavitation-based extraction techniques such as ultrasound-assisted extraction (UAE), negative-pressure cavitation (NPC) extraction, microwave-assisted extraction (MAE), and hydrodynamic cavitation extraction (HCE) have been reported (Lu et al. 2014; Zou et al. 2010). In 2010, melanin from *A. auricula* fruit bodies was extracted using cavitation-based UAE (ultrasound-assisted extraction) technology due to its numerous benefits in comparison to conventional ones such as improved extraction yield, reduced power consumption, and extraction time (Zou et al. 2010). UAE employs ultrasound pressure waves due to the resulting energy generated from these collapsing cavitational

bubbles that provide greater penetration of the solvent into the cellular material and increases mass transfer to and from interfaces. This also causes disruption of cell walls and the release of cellular materials that ultimately leads to increased extraction yield. In UAE, cavitation occurs due to the passage of ultrasound waves in the liquid medium. Whereas if it occurs, due to the pressure variations in the flowing liquid concerning the change in the geometry of constriction, then it is called hydrodynamic cavitation extraction (HCE). The limitations associated with UAE are attenuation of ultrasound waves for highly concentrated dispersed phases and lack of uniformity for dispersed extract materials (Panda and Manickam 2019). In the case of NPC, the creation of negative pressure governs cavitation. NPC extraction proved to be more effective in the extraction of heat-sensitive compounds such as polyphenols and polysaccharides (Panda and Manickam 2019). MAE uses microwave energy to heat solvents in contact with a sample to partition analytes from the sample matrix into the solvent (Lu et al. 2014; Tatke and Jaiswal 2011), but a problem with this technique is the rapid increase in temperature of the extraction mixture that may terminate the extraction process early due to the boiling of the solvent. Thus, the desired compounds are not sufficiently diffused from the material into the solvent and consequently, the extraction yield is reduced (Chuyen et al. 2018). The UAE method was reported to yield 37.33% pure melanin (Hou et al. 2019), whereas another study reported that a purification yield of 11.08% could be achieved through an MAE method, which was 40.43% higher than that obtained by alkali extraction and acid precipitation (Lu et al. 2014).

Due to the presence of various biological sources for production, structural diversity, amorphous nature, and insolubility of melanin have resulted in the non-availability of a standard method for extraction and purification of melanin. But the above-described modern extraction techniques can be alternatively used owing to numerous advantages over conventional methods like reduced energy and solvent consumption, increase in extraction yield, improvement of extract quality, reduction in extraction time, and protection of thermo-labile compounds in the extract (Panda and Manickam 2019; Tatke and Jaiswal 2011; Zou et al. 2010).

3 Purification of Melanin

Purification of melanin can be achieved by performing different steps such as redissolution, centrifugation, acid hydrolysis, precipitation, boiling, dialysis, and column chromatography followed by successive washing steps with organic solvents such as chloroform, petroleum ether, ethyl acetate, acetone, or absolute ethanol (Aghajanyan et al. 2005; Choi 2021; Dong and Yao 2012; Madhusudhan et al. 2014; Selvakumar et al. 2008; Suryanarayanan et al. 2004). Acid hydrolysis is usually performed using 6 M HCl to remove impurities like carbohydrates and protein associated with melanin pigment, whereas organic solvent aid helps in the removal of secondary metabolites. Non-hydrolyzable melanin are redissolved in NaOH and precipitated out with HCl followed by several washing steps with organic solvent

and deionized water. An additional boiling step helps to avoid the formation of melanoidins (Eskandari and Etemadifar 2021). Dialysis was reportedly used to remove salt and other impurities like low molecular weight organic and inorganic compounds (Wibowo et al. 2022). Finally, washing with absolute ethanol removes water molecules with the melanin pigment. The resulting content is lyophilized and stored as pure melanin. In 2022, one research group purified melanin with help of dialysis tubing and the resulting melanin yield was 670 mg/L, which was significantly higher than the 116 mg/L obtained from the acid precipitation method (Wibowo et al. 2022). The combination of the above-described extraction technologies has been observed to bring synergistic extraction yield compared to the conventional method paving way for the development of more advanced and efficient techniques for melanin extraction and purification.

4 Preliminary Confirmation of Melanin

The melanin pigment is insoluble in water and almost all inorganic/organic solvents. Based on this observation, a solubility test is used for the preliminary confirmation of melanin. Melanin is slightly soluble in dimethyl sulphoxide (DMSO), in water at alkaline pH, phosphate saline buffer (pH 7.2), and on the other hand, melanin gets precipitated at acidic conditions (pH 2) (Kamarudheen et al. 2019; Pralea et al. 2019). Melanin contains different functional groups in the structure like catechol, NH, and COOH that shows reactivity with certain chemicals and shows distinguishable characters helping in the identification of melanin type. When melanin is treated with hydrogen peroxide (H_2O_2), it results in decolorization due to oxidative degradation of melanin. In this reaction, the nucleophilic attack of OOH^- ions from H_2O_2 leads to the production of quinone epoxide causing bleaching of melanin (Korytowski and Sarna 1990). The reduction of $AgNO_3$ occurs when it reacts with microbial melanin leading to the formation of gray colour on the walls of the test tube due to the precipitation of melanin (Carriel et al. 2011; Lopusiewicz 2018). Similarly, when melanin reacts with $KMnO_4$, it leads to a change in color from brown to green with precipitation and decoloration of the solution. The color change is due to the reduction of $KMnO_4$ by the redox property of melanin. This reaction indicates the presence of quinone and phenol groups in the structure of melanin (Aghajanyan et al. 2005).

Chemical degradation methods are also used in the identification of different types of melanins based on the analysis of their degradation products. Melanin gets degraded by strong oxidants and reductants yielding different types of degraded products. These degraded products are further separated by chromatographic techniques and identification by different spectroscopic techniques (Dzierżęga-Lęcznar et al. 2002). Generally, gas chromatography and mass spectrometry with pyrolysis (Py-GC/MS) are used to identify pyrrole, indole, and their alkyl derivatives as pyrrole di- and tricarboxylic acids are precursors of eumelanin biosynthesis (Dzierżęga-Lęcznar et al. 2002, 2012). Likewise, in the case of pheomelanin,

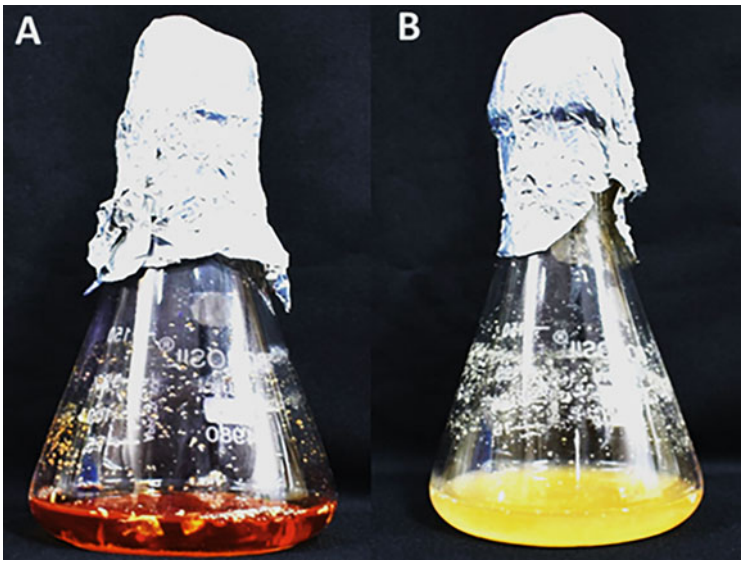

Fig. 1 Melanin synthesis inhibition: control (**a**) and treatment with kojic acid (**b**) (Ghadge et al. 2022)

precursors are thiazole or benzothiazole carboxylic acids including some other melanin pigment markers, i.e., isomeric aminohydroxyphenylalanines and aminohydroxyphenylethylamines (Donato and Napolitano 2003; Dzierżęga-Lęcznar et al. 2012; Greco et al. 2009; Ito et al. 2019).

The melanin synthesis inhibition is used to identify the enzyme involved in melanin biosynthesis by the producer strain and type of melanin. The most commonly used inhibitors are kojic acid as a tyrosinase inhibitor, tricyclazole as a DHN-melanin inhibitor, and sodium azide as a laccase inhibitor. Inhibition of melanin synthesis by kojic acid was observed for strains *Actinoalloteichus sp.* MA-32, *Streptomyces hyderabadensis* 7VPT5-5R (Fig. 1) (Ghadge et al. 2022; Manivasagan et al. 2013). The inhibition of melanin-like pigments by sodium azide was previously reported for *Bacillus weihenstephanensis*, *Bacillus subtilis* 4NP-BL (Fig. 2) (Drewnowska et al. 2015; Ghadge et al. 2020). A higher concentration of sodium azide (>50 μg mL^{-1}) led to the inhibition of bacterial growth. The inhibition of melanin synthesis is reported in the black yeasts *Trimmatostroma salinum*, *Phaeotheca triangularis* and *Hortaea werneckii* by use of tricyclazole, a specific inhibitor for polyketide melanin biosynthesis (Kogej et al. 2003).

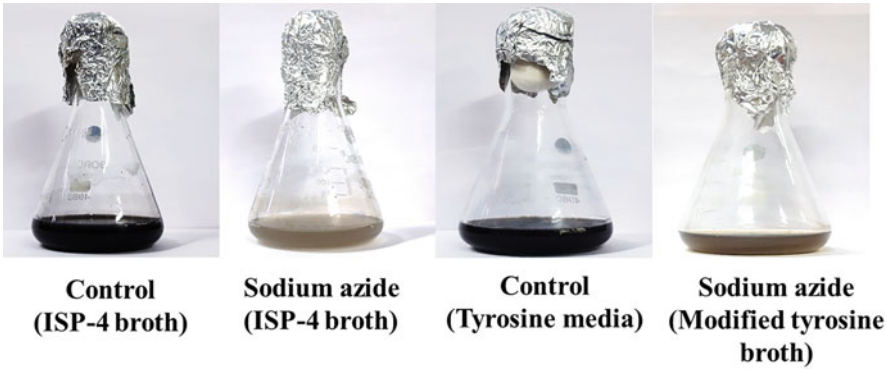

Fig. 2 Inhibition of melanin synthesis by sodium azide (Ghadge et al. 2020)

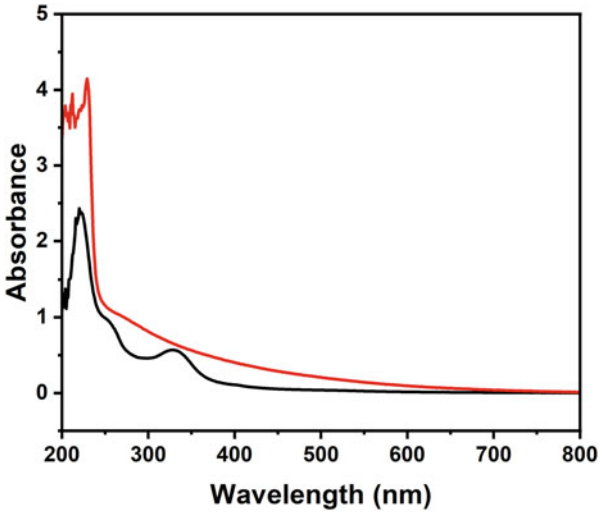

Fig. 3 UV/vis spectra of purified melanin (black) and synthetic melanin (red) (Ghadge et al. 2022)

5 Characterization of Melanin

5.1 UV–Visible Spectroscopy

UV/visible spectroscopy is the most common and widely accepted technique used for the primary identification/confirmation of melanin. The absorbance of melanin monotonically increases towards the UV region (high energy). It decreases towards the visible region (low energy radiation), which is a unique characteristic property found in melanin and this property is employed for melanin identification (Fig. 3) (Gao and Garcia-Pichel 2011). Melanin of microbial origin shows maximum absorption in the almost entire UV region (200–400 nm). The ratio of A_{650}/A_{500} is used to quantify eumelanin concentration from the mixture as well as for the differentiation

of eumelanin and pheomelanin. The ratio of melanin above 0.25 is referred as eumelanin, while a ratio below 0.15 is considered pheomelanin (Saini and Melo 2015).

As we know, photoprotection is the main function of eumelanin and it is due to broadband absorption, but its origin is still mysterious. The recent theoretical (computational) and experimental studies indicated that broadband absorption emerges from the chemical disorder of eumelanin due to the chemical diversity of building blocks at the oligomeric level and it is widely accepted (Singh et al. 2021). Based on previous studies, the oligomeric model is most acceptable for the origin of broadband absorption due to the formation of different types of chemically-modified oligomers having different configurations and conformations. These oligomers interact with each other mainly by π–π interactions, which affect the electron delocalization resulting in the absorption spectrum of eumelanin (Arzillo et al. 2012). The different studies speculated that non-covalent interactions play a major role during eumelanin oligomerization in multilevel structural organization, like stacking interactions (π–π) (Chen et al. 2013). The close association of oligomers in a multilevel organized structure determines the alteration of absorption spectra. DHI and DHICA precursors are used to study the aggregation model of eumelanin and its UV/VIS properties to understand broadband absorption (Ju et al. 2018). Their investigation revealed that monomeric units (DHI and DHICA) undergo polymerization from monomer to oligomers and then oligomers form small stacks. These stacked oligomers further oligomerize and stacked to form larger aggregates called protomolecules (Spano 2010). Their findings provided strong support to postulate that broad absorption bands due to delocalization of intrinsic π-electron within integral eumelanin oligomers and altered by other interactions such as attractive stacking between aromatic rings and aggregation in the stratified framework of complex structure (Simpson et al. 2014).

The recent studies on eumelanin supported the hypothesis of an aggregate model of eumelanin but its detailed structure is still unknown due to a lack of experimental proof. Because eumelanin precursor is redox-active and has different polymeric sites it makes it very difficult to understand its structure-property relationship. The complexity and heterogeneity of eumelanin hinder the experimental approaches to understanding its structural and optical properties (Yildirim and Bayindir 2014).

5.2 Electron Paramagnetic Resonance (EPR)

"Paramagnetism" is one of the main fundamental properties of eumelanin. Eumelanin is a redox-active macromolecule due to the presence of indolequinone group precursor molecule 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA). Eumelanin contains different paramagnetic centers, which is due to quinone groups in the structure (Gessler et al. 2014). Recent studies on eumelanin identified two types of paramagnetic centers in solid-state- and liquid-state. These centers are differentiated from each other by their *g*-factors and

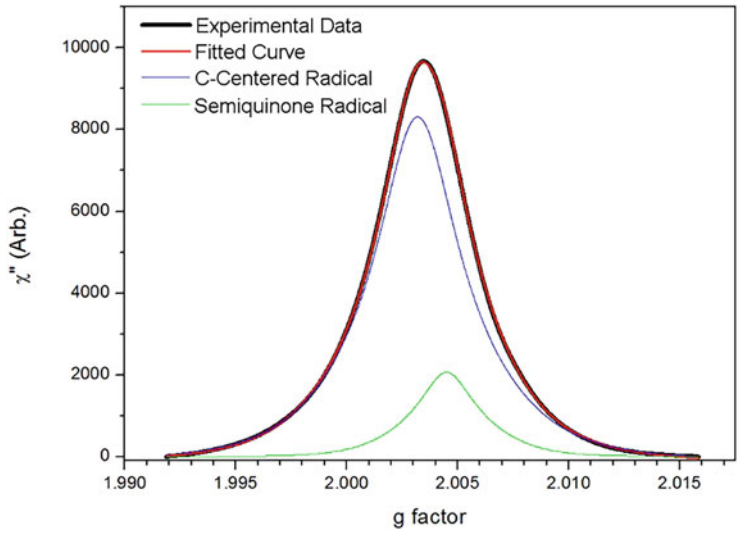

Fig. 4 Deconvolution of the integrated EPR spectrum associated with the basic sample. The spectrum is best explained by the presence of two free radicals, the more dominant carbon-centered signal ($g = 2.0032$) and the small semiquinone signal ($g = 2.0045$). Data at a water content of 14.0% and microwave power of 0.71 mW1/2 (Mostert et al. 2013)

line shapes (Paulin et al. 2019). It is hypothesized that carbon-centered radicals (CCR) come from the internal skeleton of the eumelanin structure, and it is prevented by the external environment (Paulin et al. 2021a). CCRs were mainly found in solid samples (dry powder) and they are less affected by temperature and pH. The semiquinone free radicals (SFR) are observed in the liquid state (solution) and strongly affected (change in intensity of signal) by pH. Furthermore, these two types of free radicals have constant *g*-factors, 2.003 for CCR and 2.005 for SFR (Fig. 4).

Paulin et al. 2019 performed experimental and theoretical work on the paramagnetic behavior of melanin. They used a computational model (DFT) to compare *g*-factors and hyperfine coupling constants to correlate the structure and free radical centers of melanin. The precursors of eumelanin, DHI, and DHICA, are redox-active molecules with different redox states (Fig. 5), containing both positive and negative charged states having an unpaired electronic and zwitterionic state of an odd number of electrons (Cuba et al. 2021).

The results show that the partially oxidized molecules (indolequinone and semiquinone) are associated with semiquinone free radicals (SFR), while fully reduced state (hydroquinone) and nitrogen-protonated species (DHICA) are associated with carbon-centered radicals (CCR). The positively charged species are referred to as a secondary product of eumelanin and such types of species are found in eumelanin derivatives (Paulin et al. 2019). The results revealed that the

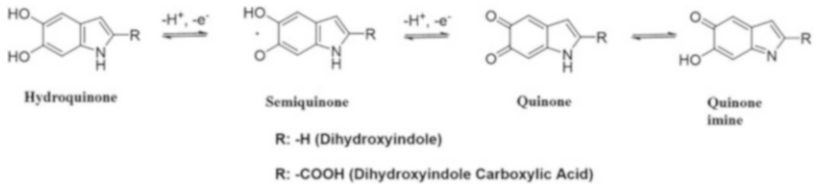

Fig. 5 Different redox forms of melanin monomeric structures: R = H (DHI) or COOH (DHICA) (Reali et al. 2021)

presence of three types of EPR signals comes from typical eumelanin structures. Based on experimental data, it is observed that the EPR signals of CCR from two precursors DHI and DHICA can be differentiated. In this work, the main focus was on the differentiation of paramagnetic species present in eumelanin. The experimental results of EPR spectra of eumelanin and its precursor molecules (DHI and DHICA) revealed that it contains three different types of free radicals, among them, two are CCRs and another is SFR. Investigation of EPR variables and energy state of different substructures of eumelanin indicated that the carbon-centered radicals (CCRs) mainly come from the reduced state of eumelanin structural organization (Paulin et al. 2019, 2021a).

Eumelanin is associated with the presence of *O*-semiquinone radicals, while pheomelanin contains *O*-semiquinoneimine radicals. EPR spectrum can distinguish different types of melanins, eumelanin shows a single line with a hyperfine shape at variable temperature (low to high) and is less affected by microwave power, while that of pheomelanin has a complex line shape with hyperfine structure arising through the interplay between free electrons with electrons of adjacent nitrogen nuclei (Zdybel et al. 2017). Natural melanin can be differentiated from synthetic melanin by observing spectral patterns and line width. These characteristic differences in line shape and width of the EPR spectrum were used to distinguish natural melanin from synthetic melanin.

5.3 Electrical Properties of Melanin

Melanin has two main properties, broadband absorption, and redox activity, which are responsible for electric charge conduction. The property of melanin to conduct electrical charge was studied in the 1970s, and its semiconducting charge behavior was demonstrated by Mott-Davis amorphous semiconductor (MDAS) theory (McGinness 1972; McGinness et al. 1974; Powell and Rosenberg 1970). The electric conductivity of melanin is due to the presence of different functional groups (carboxylates, aromatic amines, and catechols) having different redox states which generate protons and electrons during oxidation-reduction reactions. The various types of charge transport mechanisms are reported to be present in eumelanin i.e. intra- and inter-atomic interaction (H-bonding), and π–π interaction (Gouda

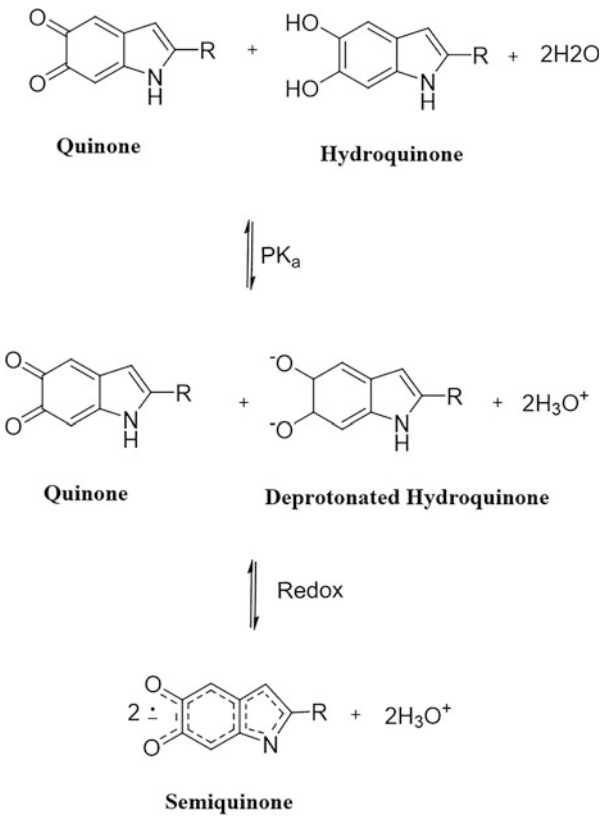

Fig. 6 The comproportionation equilibrium reaction involves two steps: hydroquinone deprotonates leading to hydronium formation; and the hydroquinone reacts with a quinone in a 1-electron redox reaction to form semiquinone radicals, a moiety of an intermediate oxidative state (Sheliakina et al. 2018)

et al. 2020). The current charge transport model of melanin was explained by comproportionation equilibrium (Fig. 6), where quinone and hydroquinone species react in the presence of water to generate semiquinone species and protons. From recent work, it is observed that the previous amorphous semiconductor model is replaced by the comproportionation equilibrium model in which the formation of free radicals (electron) and hydronium ions (protons) takes place where hydronium ions are mobile charge carriers showing hybrid ionic-electronic behavior (Mostert et al. 2012; Sheliakina et al. 2018; Tian et al. 2019).

5.4 Fourier-Transform Infrared Spectroscopy (FT-IR)

FT-IR spectroscopy utilizes the infrared part of the spectrum comprising wavelengths from 4000 to 700 cm^{-1}. Absorption of a specific wavelength in the infrared spectrum is a characteristic of functional groups. This absorption in the infrared spectrum varied by wavelength and intensity is measured by FT-IR spectroscopy and these details are used to detect the presence of different functional groups in

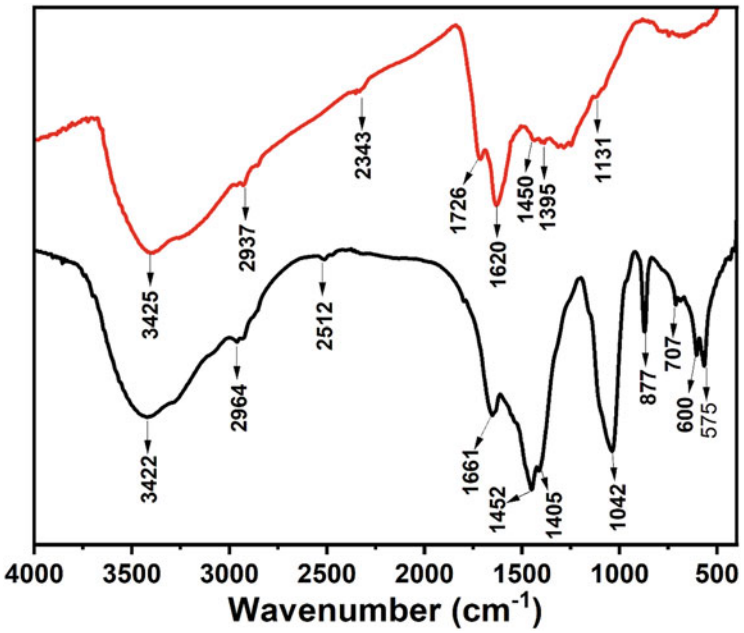

Fig. 7 FT-IR spectra of purified melanin (black) and synthetic melanin (red) (Ghadge et al. 2022)

given samples (Mbonyiryivuze et al. 2015). The complexity of melanin structure made it difficult to study using spectrometry techniques. Melanin has functional groups like amides, hydroxy, carboxylic, phenolics, aromatic rings, aliphatic carbon, and indole ring. Given the different functional groups present in the melanin, FT-IR can serve as a technique for the identification of melanin, detection of impurity, metal conjugation, and type of melanin (Sajjan et al. 2013). A careful investigation of the literature reveals some signature patterns of melanin in IR spectra. A broad peak of O–H and N–H stretching in the range of 3600–2800 cm^{-1}, a sharp peak due to stretching of CH_3 group in the range of 2900–3000 cm^{-1}. In some cases of eumelanin, CH_3 and CH_2 stretching absorption give 2 to 3 peaks in the range of 2800–3000 cm^{-1}, these peaks are generally small and fall in the broad peak of O–H and N–H stretching. Stretching of C=O, C=C, and COO– represented by a sharp deep peak in the range of 1620–1650 cm^{-1}. Next to this small peak in the range of 1500–1600 cm^{-1}, there is a characteristic peak of melanin having an indole ring due to the bending of the N–H group. Other characteristic peaks of melanin in the range of 1400–1500 cm^{-1} are due to aliphatic carbons. In this range, peaks appear due to C–H, CH_2–CH_3 bending vibration. The phenolic stretching vibration of microbial melanin gives a small peak in the range of 1200–1300 cm^{-1}. Weak peaks in the range of 900–600 cm^{-1} are due to N–H wagging, aromatic C–H, and the substitution of alkene C–H (Ammanagi et al. 2021; El-Naggar and El-Ewasy 2017; Ghadge et al. 2020; Vasanthabharathi et al. 2011) (Fig. 7).

5.5 X-Ray Photoelectron Spectroscopy (XPS)

XPS is used to analyze the elemental composition and state of elements on the surface of the solid samples. Due to the problems in solubility of the melanin, XPS is quite a handy technique to analyze the composition, hybridization, oxidation state of elements, and conformation of functional groups present in the melanin. To analyze the average surface chemistry of the sample, XPS uses low energy X-ray (Soft X-ray). This low-energy photon then generates a photoelectron from the atoms present on the sample surface. Thus, XPS utilizes the difference in the binding energy of the electrons in different atoms by measuring the kinetic energy of the photoelectron emitted from the sample surface and uses this data to generate the information about surface chemistry of the sample (Van der Heide 2011).

Selection of the energy range of photoelectrons allowed screening of one element at a time. Each atomic orbit of every element has characteristic binding energy, thus giving a specific peak for every atom. The binding energy of electrons depends on the chemical and physical environment of the atom. This helps in the identification of different types of bonds formed by elements in the material. For example, in the process of screening of C1s photoelectron, the presence of C–O–C, C=O, C=OH, –O–C=O, –O–CO–O will cause the sifting of the peak, while the main peak consists of signals of C–C, C=C, and CH (Bregadiolli et al. 2021). The values for different states of elements or chemical shifts are available in databases like 'The International XPS Database' (https://xpsdatabase.com/).

A study on the eumelanin from four different sources using XPS gives an insight into the structure of the composition and structural differences among them. Elemental composition of the natural eumelanin from sepia, crow feather, turkey feather, and human hair gives us a range of composition in eumelanin like carbon 66.3–67.4%, oxygen 18.8–22.2%, and nitrogen 8.7–12.9%. Natural eumelanin is made up of DHI, DHICA, and their derivatives, so the calculation of the ratio between C=O and O–C=O and the amount and concentration of O–C=O is directly equal to the amount of DHICA and its oxidized forms of monomers. The scanning of the C1s will give the largest peak at 285 eV, peak fitting of this peak can be done using chemical shifts for C–C(H) at 284.9 + 0.2 eV, C–OH/C–N 286.3 + 0.2 eV, C=O at 288.1 + 0.2 eV, O–C=O at 289.3 + 0.2 eV approximately (Xiao et al. 2018).

The surface layer of the material is susceptible to absorption of water, CO, and oxidative modification, which can result in the wrong quantification of the composition of melanin. The problem of surface modification can be eliminated by a cluster beam of 500 Argon ions (Paulin et al. 2021b).

5.6 Raman Spectroscopic Analysis

Peak around 1590 cm^{-1} denoted the stretching vibrations of aromatic C=C bond in the indole ring. Bands around 1690 cm^{-1} arise due to quinone C=O stretching.

Signals around 1510 cm^{-1} are related to the C=N stretching. The band around 1341 cm^{-1} is observed due to aromatic C–N stretching of the indole structure. C–O stretching of the carboxylic acid is visible at about 1220 cm^{-1} (Capozzi et al. 2005).

Four bands were found to be visible in the spectrum of pheomelanin in the range of 500–2000 cm^{-1}. A band around wavenumber 500 cm^{-1} is considered an out-of-plane deformation of the phenyl ring in the benzothiazine structure. The stretching vibrations of the C–N bond are also observed in pheomelanin at about 1150 cm^{-1}. Stretching vibrations of phenyl ring arise in the Raman spectrum at around 1490 cm^{-1}. High Raman intensity in the range of 1750–2000 cm^{-1} is considered the characteristic pattern for the pheomelanin because eumelanin shows a flat line in this region (Galvan et al. 2013).

5.7 *Nuclear Magnetic Resonance (NMR) Spectroscopy*

There are four major classes of melanin named eumelanin, pheomelanin, allomelanin, and pyomelanin. The basic moiety of the eumelanin structure is comprised of 5,6-dihydroxy indole (DHI) and 5,6-dihydroxy indole carboxylic acid (DHICA). The monomeric unit in the pheomelanin is composed of a benzothiazine ring. Allomelanin is made up of the 1,8-dihydroxy naphthalene moiety. These structural differences within the different types of melanin are observed due to variations in the precursor moiety or intermediate compounds during melanin production. Spectroscopic data is important to study the structural characteristics of melanin. ^{1}H NMR and ^{13}C NMR are widely used techniques to determine the structural features of melanin.

^{1}H NMR spectrum of eumelanin showed prominent peaks of resonance around 7.286 and 7.21 ppm (Fig. 8) which are attributed to the indole/pyrrole ring. –NH groups of melanin moiety give signals around 8.00 ppm as a singlet. Peaks in the range of 3.4 and 4.4 ppm are ascribed to the protons attached to the methyl or methylene group, which are attached to nitrogen and/or oxygen atoms. Signals at 2.2 and 2.8 ppm provide evidence for the presence of the methylene group. Signals at 1.00 and 3.00 ppm denoted the presence of the –NH group lined to the indole. Resonance signals around 0.9–1.00 ppm are described for aliphatic fragments such as CH_2CH_3 and $CH(CH_3)_2$ (Barretto and Vootla 2020; Ghadge et al. 2020). In the ^{13}C NMR spectrum, peaks in the range of 120–140 ppm are due to the aromatic carbons involved in the indole or pyrrole system. The peaks from 50–60 ppm arise due to the carbon atom linked to the nitrogen. The peaks for the methyl and methylene groups are observed within the 10–40 ppm range.

^{13}C NMR spectra of pheomelanin show characteristic peaks from 30–70 ppm which resemblance to = C–S and C–H from the aliphatic chain present in cysteine. Resonance around 170–200 ppm is attributed to the carbonyl carbon. Signals around 110–160 are observed in the spectra indicating the presence of aromatic carbons (De Souza et al. 2018).

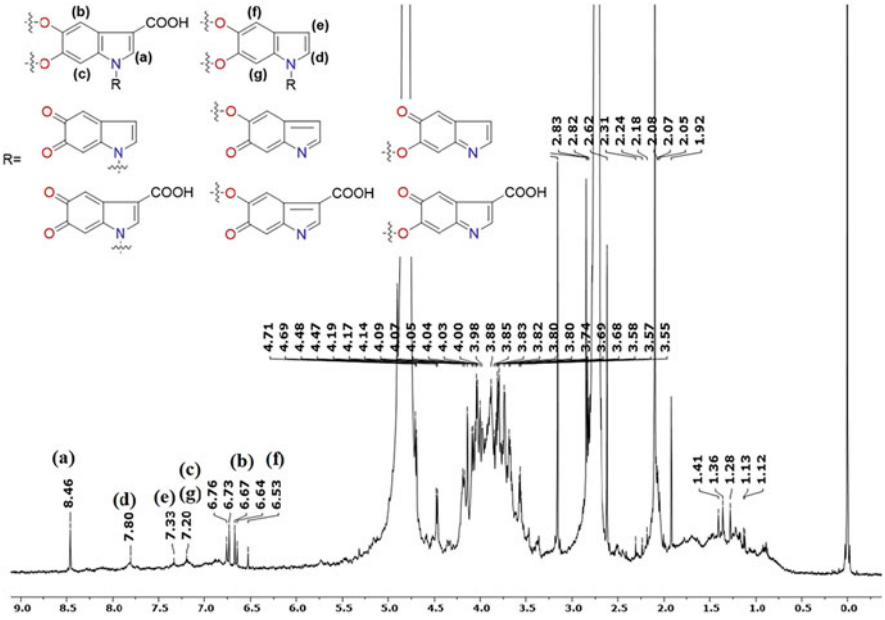

Fig. 8 ^{1}H NMR spectrum of the purified melanin (Ghadge et al. 2022)

In the past literature, allomelanin was characterized by Cross-Polarization Magic-angle spinning (CP/MS) NMR spectroscopy. ^{13}C spin echo (SE) CP/MAS NMR spectrum shows a broad range of peaks from 100 to 160 ppm due to the presence of protonated and non-protonated aromatic carbons. Peaks in the range of 20–40 ppm are due to the protonated aliphatic carbon (Singla et al. 2021). A characteristic peak of phenoxy carbon can also be found at around 152 ppm in the solid-state NMR (Zhou et al. 2019).

6 Conclusion

Microorganisms can produce different types of melanin and utilize a variety of precursor molecules for synthesis. Heterogeneity due to different sources, metabolic pathways, and precursor molecules has resulted in the complex supramolecular structure of melanin. Microbial melanin has gained interest due to its diverse functions within the host and various biological activities and also it has an advantage over other sources of melanin for production due to scalability, sustainability, and cheaper production cost. Despite such importance of melanin, its commercial utilization is restricted due to its insolubility, and low yield which results in higher production costs. The extraction and purification of melanin is a difficult job due to its diverse origin, location, and complex structure. So, there is an urgent need to

develop sustainable extraction methods which will increase the yield and solubility of melanin. The complexity and heterogeneity of melanin are the main hurdles for structure elucidation, which will need the use of different modern analytical techniques and approaches. This book chapter covers microbial melanin production, extraction techniques, and its structural properties through different analytical techniques. Due to the multifunctionality and biocompatibility of melanin, it has become a tunable biomaterial of application in various fields. Still, the fundamental structural-property relationship is not fully understood which will attract the researchers and has scope for further development.

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Exploiting Melanin-Metal Interactions for Emerging Technologies

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Abbreviations

DHI	5,6-dihydroxyindole
DHICA	5,6-dihydroxyindole 2-carboxylic acid
HOMO	Highest Occupied Molecular Orbital
LUMO	Lowest Unoccupied Molecular Orbital
OECT	Organic ElectroChemical Transistor
PEDOT:PSS	Poly(3,4-ethylenedioxythiophene)-poly(styrenesulfonate)
WEEE (e-waste)	Waste electrical and electronic equipment
XPS	X-ray Photoelectron Spectroscopy

1 Introduction

Electronics have dramatically impacted the everyday life, at the global level. The use of organic electronic materials extracted from natural sources (biosourced) opens new venues for electronics: less relying on critical chemical elements and eco-designed in terms of end-of-life scenarios, possibly including compostability (Santato and Alarco 2022). Organic electronic materials can sustain ionic and electronic transport, with implications in the concept itself of semiconductivity (Reali et al. 2021). Melanins are a family of biopigments relevant for sustainable organic electronics. Among melanins, eumelanin has been the member of the family most investigated by physicists, materials scientists and physical chemists (Reali et al. 2020, 2021). Eumelanin features a range of functional properties, such as ionic

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and electronic transport, redox activity, metal binding affinity, biocompatibility, and biodegradability (Di Mauro et al. 2017; Liu et al. 2004). These properties are relevant for optical, electrochemical, electronic, and metal recovery applications. This chapter will review the chemical, structural and physicochemical properties of eumelanin and its interactions with metal ions, focusing on recent developments and building on the state-of-the-art in this same field, we proposed in 2017 (Di Mauro et al. 2017).

2 Chemical Structure and Physicochemical Properties of Eumelanin

The term melanin indicates a set of indole/quinone-based natural pigments resulting from biochemical syntheses taking place in different living organisms (animals, plants, fungii, and bacteria) (Galeb et al. 2021; Xie et al. 2019; Cao et al. 2021).

Based on their chemical precursors, these pigments can be organized into five categories: eumelanin, pheomelanin, neuromelanin, pyomelanin, and allomelanin (Fig. 1).

In all cases, except for allomelanin, the pigments are obtained through the oxidation/polymerization of the amino acid tyrosine molecules. Allomelanins are synthesized from phenolic compounds by fungi and plants through a process of biosynthesis (Singla et al. 2021).

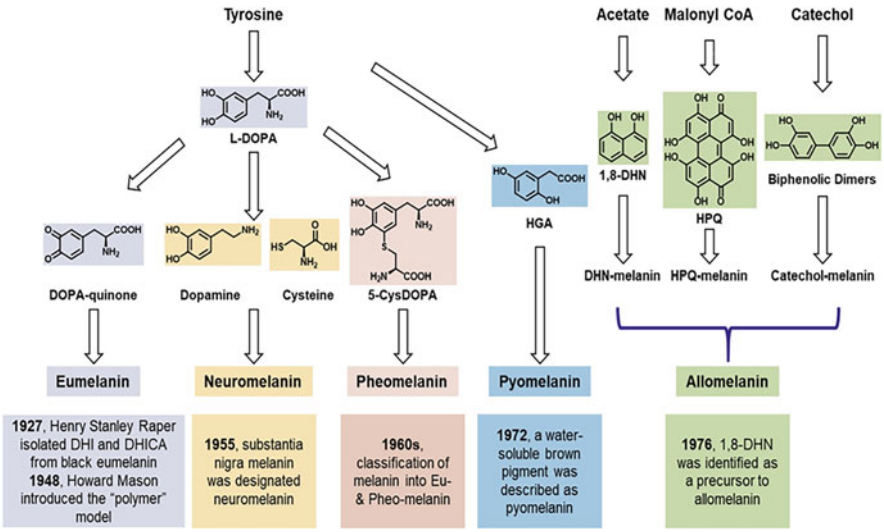

Fig. 1 Biosynthetic pathways of the structures of melanin: eumelanin, neuromelanin, pheomelanin, and the two nitrogen-free analogues, pyomelanin and allomelanin. Compilation of converging data for pathways from a wide range of literature sources with a historical timeline highlighting melanin discoveries. Adapted from ref. (Cao et al. 2021)

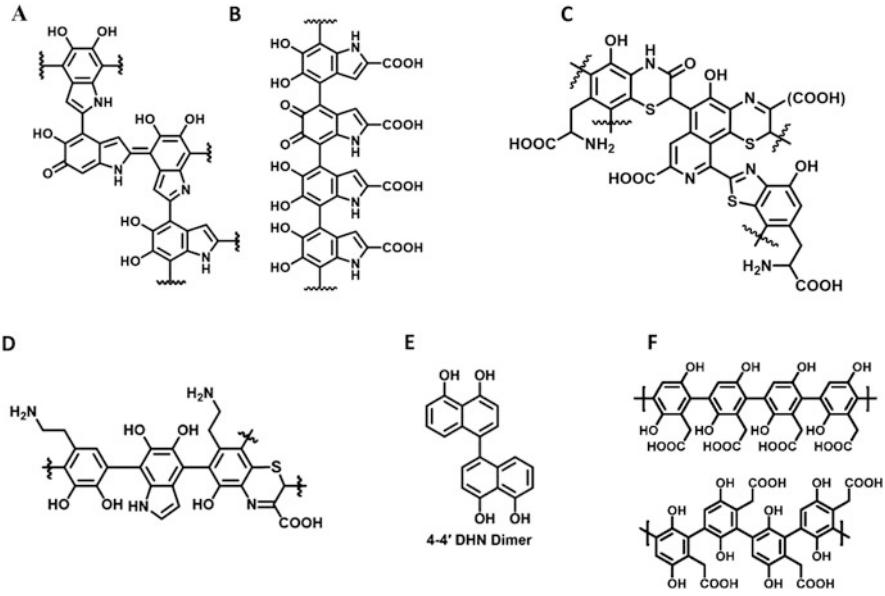

Fig. 2 Potential structures of natural melanins: (**a**) DHI eumelanin and (**b**) DHICA eumelanin; (**c**) Pheomelanin; (**d**) Neuromelanin; (**e**) Dimer of allomelanin; and (**f**) Two possible structures of pyomelanin. Adapted from ref. (Cao et al. 2021)

For any of these melanin categories, as given in Fig. 2, it is necessary to use rigorous extraction methods to isolate the melanin component from all the other components present in the medium where the biosynthesis took place.

The preparation processes and sampling techniques can drastically change the chemical composition of the extracted sample, thus affecting the physicochemical properties of the extracted samples. (Liu and Simon 2003; Madaras et al. 2010). For example, eumelanin extracted from the ink sac of cuttlefish using the "syringe" method brings about an ink including L-DOPA (L-3,4-dihydroxyphenylalanine), dopamine, and taurine. In contrast, the "milking" method bring about an ink including tyrosinase and epinephrine (Madaras et al. 2010; Derby 2014).

2.1 Eumelanin

Within the melanin family of biopigments, eumelanin has been the most explored and studied, because of its application in various fields, such as electrochemical energy storage, bioelectronics and green electronics (João Paulin and Graeff 2021). Eumelanin is a natural biomacromolecule composed of two building blocks: 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole 2-carboxylic acid form (DHICA). The molecular ratio between DHI and DHICA building blocks affects

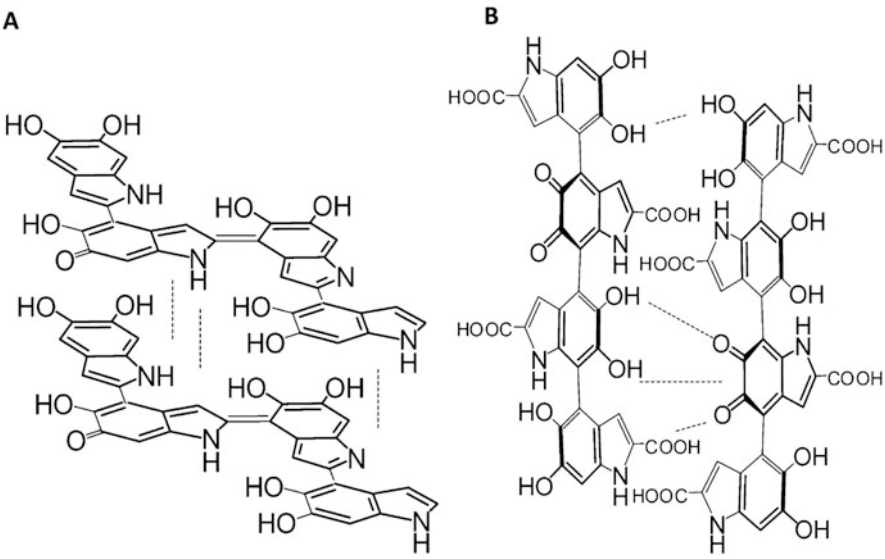

Fig. 3 Supramolecular structures of DHI- and DHICA-melanins. (**a**) DHI melanin represented as planar oligomeric scaffolds, (**b**) DHICA melanin is made up of twisted linear oligomer structures featuring atropisomerism. Adapted from ref. (d'Ischia et al. 2014)

the physicochemical properties of eumelanin (Terranova and Tamburri 2021). The presence of a quinone group in the building blocks, besides the presence of the carboxylic group in one of the two building blocks, is among the distinctive molecular features of eumelanin.

The carboxylic group is essential to differentiate the structure of eumelanin from that of synthetic polydopamine, so explored in the last decade. The absence of the carboxylic group brings about planarity in the molecular structure of polydopamine. As opposed to that, eumelanin features a structure with twists in its carbon backbone, where it is possible to observed atropisomerism by adjacent DHICA moieties (Pezzella et al. 2002; d'Ischia et al. 2014). The existence of these stable conformational isomers is due to the sizeable torsional barrier along the inter-unit bond (Fig. 3). In agreement with that, in the ultraviolet (UV)-visible spectra, no intense absorption bands are observed above 400 nm for DHICA-based melanin structures whereas intense absorption bands are observed for the DHI-based melanin structures in the visible region (Micillo et al. 2016).

2.2 *Eumelanin vs Other Melanins*

The presence of the nitrogen atom also permits to differentiate among different melanin structures. Allomelanin and pyomelanin are nitrogen-free melanins whereas in eumelanin the nitrogen atom is part of a pyrrole ring and in pheomelanin it is part

of a thiazine ring. Neuromelanin results from the combination of 5-S-cysteinyl-dopamine and dopaminochrome (Fig. 2d).

It is worth noticing that, in general, the presence of the nitrogen atom in aromatic heterocycles causes the non-bonding electron pair of the nitrogen to occupy a sp^2 hybrid orbital. Therefore, the non-bonding electrons pair will be in orbitals with significant "s" character, close to the nucleus (and as such less prone to engage in chemical bondings). Despite reports on the participation of nitrogen atoms in chelation bondings, the effects of sp^2 hybridization should be taken into consideration when studying melanin-metals interactions.

2.3 Chemical and Physical Disorder

Eumelanin features chemical disorder due to its two building blocks, several polymerization sites connecting the building blocks, and three redox states bringing about comproportionation and tautomeric equilibria (Fig. 4). The chemical disorder is associated to physical disorder, in the sense that several supramolecular organizations are possible considering the different chemical species available in the macromolecular eumelanin biopigment.

2.4 Functional Properties of Eumelanin

Beyond applications as UV–Vis absorbers, eumelanin features metal ion binding properties, radical scavenging activity against reactive oxygen species, antioxidant activity, and charge transfer/charge carrier (electronic and ionic) transport properties. In what follows, we will discuss charge transfer and electrochemical properties.

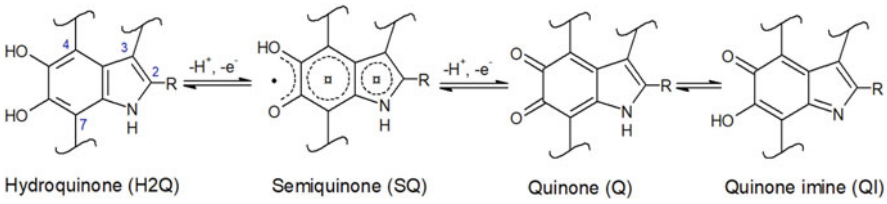

Fig. 4 Molecular structures of DHI and DHICA, with R is –H in DHI and –COOH in DHICA. The redox forms of DHI and DHICA are: hydroquinone (H2Q), semiquinone (SQ), quinone (Q) and quinone imine (QI) (the tautomer of Q). The building blocks can polymerize into eumelanin oligomers and polymers at different sites of the monomers (shown as 2, 3, 4, 7 in the figure). Adapted from ref. (Di Mauro et al. 2017)

3 Electrochemical Properties of Eumelanin: Focus on Metal Ions

When dealing with redox-active materials and devices, electrochemistry helps to shed light on electronic properties and chemical changes in materials upon charge transfer processes (Zhu and Shi 2019). Most of natural redox active materials tend to be non-soluble in aqueous media and form unstable colloidal suspensions. In addition, their immobilization on the surfaces of working electrodes can be challenging.

3.1 Aspects of Redox Properties in Eumelanin, in Presence of Alkaline and Ammonium Ions

Eumelanin can feature proton-coupled electron transfer (PCET) (Costentin et al. 2010). Considering its quinone functionality (Fig. 4), it is reasonable to make the hypothesis that, during an electron transfer process in aqueous media, $2H^+$ and $2e^-$ are simultaneously transferred.

For *Sepia* melanin (eumelanin extracted from the ink sac of cuttlefish) (Kim et al. 2014; Xu et al. 2017; Gouda et al. 2020), Kim et al. report values between −0.2 and 0.25 V vs. Ag/AgCl (Kim et al. 2014) whereas Xu et al.(Xu et al. 2017) report values of −0.06 V and 0.15 V vs. Ag/AgCl. These potentials are expected to depend on the pH value of the electrolyte (Gouda et al. 2020), although some authors report on potentials independent on pH (Serpentini et al. 2000). The interaction with alkaline metal ions produces shifts in the redox potentials, likely because the ions interact with the binding sites of eumelanin or intercalate within the supramolecular π–π stacks (Xu et al. 2017; Borghetti et al. 2010; Tian et al. 2019).

The cyclic voltammetries for eumelanin in presence of different alkaline and ammonium ions (Figs. 5a, b) show the presence of various redox signals. Voltammograms obtained with DHICA-melanin are better resolved compared to DHI-melanin, possibly because in DHICA-melanin, electron transfers, and ion exchanges are more localized than in DHI-melanin. Instead, voltammograms of DHI-melanin show higher values of the current owing to the more efficient π-π stacking in DHI-melanin concerning DHICA-melanin (Xu et al. 2017; Lucia et al. 2013).

Tian et al. studied the mechanisms behind electron and ionic transfer in eumelanin pigments immersed in aqueous electrolytes including the monovalent cations Li^+, Na^+ and K^+ (Tian et al. 2019). Their research succeeded in revealing structure-electron transfer (redox) property relationships, beyond the expected redox signals associated to the quinone/hydroquinone redox couple. In particular, redox processes were associated to specific interactions between metal ions and carboxylates pendant groups or aromatic amines. Further, the effect of the size of the metal ions was investigated.

On the one hand, there is a difference between natural and synthetic eumelanin, in terms of redox properties. For natural eumelanin, redox signals may vary from

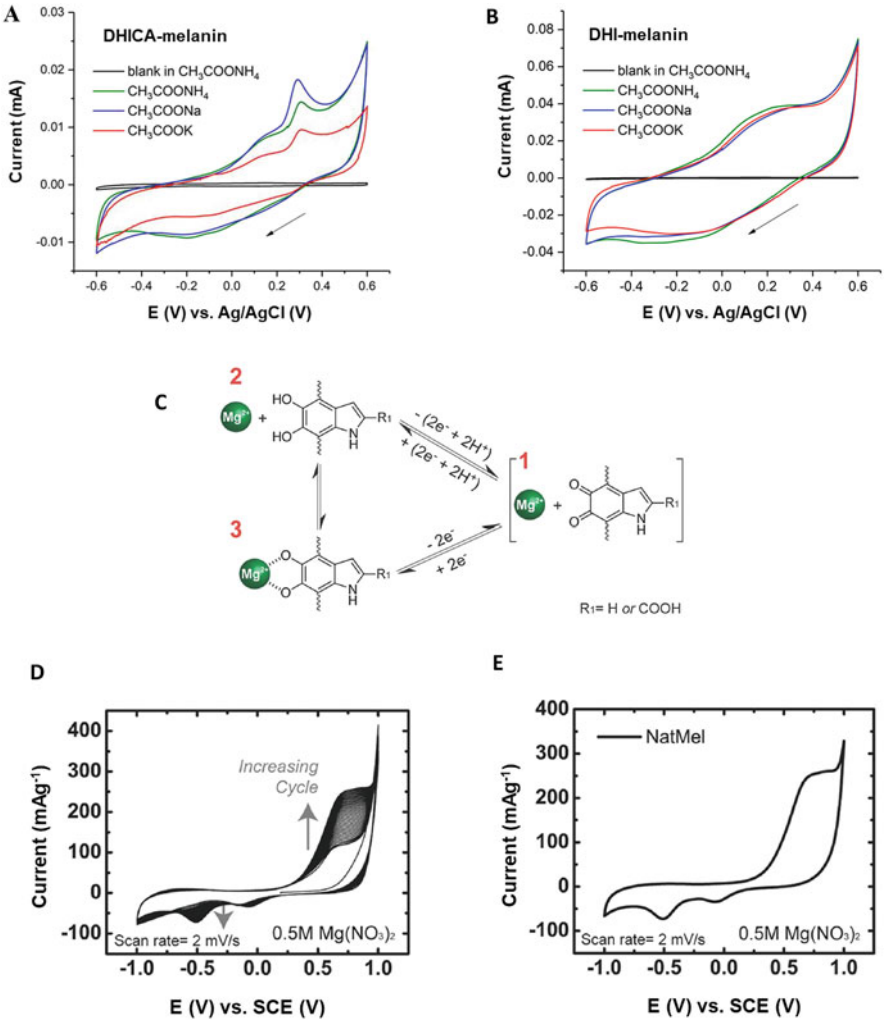

Fig. 5 Electrochemical behavior of eumelanin. DHICA-melanin (**a**) and DHI-melanin (**b**) in the presence of monovalent ions, $\nu = 5$ mV/s, acid buffers. Adapted from ref. (Xu et al. 2017). (**c**) Mechanism proposed for the interaction/coordination of Mg^{2+} ions in the catechol groups in Sepia melanin during voltammetry cycles. States 1 and 3 represent the melanin in the direction of oxidation and reduction states, respectively. State 2 represents an intermediate state of a catechol group before forming a coordination bonding. (**d**) and (**e**) represent the cyclic voltammetries of Sepia melanin in the presence of an electrolyte based on Mg^{2+} ions, 0.5 M $Mg(NO_3)_2$. Adapted from ref. (Kim et al. 2014)

sample to sample due to the different ratios of DHI:DHICA. On the other hand, for synthetic eumelanin, redox signals are more reproducible. Here, however, heterogeneities can manifest if, for instance, during the polymerization process not

all quinone groups are equally oxidized (Rózanowska et al. 1999; Mostert 2021; Panzella et al. 2013).

3.2 Redox Properties (Multivalent Ions)

Currently, multivalent ions (such as Mg^{2+}, Ca^{2+}, Zn^{2+}, etc.) are investigated to replace Li-ions in energy storage devices with high energy densities. Eumelanin presents different binding sites to interact with these multivalent ions (Hong and Simon 2007). For divalent ions, catechol groups are responsible for chelation under an electrochemical regimen (Xu et al. 2017; Kim et al. 2014). In an interesting electrochemical fingerprinting study aiming at assessing structural changes in Sepia melanin-based electrodes during cycling, Kim et al. reported on porphyrin-like structures formed by tetramers of Sepia melanin building blocks (Kim et al. 2016).

4 The Effect of Metal Ions on the Electronic Transport of Eumelanin

In the 1970s, McGinness et al. observed a reversible electrical switching in hydrated eumelanin pellets (McGinness et al. 1974). This observation led to the description of the electrical behavior of eumelanin within the amorphous semiconductor model (McGinness et al. 1974; Davis and Mott 1970). More recent experimental results challenged such description proposing the possibility of predominant electronic transport in dry eumelanin and mixed ionic electronic transport in wet eumelanin (Mostert et al. 2012; McGinness et al. 1974; Davis and Mott 1970).

4.1 Monovalent Cations

Borghetti et al. studied the morphological and electrical properties of eumelanin after mixing with potassium-including salts (Borghetti et al. 2010). They both drop cast and electrodeposited eumelanin films on ITO and Au substrates from a mixture of potassium bromide and eumelanin-dimethyl sulfoxide (DMSO) solution, to study the effect of the salt on the formation of eumelanin aggregates. They propose that the interaction between potassium cations and nitrogen atoms in the pyrrole ring brings about additional electronic states in the valence band, as indicated by a transfer of spectral weight involving the HOMO level in XPS spectra, thus increasing the number of possible applications of the multifunctional eumelanin biomacromolecule.

4.2 Multivalent Cations

Mostert et al. reported that the modulation of the proton concentration in eumelanin by the chelation of the transition metal ion Cu^{2+} induces a modulation of the conductivity (Mostert et al. 2020). The modulation of the proton conductivity was characterized using Electron Paramagnetic Resonance (EPR). For this study, $CuCl_2{\cdot}2H_2O$ was added to a eumelanin solution (eumelanin powder obtained from D–L, dopa, dissolved in water and NH_3) and then processed in thin film form. Cu^{2+} was chelated by hydroquinone/quinone moieties of melanin. The presence of Cu^{2+} increases its conductivity due to the increase in proton concentration in melanin. Afterwards, Organic Electrochemical Transistors (OECTs) were fabricated with the melanin-based copper-including quasi solid-electrolyte as the ionic gating medium and PEDOT:PSS as the transistor channel material. The enhancement of the proton conductivity in melanin is relevant for proton-to-electron transducing devices.

5 On the Interactions Between Eumelanin and Metal Electrodes

Wünsche et al. reported on the interaction between gold electrodes and hydrated eumelanin films deposited on gold electrode-pre-patterned SiO_2 substrates (Wünsche et al. 2013). They showed that interaction between Au and eumelanin under electrical bias in wet environment results in the formation of Au-eumelanin nanoaggregates and dendrites. These dendrites can bridge one electrode to the other after the bias, leading to a dramatic increase of the current. The formation of the *bridging* dendrites following the dissolution of the Au electrodes was attributed to both the metal-binding properties of phenolic hydroxyl groups in eumelanin and Cl^- (present low amounts of in eumelanin). It is worth mentioning that eumelanin also features reducing properties that can bring, chemically (i.e., in absence of electrical bias, gold cations to metallic gold. Di Mauro et al. (2016) later studied in detail the chemical and structural changes occurring at interfaces between metal electrodes (Pd, Cu, Fe, Ni and Au) and hydrated films of eumelanin, under bias (Di Mauro et al. 2016, 2019).

6 On the Possibility to Use Eumelanin in Metal Extraction from E-Waste

The field of electronics has profoundly modified the life quality of everyone, from information and communication technologies to education and industrial production (Patwa et al. 2021). Unfortunately, planned obsolescence and rapid upgrading of consumer electronics have led to the dramatic accumulation of waste electrical and

electronic equipment (WEEE). Globally, about 50 million tons of electronic-waste (e-waste) are produced per year, with detrimental effects on human health and the environment (Dar et al. 2020; Tchounwou et al. 2012).

The composition of e-waste includes organic and inorganic materials such plastics, flame-retardants, and metals (Du et al. 2023). The global recycling rate for e-waste is about 20% (31% in Europe and North America, 12% in Eastern and South Eastern Asia, 5% in Central and Southern Asia, 4% Sub-Saharan Africa and 1% Latin America) (Boubellouta and Kusch-Brandt 2022). A relevant portion of e-waste is exported, sometimes illegally, to sub-Saharan and South-East Asian countries.

Precious metals, e.g., gold, palladium and platinum, have high economic value by considering their primary application in the electrical and electronics industry (Rafiee et al. 2021; Gunarathne et al. 2022). Recovering precious metals from e-waste represents an important economic opportunity.

There are several physicochemical and biological methods to extract metals from e-waste.

6.1 Hydrometallurgy

In hydrometallurgy, liquid chemistry based on the use of different chemicals (cyanide, thiourea, thiosulfates and acids) is employed to extract metals. After leaching (where solutions are used to solubilize the metal-containing materials by converting them into soluble salts), metals are usually further processed for purification and extraction (Gaydardjiev 1998; Whitworth et al. 2022). Cyanide ions (CN^-) and *aqua regia* are used to recover gold in hydrometallurgy (La Brooy et al. 1994; Syed 2012).

6.2 Biohydrometallurgy

The biohydrometallurgical method is based on the use of different bacteria including chemolithoautotrophic bacteria, heterotrophic bacteria and fungii, such as *Aspergillus Niger* and *Penicillum simplicissimum (*Esmaeili et al. 2022*),* to transform insoluble metal oxides/sulfides into soluble metal ions for their subsequent recovery (Gu et al. 2018). There are different types of bacteria that have been used for bioleaching, such as mesophilic (temperature range 25–35 °C) and thermophilic (50 °C) (Gu et al. 2018; Kaksonen et al. 2017). A frequently used microorganism in sulfide ores bioleaching is *Acidithiobacillus ferrooxidans (*Watling 2006*).*

Bioleaching has been used in metal mining from the decades (Ji et al. 2022). Recently, this low cost and environmentally friendly method has been employed to extract heavy metals from ash and sewage sludge for bioremediation purposes (Gu et al. 2018).

6.3 Pyrometallurgy

Pyrometallurgy is based on the thermal treatment of materials, such as minerals and ores, to obtain precious metals (Harvey et al. 2022; Zhu et al. 2022). Compared to hydrometallurgy, it involves partial or complete conversion of chemical compounds into their elemental form. For example, pyrite is converted into pyrrhotite and elemental sulphur (Whitworth et al. 2022). During the pyrometallurgy process, different oxides and reducing agents are being used. For instance, to extract platinum-based metals (PBMs), lead oxides are used (Kim et al. 2013; Peng et al. 2017).

6.4 A Possible Perspective on the Interaction of Melanin with Metals for e-Waste Recovery

There are number of physicochemical mechanisms that can be exploited for the extraction of metals with melanin (Di Mauro et al. 2017; Meredith and Sarna 2006; Pilas et al. 1988; Hong et al. 2007).

We propose that in the future natural chelating agents could be used to promote metal recovery in urban mining, to recover precious, critical or strategic metals (Electronic Waste: Recycling and Reprocessing for a Sustainable Future, Maria E. Holuszko (Editor), Amit Kumar (Editor), Denise C. R. Espinosa (Editor) ISBN: 978-3-527-34,490-1).

Literature reports that, melanin extracted from squid ink features high adsorption tendency for lead (Xue et al. 2009). Systematic studies are needed to shed light on the effect of the source of melanin and its molecular and supramolecular structures (as well as possible presence of other chemical compounds in the natural or synthetic melanin material) on its binding affinity to metals, for metal recovery purposes. For instance, eumelanin prepared by L-DOPA can remove 95% of initial lead present in the investigated sample, a percentage dramatically higher than that one observed with eumelanin extracted from human hair (Sono et al. 2012). Results reported by Darwish et al. show that synthetic melanin nanoparticles (5,6-diacetoxy indole precursor that is hydrolyzed in situ into dihydroxy indole (DHI)) adsorb different metal ions; here highest adsorption values from 50 ppm solutions were observed for Co^{2+}, Ni^{2+} and Zn^{2+} and lowest for Cu^{2+}, Cd^{2+} and Pb^{2+} (Darwish et al. 2021).

Well beyond eumelanin, we wish to encourage the research community active in the field of metal recovery and water and soil remediation to explore, at large scale, the use of biosourced organic chelating agents considering their abundance, low cost, biodegradability and, possibly, biocompatibility.

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Fundamentals and Applications of Optically Active Melanin-Based Materials

Ming Xiao and Weiyao Li

Abstract Melanin is a group of ubiquitous biological pigments. It has excellent photonic properties such as broadband light absorption and high refractive index, combined with other physicochemical functions like free radical quenching and metal chelating capabilities. In this chapter, we focus on the optical functions of melanin. We will first discuss chemical and physical structures in five different types of melanin. Next, we divide melanin's photonic properties into two parts. One is about broadband absorption, where we explore the mechanism behind it and how absorption leads to various applications such as UV protection, solar desalination, and photothermal therapy. The other is related to scattering, which includes the scattering from single particle and aggregates of particles. In both scenarios, structural colors can be produced. We believe this chapter will provide a clear understanding of melanin's optical properties and insights into the rational design of melanin-based optically active materials.

1 Introduction

Melanin is a family of dark polymeric pigments widely found in nature, ranging from animals, plants, and fungi to prehistoric organisms. It possesses superior multifunctional properties despite the fact that its precise hierarchical chemical structure remains unclear. These include a high refractive index, broadband absorption across UV to near IR region, radical quenching ability, strong metal chelating ability, and protection against high-energy radiation. In this chapter, we focus on the optical properties of melanin-based materials and, broadly speaking, how melanin interacts with electromagnetic waves. First, we introduce the chemical structures of melanin and make classifications based on the chemical structure of monomer

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precursors. In the next two sections, we discuss absorption- and scattering-related properties. In the absorption section, we summarize the fundamentals of melanin's broadband absorption and how melanin dissipates absorbed energy, which is related to different applications, like UV protection, photothermal therapy, and fluorescent imaging. The scattering section includes the scattering from single particles and assemblies of particles, with detailed summary on summarize how melanin particles assemble into structurally colored materials. In the end, we present our views on the challenges and opportunities in this field.

2 Chemical Structure

In contrast to proteins or nucleic acids, melanin biosynthesis is not a genetic or sequence-controlled process. It starts with various monomeric precursors and ends with heterogeneous, hierarchical polymeric structures (Cao et al. 2021). The unique optical properties of melanin rely on the complexity of its molecular units, chemical structures, and physical packings. In this section, we will discuss five types of melanin and their complex hierarchical structures.

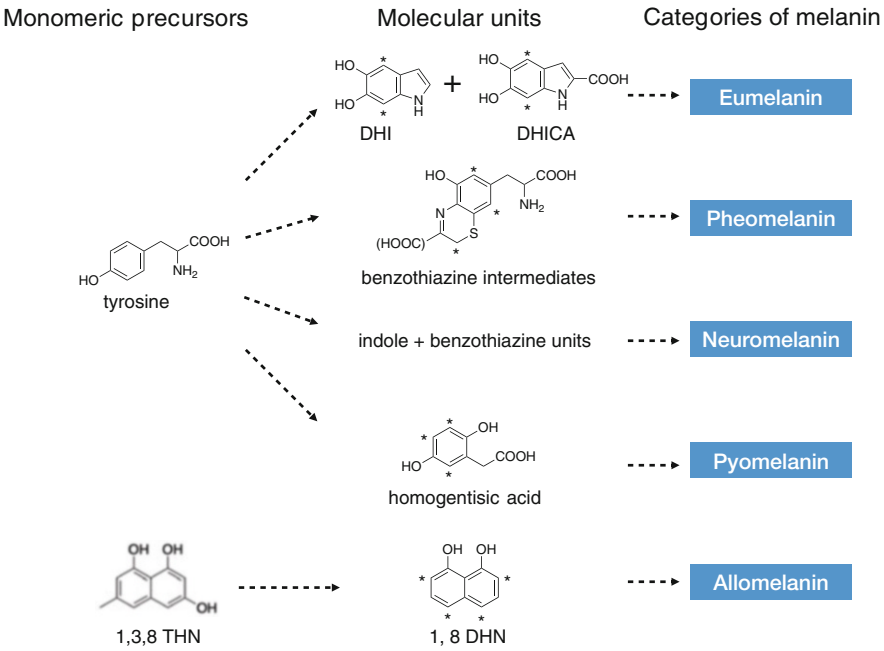

Fig. 1 Classifications of melanin based on the chemical structure of molecular unit. The star symbols indicate possible chemical bonding sites to other molecular units

2.1 Classifications of Melanin

There are five categories of melanin based on molecular units in their macromolecular structures: eumelanin, pheomelanin, neuromelanin, pyomelanin, and allomelanin (Fig. 1). They share similarities such as displaying dark colors (brown to black), containing conjugated aromatic structures, and being insoluble in most solvents. However, their chemical structures are quite different from each other, which will be discussed later.

Eumelanin, pheomelanin, and neuromelanin are mostly found in animals. They are produced initially from tyrosine by a series of enzymatic reactions in cells (commonly melanocytes). In their biosynthetic paths, tyrosinase oxidizes tyrosine to dopaquinone (DQ) and DQ polymerizes to eumelanin with two main building blocks 5,6-dihydroxyindole (DHI) or 5,6-dihydroxyindole-2-carboxylic acid (DHICA). The cysteine in the cell promotes the production of pheomelanin by forming cysteinyldopa and then benzothiazine intermediates, which prohibits the formation of eumelanin (Wakamatsu et al. 2021). Neuromelanin also originates from tyrosine, which is produced in the substantia nigra and locus coeruleus of brains. Neuromelanin is often mixed with other living tissues and it contains both indole and benzothiazine as molecular units. Pyomelanin and allomelanin are nitrogen-free melanins, which are produced mostly in fungi, bacteria, or plants. Pyromelanin biosynthetic process involves catabolism of tyrosine to homogentisic acid, while allomelanin is produced directly from nitrogen-free precursors such as 1,8-dihydroxynaphthalene (DHN).

In addition to biosynthesis in living tissues, melanin can be made in the laboratory through chemical oxidation or enzymatic oxidation using corresponding monomeric precursors. Synthetic melanin has dark coloration similar to natural melanin. Eumelanin, pheomelanin, and neuromelanin can be synthesized starting from various precursors such as tyrosine, dopamine, L-3,4-dihydroxyphenylalanine (L-DOPA), DHI, and DHICA via enzymatic reaction or oxidations (d'Ischia et al. 2013; Li et al. 2019; Napolitano et al. 2013; Wakamatsu et al. 2003). Both allomelanin and pyomelanin, can be synthesized in vitro from DHN and homogentisic acid, respectively (Zhou et al. 2019; Schmaler-Ripcke et al. 2009). More details on the synthesis of different types of synthetic melanin can be referred to in a recent review from Gianneschi group (Cao et al. 2021).

Although the chemical structure of main molecular units in melanin has been intensively studied, melanin's polymeric structure has not been fully revealed. Melanin's monomeric precursors have more than two reactive sites, making its polymerization complicated. In the synthesis of eumelanin, the rearrangement of dopachrome to form DHI or DHICA not only diversifies the monomeric structures but also provides various arrangements of units in the polymeric chains (Swift 2009). In the pheomelanin, the cysteine not only attaches to a different site on DOPA, but also splits a fraction of the monomers to form benzothiazines, rather than indole-like eumelanin, which adds another level of complexity. Neuromelanin is produced from two monomeric precursors and has a more chemically disordered structure.

Pyomelanin can be made from the polymerization of homogentisic acid, which is a degradation product from tyrosine (Schmaler-Ripcke et al. 2009; Frases et al. 2007). However, the chemical structure of pyomelanin is less studied compared to other types of melanin. Both natural and synthetic allomelanin can be made from a universal monomeric precursor, DHN. Unlike dopachrome, DHN has fewer chances to rearrange, but allomelanin structure is also disordered due to various linkages in DHN dimers and mutations of DHN by reduction, oxidation, and dehydration (Zhou et al. 2019; Jackson et al. 2009).

2.2 *Complex Hierarchical Structure of Melanin*

Melanin often comes in the form of particles ranging from tens of nanometers to a few microns. Although its insolubility in most solvents makes it a challenge to quantify its chemical structure, it is clear that melanin has a complex hierarchical structure ranging from molecular to micrometer scales. The structure of eumelanin is the most widely studied. As the monomer precursor, the tyrosine converts to molecular units, DHI and DHICA. Several molecular units form planar oligomers that are called protomolecules and some models suggest that protomolecules are porphyrin-like tetramer structures (Chen et al. 2013; Kaxiras et al. 2006). These planar protomolecules stack with a spacing of 3–4 Å and stacked protomolecules pack with ordered or disordered structures to form primary nanoparticles 10–60 nm. Higher geometric packing order of these protomolecules will lead to broadening and red-shifts of the absorption band (Ju et al. 2018). The primary particles assemble into submicron-sized particles, ranging from solid spheres, solid rods, or hollow rods in natural melanin (Fig. 2) (Xiao et al. 2018).

As a model synthetic eumelanin, polydopamine has been utilized to aid in revealing the mysterious structure of natural eumelanin. Polydopamine, made via oxidation polymerization of dopamine, contains various molecular units, including uncyclized dopamine and DHI, which provide various chromophores to absorb light (Della Vecchia et al. 2013; Bisaglia et al. 2007). Although there is still some debate, it is mostly agreed that polydopamine particles are composed of linear polymer

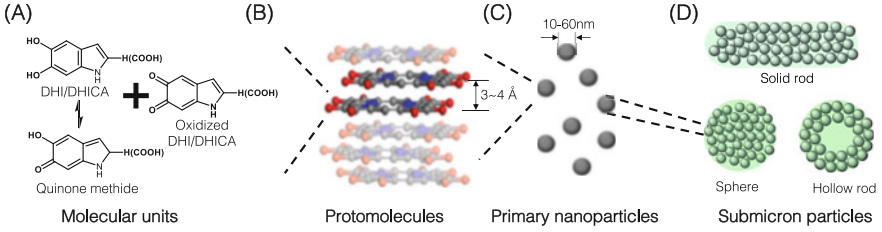

Fig 2 A hierarchical structure model for eumelanin: (**a**) melanin molecular units, (**b**) stacked protomolecules, (**c**) primary particles, and (**d**) melanin particles with different morphologies. Adapted from Xiao et al. (2018)

chains that are bound together via physical interactions such as hydrogen bonding, cation-π interaction, and π-π stacking (Büngeler et al. 2017; Li et al. 2015; Hong et al. 2018). The DHI units propagate through the six-membered ring to increase the molecular sizes in polydopamine (Li et al. 2019). Using matrix-assisted laser desorption/ionization mass spectroscopy, Reale et al. found that oligomers made from DHI can reach up to 30 units (Reale et al. 2012).

3 Absorption-Related Properties and Applications

Melanin can absorb light broadly from UV up to near-infrared (200–1700 nm) with decayed absorptivity at longer wavelengths (Li et al. 2020). As organic polymers, their broadband absorption is unique, making them ideal for black pigments and UV-protecting agents. Most of the light absorbed by melanin is dissipated as heat, and this photothermal effect can be used for saltwater desalination or photothermal therapy. In addition, melanin can be modified to fluoresce, expanding its applications. In this section, we will discuss the broadband absorption of melanin, the underlying mechanism of absorption, and related applications.

3.1 Optical Absorption

Melanin has a broadband absorption, unlike other pigments that often have significant absorption peaks at certain wavelengths. Its absorption spectrum varies with its chemical structure. Quantifying melanin absorption is challenging for several reasons. First, melanin is insoluble in most solvents without chemical degradation, so it is difficult to conduct conventional absorption measurements on solvent-casting melanin films. Second, melanin from biological systems is often mixed with other cellular components, making the purification process tremendously difficult. Third, melanin, often in the form of micron-scale or even larger particles, scatters light, which overestimates light absorption. Therefore, there is no perfect way to accurately measure the absorption of natural melanin. Stavenga et al. measured the refractive index of intact barbule cells that contained multilayered natural melanin particles and used an optical model to obtain a best-fitting value (Stavenga et al. 2015). Based on this method, they reported that the imaginary index varies from 0.127 to 0.029 at wavelengths of 400–800 nm (Fig. 3a).

Synthetic melanin can be chemically prepared in a more controlled manner, removing some barriers to absorption measurements. Xiao et al. measured melanin nanoparticles made from dopamine and the imaginary index decreased from 0.33 to 0.09 at 400–800 nm (Fig. 3a). To greatly reduce the scattering effect of the particles, they used dilute solutions (10^{-4}–10^{-3}% v/v) and small particle sizes (120 nm) (Xiao et al. 2015). In addition to melanin nanoparticles, there are a few reports on quantifying the absorption of synthetic melanin films. Bothma et al. fabricated

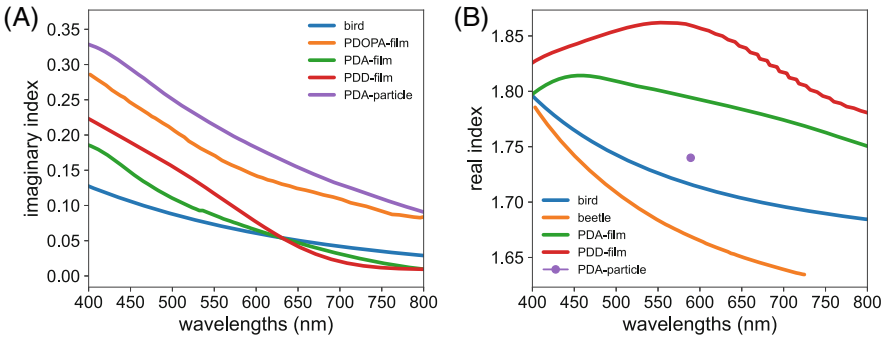

Fig. 3 Refractive indices of various types of melanin. (**a**) The imaginary part of refractive indices of melanin in bird feathers (Stavenga et al. 2015), PDOPA melanin films (Bothma et al. 2008), PDA and PDD melanin films (Li et al. 2020), and PDA melanin nanoparticles (Xiao et al. 2015). (**b**) Real part of refractive indices of melanin in beetle scales (Yoshioka and Kinoshita 2011), bird feathers (Stavenga et al. 2015), PDA and PDD films (Li et al. 2020), and PDA melanin nanoparticles (Xiao et al. 2015)

device-quality melanin films with DOPA and used an integrating sphere to measure absorption. We can calculate the imaginary indices of the PDOPA melanin film as 0.286–0.083 based on the absorption coefficient (Fig. 3a) (Bothma et al. 2008; Xiao et al. 2020). Li et al. made two types of smooth melanin films through oxidation polymerization at the water-air interface. One is polydopamine (PDA) and the other is poly(dopamine-L-DOPA) (PDD). They used ellipsometry to measure imaginary indices of PDA and PDD melanin films to be 0.185–0.0096 and 0.222–0.0094 (Fig. 3a) (Li et al. 2020). The reported synthetic melanin shows higher absorption than natural melanin, probably because natural melanin is mixed with non-absorbing components like proteins, lipids, or polysaccharides.

Understanding the mechanism behind melanin's broadband absorption will guide the rational design of novel black pigments. Different levels of heterogeneities contribute to melanin's broadband absorption. The first level is the chemical structure variation as mentioned in Sect. 2. Take eumelanin, for example, it is made of mixtures of DHI and DHICA units. These molecular units make protomolecules with different degrees of polymerization. DHI and DHICA have different connecting sites, leading to different chemical structures in protomolecules. Thus, the eumelanin contains different protomolecules that have absorption peaks at different wavelengths and the supposition of these peaks leads to broadband absorption (Tran et al. 2006). The second level of heterogeneity is the variation in redox states. The indole groups in melanin have a fully reduced state (hydro-quinone), half-reduced state (semi-quinone), and oxidized state (quinone). Density functional theory calculations show that the absorption peak of the reduced protomolecules is red-shifted as compared to the oxidized form and the half-reduced form exhibits intermediate behaviors with a broader absorption (Stark et al. 2005). On the third level, the stacking of planar melanin protomolecules plays a significant role in its absorption behavior. Eumelanin protomolecules pack with a spacing of 3–4 Å. Chen et al.

combined experiments and simulations to demonstrate excitonic coupling from geometric packing is a key reason for broadband optical absorption (Chen et al. 2014). Later, Ju et al. demonstrated that higher geometric packing ordering efficiently broadens and red-shifts the absorption band (Ju et al. 2018). Taken together, the unique blackness of melanin is a synergistic effect of heterogeneity in chemical structure, redox states, and geometric packing.

3.2 Black Additives

The basic function of melanin is to provide dark colorations in animals and fungi and the colors include black, brown, or grey colors (Evayanti and Artaria 2019; Itou et al. 2019; Surmacki et al. 2021; Mattoon et al. 2021). Dark pigmentations induced with melanin have significant biological functions such as warning color, camouflage, and courtship. Some animals like insects generate black patterns with melanin for aposematic signals, (Liu et al. 2014) while some like cephalopods generate dynamic patterns for camouflage by controlling the melanin density and distribution rapidly (Mäthger et al. 2009). Melanin patterns are also critical in sexual selection for animals like butterflies (Wiernasz 1995). In addition to exterior dark patterns, cuttlefish ejects inks for anti-predator defense (Derby 2014). The inks are a primary source of natural eumelanin used in research.

Melanin can be used as biocompatible hair dyes due to its excellent binding ability. Through the deposition of polydopamine or co-deposition of polydopamine and cysteine, one can obtain melanin-dyed dark hair with different hues (Battistella et al. 2020; Dong et al. 2019). The colors of the dyes can be tuned by doping different types of metals or melanin precursors (Fig. 4a). The melanin hair dye can be even made permanent using a pre-grafting method (Zheng et al. 2022).

Melanin can enhance color saturation by absorbing incoherent backscattering light. Advantages of melanin over other conventional broadband absorbers include biocompatibility, defined and controllable particle sizes, and tunable absorption. Non-iridescent structural colors in bird feathers are caused by coherent light scattering in the spongy layer, and the underlying amorphous melanin layer absorbs incoherent light scattering and enhances color saturation (Saranathan et al. 2012; Shawkey and Hill 2006). Synthetic melanin particles have been extensively doped into photonic colloidal packings to enhance color saturation, and the required doping ratio is as low as 0.5% w/w (Cai et al. 2014; Lee et al. 2019).

3.3 Protection from Electromagnetic Radiation

Melanin has a gradually descending absorption band from UV (200 nm) (Wang et al. 2017) to near-infrared (NIR, 900 nm) (Li et al. 2020) with two broad absorption humps in the mid-infrared range (MIR, 2.5–25 μm). Although some melanin

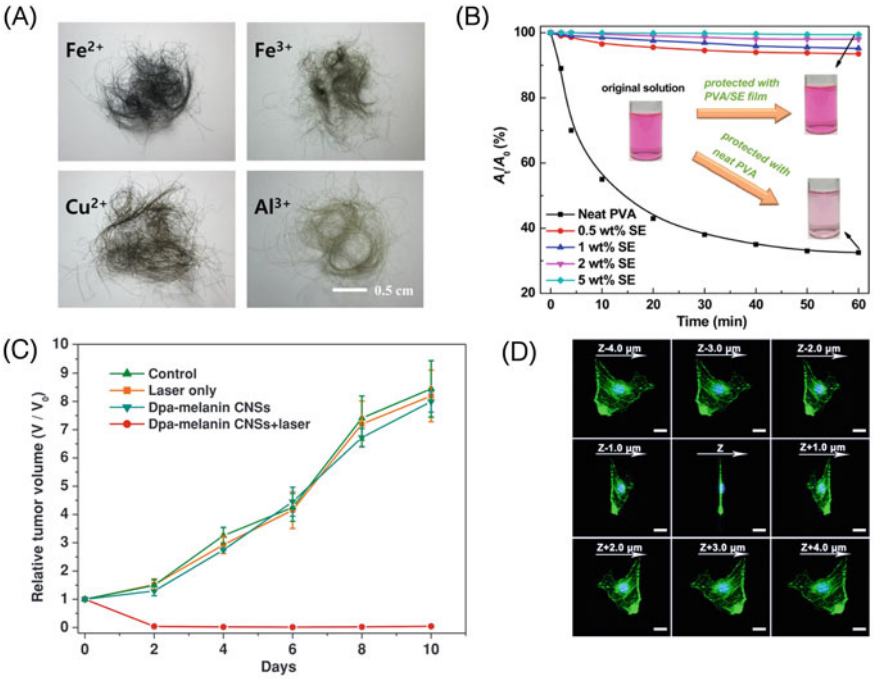

Fig. 4 Absorption-related applications of melanin in various fields. (**a**) Hairs are dyed with polydopamine melanin catalyzed by different metal ions. Reproduced with permission (Im et al. 2017). Copyright 2017, the American Chemical Society. (**b**) The absorption curves of rhodamine solutions change with the time of UV irradiation. The solutions are covered by PVA film that contains different concentrations of sepia melanin. Reproduced with permission (Wang et al. 2016). Copyright 2016, the American Chemical Society. (**c**) 4 T1 tumor growth curves of the mice after different treatments show the efficacy of photothermal therapy. Reproduced with permission (Liu et al. 2013). Copyright 2013, WILEY-VCH. (**d**) Confocal microscope images of A549 Cells stained with in situ synthesized PDA nanoparticles. Reproduced with permission (Ding et al. 2017). Copyright 2017, the American Chemical Society. Scale bars, 10 μm

can provide protection from high energy radiation like X-ray and γ-ray (Cao et al. 2020; Rageh et al. 2015), there is still a lack of more reliable quantitative evidence of absorption in such a high energy range.

UV light that reaches the earth's surface is mostly UVA and UVB (290–400 nm), which can cause DNA damage and even skin cancer in some extreme cases. Humans with darker skin pigmentation have less oxidative stress and a lower chance of sun-induced skin cancers (Shih et al. 2020; Brenner and Hearing 2008). Birds with melanin-enriched skins tend to be more common in regions with abundant sunshine, suggesting that UV protection is related to dark bird skins (Nicolaï et al. 2020). Melanin in skin, hair, and eye can absorb UV light and quench free radicals generated by UV radiation (Shih et al. 2020; Herrling et al. 2008; Hu et al. 2008). Melanin can release its absorbed energy from UV radiation to heat at a time scale of 160 fs, reducing the chance of excited state splitting to free radicals that will cause

damage to the living tissues (Corani et al. 2014; Qu et al. 2000). Corani et al. and Panzella et al. reported that DHICA melanin dissipated UV and scavenged free radicals faster than DHI melanin, suggesting the DHICA component played a major role in eumelanin's UV protection (Corani et al. 2014; Panzella et al. 2013). Melanin is doped into polymer films to enhance the resistance to UV exposure. A polyvinyl alcohol film doped with sepia melanin can efficiently protect rhodamine dyes from photodegradation (Fig. 4b) (Wang et al. 2016). In addition, doping sepia melanin largely decreases the UV damage to polymer mechanical properties.

Along with the photoprotection of surrounding tissues, the melanin structure may also undergo photodegradation when exposed to visible and UV light. Though melanin releases hydroxyl radicals to quench other free radicals upon visible and UV irradiation (Qu et al. 2000), a study showed that visible light can disrupt the structure of melanin and reduce the antioxidative capacity (Zareba et al. 2006). Li et al. studied the UV degradation behaviors of dopamine melanin, L-DOPA melanin, and sepia melanin. They demonstrated that strong UVA irradiation (2.43 ± 0.02 W/cm^2) degraded a fraction of the six-membered benzyl ring to furo[3,4-b]pyrrole and CO_2 while the pyrrole ring of the indole unit was still intact for all three types of melanin (Li et al. 2019).

3.4 Photothermal Effect

The absorption of light by melanin mainly converts into heat, and this photothermal effect leads to many applications. Liu et al. made a photothermal therapeutic (PPT) agent based on melanin nanoparticles for in vivo cancer therapy. With a photothermal conversion efficiency of 40%, the PPT agent can damage tumor cells under 808 nm lasering without affecting healthy tissues (Fig. 4c) (Liu et al. 2013). Later, Yang et al. developed a new type of synthetic melanin by copolymerizing arginine and dopamine, which significantly increased the photothermal conversion efficiency at 808 nm light (Yang et al. 2019).

In addition, the photothermal effect of melanin can be used for solar desalination. The solar energy absorbed by melanin can be harvested to evaporate brine to produce pure water. Zou et al. constructed donor-accepter pairs in synthetic melanin molecules to boost the absorption and used optimized melanin nanoparticles for solar desalination (Zou et al. 2020). When applied to thermoelectric devices, melanin can convert solar energy into heat, generating voltages up to 229 mV through the thermoelectric effect (Bai et al. 2022).

3.5 Fluorescence

Melanin is usually not fluorescent because it can absorb self-emitted light. Synthetic melanin made from dopamine can efficiently quench various fluorescent dyes that

are attached to the surface of melanin nanoparticles (Qiang et al. 2014; Ma et al. 2016). The fluorescence quenching mechanism is assumed to involve Forster resonance energy transfer and/or photoinduced electron transfer. To make melanin fluorescent, one needs to eliminate the aggregation-induced quenching effect by disrupting the stacking of planar melanin protomolecules. Researchers have used chemical oxidation (H_2O_2, $NaIO_4$, $KMnO_4$) to disrupt the stacking or degrade melanin particles in alkaline solutions to decrease particle sizes (Lin et al. 2015; Yin et al. 2018; Zhang et al. 2012; Xue et al. 2018).

Fluorescent melanin can be used as a novel biocompatible and surface-active dye for sensors and biomedicine. Melanin has strong chelating capabilities with metal ions, and its fluorescence disappears after binding with metal ions. Taking advantage of this pheonomenon, researchers have designed fluorescent probes to detect Fe^{3+} or Pb^{2+} (Yin et al. 2018; Zhang et al. 2020). Another important application is to use melanin nanoparticles for cell imaging (Yang et al. 2016; Ding et al. 2017; Zhang et al. 2019). Ding et al. demonstrated that polydopamine nanoparticle (5–10 nm) was synthesized in situ within the nucleus of living cells, and this biomimetic nuclear dye showed high quantum yield (~35.8%), high photostability, low cytotoxicity, and long-term fluorescence tracking property (Fig. 4d) (Ding et al. 2017).

4 Scattering-Related Properties and Applications

Natural and synthetic melanins are often particulate shapes. When the size of melanin particles is comparable to the wavelength of light, they can scatter light and produce various optical effects. By controlling the size and packing of melanin particles, one can manipulate their scattering for different applications, for example, producing structural colors, monitoring the state of melanin-containing cells, or making a nanoprobe for Raman imaging.

The scattering property strongly depends on a material's refractive index. The refractive index of melanin typically reported to be as high as 2.0, is much higher than most common polymers (1.4–1.6). Recent efforts have been made to measure the refractive index of melanin, however, the reported index varies depending on the morphology and chemistry of melanin and measurement methods (Fig. 3b). Yoshinoka and Kinoshita determined the real refractive index of melanin in the Jewel beetle's elytra to be 1.65–1.78 from a typical multilayered model using experimental data (Yoshioka and Kinoshita 2011). Later, Stavenga et al. reported 1.7–1.8 for melanin in damselfly wings and bird feathers by fitting several parameters to match theoretical calculation with experimental data from interference microscopy (Stavenga et al. 2012, 2015). Xiao et al. measured the refractive index of synthetic melanin (PDA) nanoparticles to be 1.74 at 589 nm (Xiao et al. 2015). Li et al. used ellipsometry to measure indices of films for two different synthetic melanin: PDA melanin from only dopamine and PDD melanin from a mixture of dopamine and L-DOPA. PDA film has a maximum index of 1.81 at 485 nm and PDD film has a maximum index of 1.86 at 550 nm (Li et al. 2020). Despite some

discrepancies among different measurements (Fig. 3b), the real part of the refractive index ranges from 1.65 to1.85 across the visible wavelengths, which is higher than common polymers.

4.1 Single Particle Scattering

Melanin particles cannot dissolve in solvents completely without a degradation reaction catalyzed by a strong base like NaOH. When measuring the absorption of melanin, the scattering of melanin particles cannot be completely avoided. Riesz et al. quantified that the contribution of scattering in dilute eumelanin solutions (0.0025% w/w) is less than 6% to the total optical attenuation in the UV regions (210–325 nm), and contribution at wavelengths of 325–800 nm is negligible (Riesz et al. 2006). They obtained a perfect match between calculations and experiments when fitting the scattering of eumelanin to Raileigh scattering with assuming particle sizes to be 38 nm. This suggested that small particle size and a very dilute solution is the key to minimizing the scattering effect when measuring the real absorption. When particles become larger or concentrations are not dilute enough, the scattering contributes more to the total extinction.

One can exploit the scattering of individual melanin particles for new applications. For example, Song et al. simulated wavelength-dependent scattering of melanosomes in retinal pigmented epithelial cells and found that optical properties of melanin particles changed when they were bleached. This method can be used to detect the changes in retinal pigmented epithelial cells (Song et al. 2017). When melanin particles are monodisperse and fall in sizes comparable to wavlengths of visible light, they can generate structural colors by Mie scattering from individual particles (Cho et al. 2017). The high absorption of melanin attenuates multiple scattering so that resonate Mie scattering of monodisperse melanin particles dominates. The Mie scattering makes structural colors and different colors are obtained depending on the size of melanin particles (Fig. 5a).

4.2 Scattering from Assemblies of Particles

The assemblies of melanin particles can generate more interesting optical phenomena. In bird feathers, natural melanin particles organize into various ordered packings, like multilayered structures, square packing, or hexagonal packing. These ordered structures made from melanin particles can selectively reflect light at certain wavelengths, producing structural colors. These types of colors depend only on the nanostructures, but not the chemical structure, in contrast to colors of pigments or dyes. We will summarize different types of assembled photonic structures that are made of melanin particles.

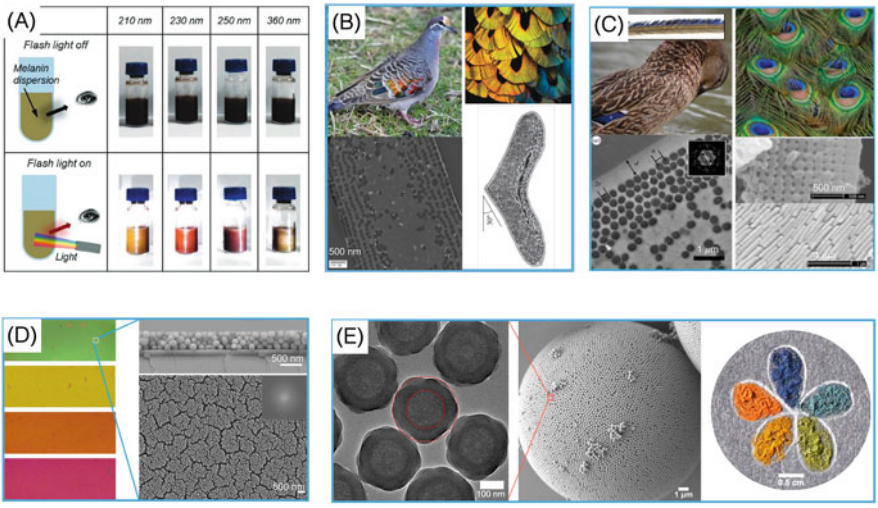

Fig. 5 Structural colors due to scattering from individual and assembled melanin particles. (**a**) Dilute melanin nanoparticle solutions (0.2% w/w) show different colors depending on the particle sizes, which is due to Mie scattering. Reproduced with permission (Cho et al. 2017). Copyright 2017, WILEY-VCH. (**b**) Photos and transmission electron microscopy images of structurally colored bird feathers that contain multilayered melanin particles. From left to right, common bronzewing (Reproduced with permission (Xiao et al. 2014). Copyright 2014, the Optical Society) and bird of paradise (Reproduced with permission (Stavenga et al. 2011). Copyright 2011, the Royal Society). (**c**) Photos and transmission electron microscopy images of structurally colored bird feathers where melanin particles form 2D ordered structures. From left to right, 2D hexagonal packing in mallard feathers (Reproduced with permission (Eliason and Shawkey 2012). Copyright 2012, the Royal Society) and 2D square packing in peacock feathers (Reproduced with permission (Zi et al. 2003). Copyright 2003, the National Academy of Sciences, USA). (**d**) Structural colors from films made of synthetic melanin particles. Reproduced with permission (Xiao et al. 2015). Copyright 2015, the American Chemical Society. (**e**) Core-shell melanin particles (melanin as cores) assemble into 3D photonic balls to produce bright and noniridescent structural colors (Xiao et al. 2017)

1. Layered Structures

Melanin particles assemble into a single layer or multiple layers in some bird feathers. When the layer thickness and spacing are hundreds of nanometers, they can reflect light with interference, producing structural colors. The simplest case is the iridescent colors in feathers of blue-black grassquits (*Volatinia jacarina*), where a 127-nm-thick keratin layer and a 422-nm-thick-melanosome layer form a two-layered structure (Maia et al. 2009). Another example is the tree swallow mantle feathers containing a keratin layer (148 nm) and a melanosome layer (173 nm). The keratin layer can absorb water and its thickness increases under high humid conditions, leading to color changes in these feathers (Eliason and Shawkey 2010).

In some bird feathers, there are multilayers of melanin particles dispersed in the keratin matrix with ~100 nm spacing. For example, common bronzewing (*Phaps*

chalcoptera) feathers contain 6–7 layers of melanin particles with equal spacings in the keratin matrix (Fig. 5b) (Xiao et al. 2014). The slight variations in both thickness and spacing of the melanin layer lead to a full-color spectrum from blue to red (462–647 nm) across a single feather. The bird of paradise (*Parotia lawesii*) has uniquely boomerang-shaped barbules in which reflectors are composed of multilayer melanin particles crossing at a 30 ° angle (Fig. 5b) (Stavenga et al. 2011). This boomerang-shaped geometry increases the angle dependence of the colors, which is biologically beneficial to mating.

Bird feathers have inspired researchers to assemble synthetic melanin particles into structurally colored films. Xiao et al. synthesized monodisperse melanin nanoparticles from dopamine and assembled them into hundreds of nanometers of films to produce structural colors (Xiao et al. 2015). Their colors vary with the film thickness (Fig. 5d). The high color saturation is due to the broadband absorption of melanin. In addition, they found the melanin nanoparticle films could reversibly absorb and desorb water, and melanin films change colors dramatically upon humidity variations (Xiao et al. 2016).

2. 2D Packing of Particles

Natural melanin particles in bird feathers are often rod-like shaped with hundreds of nanometers in diameter and several microns in length. They can be monodisperse with controlled aspect ratios (Xiao et al. 2018), however, it remains challenging to synthesize such rod-like melanin particles with controlled size and aspect ratios in the laboratory. In bird feathers, the rod-like melanin particles prefer to align parallel to the inner surface of barbules due to depletion attraction (Maia et al. 2011). Being monodisperse and rod-like shaped, natural melanin particles readily self-organize into 2D crystalline structures, square or hexagonal lattices, producing bright and iridescent structural colors (Fig. 5c). For example, iridescent peacock barbules contain 2D square packed melanin rod-like particles and different colors of these feathers originate from the variation in lattice spacing (Zi et al. 2003). In mallard feathers, melanin particles form a non-close packed 2D hexagonal lattice (Fig. 5c), resulting in a broader and larger reflectance peak, and thus brighter colors (Eliason and Shawkey 2012).

3. 3D Packing of Particles

Spherical particles can pack in crystalline or short-range correlated structures that can produce structural colors (Takeoka 2012). Natural melanin particles are spherical, rod-like, or irregular shapes, however, monodisperse spherical melanin particles are rarely found in nature. Nowadays, there are quite a few approaches to synthesizing monodisperse melanin particles from different monomers (Xiao et al. 2015; Cho and Kim 2015). This fundamentally supports recent research where synthetic melanin particles are assembled in various ways to produce structural colors.

Melanin has high broadband absorption with a reported absorption coefficient of about 1/μm (Bothma et al. 2008). Based on the beer-lambert's law, a melanin film of 5 μm absorbs more than 99% of light. This suggests that if one prepares 3D packing of pure melanin particles, black colors will be obtained due to too much absorption.

This issue has been resolved by using core-shell melanin particles, co-assembling melanin and other non-absorbing particles, or copolymerization melanin with other polymers.

There are two types of core-shell melanin particles. The first type is with melanin shells. Kishikawa and co-authors coated synthetic melanin (polydopamine) onto polystyrene nanoparticles with melanin shell thickness from 2.5 to 22 nm. They drop cast solutions of core-shell particles to 3D ordered assemblies with structural colors (Kawamura et al. 2016). When the melanin shell thickness increased, the surface of core-shell particles became rougher, prohibiting the formation of crystalline structures. Later, they assembled those core-shell particles to different morphologies, micron-sized photonic balls via membrane emulsification method and photonic fibers via a microfluidic approach (Kohri et al. 2018). Kohri et al. made ellipsoidal melanin particles by stretching uncross-linked polystyrene core and melanin shell particles. When depositing structurally colored films using these particles, they found higher aspect ratios caused a blue shift of reflected spectra (Kohri et al. 2019).

The second type is with melanin cores. Xiao et al. used optical simulations to demonstrate that higher reflectance and brighter colors could be achieved from 3D photonic assemblies made of core-shell nanoparticles with high refractive index core and low index shell. Based on this theory, they used a sol-gel method to coat a silica layer (index = 1.45) onto the surface of synthetic melanin nanoparticles (index = 1.74) to make core-shell nanoparticles with high index core and low index shell. They used a one-pot emulsion method to assemble these core-shell particles into photonic balls that showed bright colors (Fig. 5e). The colors were tuned either by varying core size and shell thickness or by mixing two sizes of core-shell nanoparticles.

Co-assembling melanin with other non-absorbing particles can avoid too much absorption. Random mixing is not always obtained when assembling two types of particles. Xiao et al. demonstrated that melanin particles completely segregate at the outmost layer of photonic balls during co-assembly of melanin and silica particles in an emulsion approach (Xiao et al. 2019). With interfacial tension measurements and molecular dynamic simulations, they revealed that the surface enrichment was caused by the larger interfacial contact angle of melanin than that of silica particles. Inspired by this, Patil et al. used molecular dynamic simulations and numerical optical calculations to systematically explore how the degree of surface enrichment influences the colors of photonic balls (Patil et al. 2021). By decreasing the surface enrichment of melanin particles, the reflectance peak not only broadens but also shifts to shorter wavelengths.

Liu et al. synthesized melanin-doped polymeric nanoparticles by adding dopamine chloride to mixtures of monomers (methyl methacrylate, divinylbenzene, and 2-hydroxyehtyl methacrylate) during emulsion polymerization. To create a non close packing, they dispersed these melanin-doped nanoparticles in 2-hydroxyethyl methacrylate (HEMA) solvent and crosslinked HEMA to produce a photonic hydrogel film. By controlling the melanin concentration in these nanoparticles, they obtained sufficient absorption to produce high color saturation without disrupting the packing order (Liu et al. 2020).

4.3 Inelastic Scattering

When the light interacts with melanin, most light is scattered without changing frequency. If the frequency of the scattered light is different from that of the incident light, inelastic scattering (also called Raman scattering) happens. The frequency of Raman scattered light is often smaller than the incident light. The energy difference between incident light and scattered light is determined by the molecular vibration energy, which represents the characteristic information of molecular structures. Raman spectroscopy can detect functional groups, just like IR spectroscopy. Melanin is Raman active with two Raman peaks at around 1380 cm^{-1} and 1580 cm^{-1}, similar to G and D bands in graphene and graphite (Huang et al. 2004; Capozzi et al. 2005). The first peak originates from the linear stretching of C-C bonds within the aromatic rings, and the second peak originates from the in-plane stretching of the rings. These peaks are distinguishable from other biological materials, like proteins, polysaccharides, and carotenoids, which provides an invasive method to identify the existence of eumelanin in feathers, hairs or even fossils (Galván et al. 2013; Li et al. 2018; Peteya et al. 2017). Noble metal substrates (like gold, and silver) can largely enhance Raman signal intensity of melanin due to plasmonic resonances. Ju et al. recently coated melanin onto gold nanoparticles and used the core-shell particles as nanoprobes for Raman imaging (Ju et al. 2015).

5 Outlook

Melanin is a group of dark pigments and it interacts with electromagnetic waves in a unique way. Its special optical properties include broadband absorption across UV to near IR, the capability to rapidly dissipate most absorbed energy to heat, and a high refractive index. These properties make melanin widely used in fields like biocompatible hair dyes, UV protection, photothermal therapy, and structural coloration. Here we conclude by highlighting the challenges and opportunities in the study of melanin's photonic properties and optic-related applications. First, there is still a lack of understanding in the quantitative relations between melanin's hierarchical chemical structure and its broad absorption spectra. This includes the challenges of precisely quantifying melanin's chemical structure and revealing the complex supramolecular interactions in melanin. This quantitative relation will provide a roadmap to developing novel melanin-like materials with tuned absorption. Second, it is still unclear how melanin particles organize into ordered or disordered photonic structures in bird feathers and other living creatures. Addressing this challenge may change the current approaches to fabricating photonic materials. Third, we need to integrate melanin's excellent photonic properties with its biocompatibility, radical quenching, and metal binding capabilities so that we can create multifunctional materials for broader applications. To this end, we believe further development of

this field requires joint efforts from biologists, chemists, physicists, and materials scientists.

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