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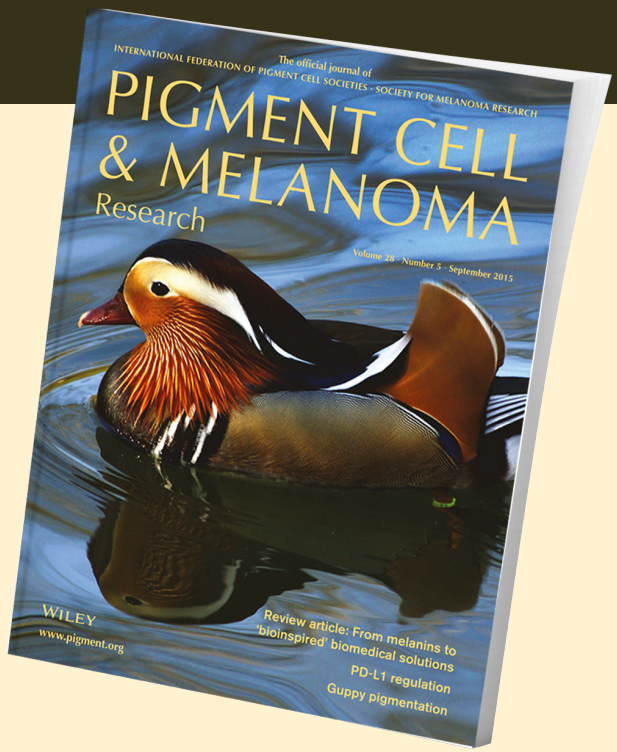

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Pigment Cell Melanoma Res. **28**; 520–544 REVIEW

Melanins and melanogenesis: from pigment cells to human health and technological applications

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Summary

During the past decade, melanins and melanogenesis have attracted growing interest for a broad range of biomedical and technological applications. The burst of polydopamine-based multifunctional coatings in materials science is just one example, and the list may be expanded to include melanin thin films for organic electronics and bioelectronics, drug delivery systems, functional nanoparticles and biointerfaces, sunscreens, environmental remediation devices. Despite considerable advances, applied research on melanins and melanogenesis is still far from being mature. A closer intersectoral interaction between research centers is essential to raise the interests and increase the awareness of the biomedical, biomaterials science and hi-tech sectors of the manifold opportunities offered by pigment cells and related metabolic pathways. Starting from a survey of biological roles and functions, the present review aims at providing an interdisciplinary perspective of melanin pigments and related pathway with a view to showing how it is possible to translate current knowledge about physical and chemical properties and control mechanisms into new bioinspired solutions for biomedical, dermocosmetic, and technological applications.

Introduction

Melanin-producing cells, the melanocytes, are endowed with unique and fascinating properties that make them a most valuable source of inspiration for new exciting approaches and solutions to important biomedical and technological problems. Much of the uniqueness of pigment cells is due to the pigment itself, melanin, as a most distinguishing trait that has attracted the attention of scientists and clinicians since a long time.

Over the past decade, melanins and melanogenesis have been increasingly appreciated outside the pigment cell community, as a source of novel research opportunities in the fields of biomedicine, dermocosmetics, nanotechnology, and materials science. The burst of interest in polydopamine is a paradigm example of this trend (Liu et al., 2014). Following a preceding consensus review (d'Ischia et al., 2013), this study has been conceived as a means of bringing together for the first time researchers from broadly diverse disciplines and from both academic and industrial settings to provide an interdisciplinary and intersectoral perspective of melanins and melanogenesis. Far from being comprehensive, this study aims mainly to offer a careful selection of background information on melanin biological roles and functions that is directly relevant to the

main objective of this review, that is, to stimulate the interests of academic and industrial laboratories active in the fields of human health and sustainable technology (Figure 1). For those important aspects of pigment cell research that do not fall entirely within the scope of the study, such as human cutaneous melanoma or non-cutaneous melanogenesis, and for a more detailed discussion of melanins, melanogenesis, and their applications, the readers are referred to a number of reviews (e.g. Galván and Solano, 2009; Prota, 1992; Riley, 1997; Solano, 2014) and an excellent book (Borovansky and Riley, 2011).

Terminology

The term *melanins* denotes pigments of diverse structure and origin derived by the oxidation and polymerization of tyrosine in animals or phenolic compounds in lower organisms. *Eumelanins* are the black-brown subgroup of insoluble melanin pigments derived at least in part from the oxidative polymerization of L-DOPA via 5,6-dihydroxyindole intermediates. *Pheomelanins* are yellow-to-reddish brown subgroup of melanin pigments derived from the oxidation of cysteinyldopa precursors via benzothiazine and benzothiazole intermediates. It is noticed that very often the terms melanin and eumelanin are used as synonymous, although this practice is not recommended. Melanocytes are responsible for the synthesis of melanin within specialized membrane-bound organelles termed *melanosomes*, and the subsequent transfer of the melanosomes to surrounding epidermal cells, the keratinocytes. Melanosomes are thus distinct from '*melanin granules*' which refer to the pigment particles produced by enzymatic or chemical reactions. '*Melanogenesis*' is the complex process of melanin synthesis in nature or, by extension, in an in vitro system. A more in-depth discussion of melanin nomenclature, definitions, and classification is given in the previous consensus paper (d'Ischia et al., 2013).

Biological roles and functions of melanins and melanogenesis

Skin and hair

Variation in human skin and hair color is a conspicuous determinant of human phenotypes and is largely due to melanin pigmentation. Photoprotection of the skin is apparently one of the most important biological functions of melanin pigments. Subjects with white skin are approximately 70 times more likely to develop skin cancer than subjects with black skin, suggesting that higher levels of constitutive pigmentation decrease susceptibility to the deleterious effects of ultraviolet radiation (UVR) (Brenner and Hearing, 2008; Coelho et al., 2009). Usually, black skin and Asian skin contain higher levels of melanin than fair skin (Tadokoro et al., 2003). Although epidermal melanin accounts for a skin protection factor of only 2–3, it provides sufficient photoprotection against UVR-induced damage of nuclear DNA by shielding nuclei by supranuclear melanin caps in melanocytes and keratinocytes (Kobayashi et al., 1998). Other important properties of eumelanin include its function as a free radical scavenger reducing the production of reactive oxygen species (ROS) (Meredith and Sarna, 2006).

The genetic basis for diversity of human hair color, which may range from black to dark brown, light brown, blond to red, has been extensively investigated (for a review, see Ito and Wakamatsu, 2011; Sturm and Duffy, 2012). Chemical studies (Ito et al., 2011a; Wakamatsu et al., 2003) showed that the melanin composition (chemical phenotype) correlates fairly well with hair color (visual phenotype). Thus, eumelanin contents decrease in human hairs of black, dark brown, brown, light brown, blond, and red color, in that order, while pheomelanin

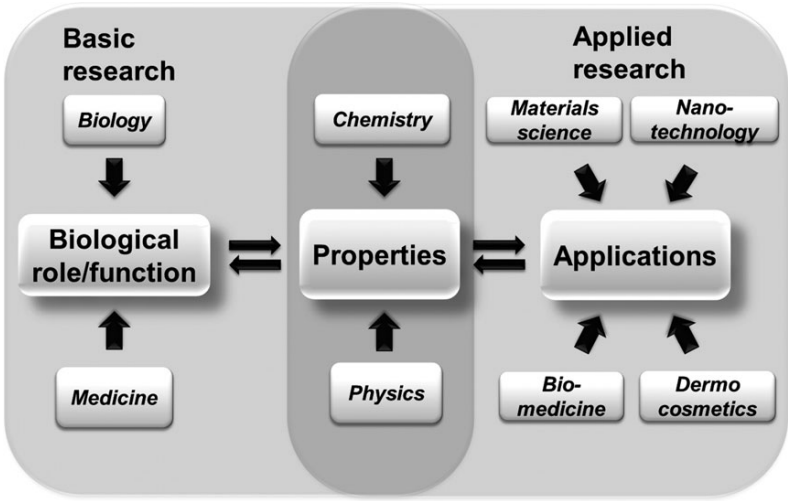

Figure 1. Melanins and melanogenesis: Elucidating chemical and physical properties is central to unravel biological roles and functions and to translate clues from pigment cells to innovative applications.

levels remain low but constant, except in the case of red hair, which contains similar levels of pheomelanin and eumelanin (Ito et al., 2011b).

Melanins, in particular pheomelanin, can also have toxic properties, especially upon exposure to UVR. Pheomelanin is prone to photodegradation (Chedekel, 1982; Greco et al., 2009; Wakamatsu et al., 2012a) and is believed to contribute to the damaging effects of UVR because it can generate hydrogen peroxide and superoxide anions (Chedekel et al., 1978; Felix et al., 1978; Simon and Peles, 2010) and might cause mutations in melanocytes (Harsanyi et al., 1980). Several studies have addressed the possible roles of eumelanin or pheomelanin in inducing DNA damage and ultimately melanomagenesis both with and without UVR. Mitra et al. (2012) showed that using a conditional, melanocyte-targeted allele of the melanoma oncoprotein BRAF (V600E) into yellow mice carrying the $MC1R^{e/e}$ allele of the *MC1R* gene, a high incidence of invasive melanoma was observed without UVR. On the other hand, selective absence of pheomelanin synthesis was proved to be protective against melanoma development. In this connection, Panzella et al. (2014) demonstrated that pheomelanin can induce antioxidant depletion by a redox cycling mechanism without UVR.

Eyes

Mammalian eye contains melanin-producing cells, which are present in different tissues and have different embryonic origin (Hu, 2008; Sarna, 1992). While the pigment epithelial cells of the retina (RPE), the iris, and the ciliary are derived from the neural ectoderm, the uveal melanocytes of the choroid, the stroma of the iris, and ciliary body are developed from the neural crest. The human pigment epithelium is typically densely pigmented in all races and in all eye colors, and it mainly contains eumelanin. Melanosomes in the human RPE are formed early in the fetal development, and although pigment deposition in these organelles may continue for some time after birth, there is very little metabolic turnover of the formed pigment granules. Pigmentation in human uveal melanocytes exhibits significant variation; these cells contain both eumelanin and pheomelanin, and their relative content and total amount depend on the race and iris color (Prota et al., 1998; Wakamatsu et al., 2008; Wielgus and Sarna, 2005).

The biological role of ocular pigmentation is only partially understood. There is little doubt that melanin in the iris and the RPE acts as an optical absorber, which prevents the retina from excess of light that impinges on the eye and reduces spurious signals that could arise from light reflection of the surface of the Bruch's membrane. Interestingly, the incidence of two important eye diseases, uveal melanoma and age-related macular degeneration (AMD), appears to be correlated with the pigmentation of the iris. A population-based study revealed that the incidence of uveal melanoma was highest in non-Hispanic white followed by Hispanics, Asians, and Blacks (Weis et al., 2006). Although the relationship between iris color and AMD is not so conclusive as that in uveal melanoma, AMD is an order of magnitude more prevalent in the white population than in darkly pigmented races (Friedman et al., 1999).

Although the correlation between AMD and pigmentation of the iris is supported by convincing epidemiological data, whether or not such a correlation can be viewed as causal is still unclear. Nevertheless, it has been postulated by many researchers that excess of light that reaches the retina is a contributing factor to AMD. Therefore, light-pigmented iris, which transmits more light, could be responsible for enhanced risk of developing AMD.

Acting as an antioxidant, uveal and RPE melanin can also protect the pigmented tissues against oxidative damage, induced by photic or oxidative stress (Meredith and Sarna, 2006; Sarna, 1992). Indeed, it was recently demonstrated that phagocytized porcine RPE melanosomes protected ARPE-19 cells, a human RPE cell line, against oxidative stress induced by hydrogen peroxide (Burke et al., 2011).

Metal accumulation, especially iron, has been reported in eye melanosomes, suggesting that melanosomes may contribute to iron homeostasis (Kaczara et al., 2012). The lack of metabolic turnover of melanin in post-mitotic cells, such as RPE, raises an intriguing question whether antioxidant and photoprotective abilities of this pigment decrease with aging and, if so, what physicochemical modifications of melanin are responsible for such changes. EPR (Sarna et al., 2003) and chemical analysis (Ito et al., 2013) of human RPE melanin and of bovine RPE melanosomes exposed to intense blue light demonstrated an age-related loss of the content of human RPE melanin, which undergoes progressive modifications through oxidative cleavage and cross-linking. These changes are most likely induced by in situ chronic photooxidation of the melanin. Chloroquine can be detected in eye melanin 1 year after administration, a phenomenon possibly associated with retinopathies.

Human brain

Neuromelanin (NM)-containing neurons are ubiquitous in human brain and occur with the highest number in substantia nigra (SN) and locus coeruleus (LC), regions that are the main targets of Parkinson's disease (PD) (Cowen, 1986; Zecca et al., 2008a). The NM pigments are found also in neurons of monkeys, dogs, horses, rats, and frogs (Barden, 1971; DeMattei et al., 1986).

NM pigments are contained within double membrane organelles along with lipid bodies and proteins (Duffy and Tennyson, 1965; Sulzer et al., 2008; Zecca et al., 2008a). Recent investigations show that these NM-containing organelles are a special type of lysosomes derived from fusion with autophagic vacuoles. NM deposition begins very early in life, and the pigment concentration increases linearly with age reaching values up to 3.7 μg/mg tissue

between 50 and 90 years of age in SN and up to 3.1 μg/mg tissue in LC (Zecca et al., 2002, 2004). In PD, a preferential loss of SN dopamine neurons containing NM occurs with respect to non-pigmented neurons, and it has been long discussed if NM can increase neuronal vulnerability in PD (Hirsch et al., 1988; Hornykiewicz and Kish, 1987; Marsden, 1983). NM concentration is 50–60% lower than in age-matched controls, due to the loss of NM-containing neurons (Zecca et al., 2002, 2004). A relationship between the vulnerability of dopamine neurons and their NM content was reported (Kastner et al., 1992).

NM pigments are comprised of spherical aggregates which are ~20–400 nm in size, with varying shapes, composed of smaller spherical substructures of ~30 nm in diameter (Bush et al., 2006, 2009). The structure proposed for the melanic portion of NMs is that of a pheomelanin core surrounded by eumelanin, forming spherical electron-dense aggregates. The oxidation potential of NM surface is not sufficiently reductive to cause a high level of oxidative stress, attributed to the presence of eumelanin on the surface (Bush et al., 2006). In NM structures, there are melanic groups bound to proteins and aliphatic chains (Zecca et al., 1992, 2000). The melanic groups, composed of dihydroxyindole and benzothiazine rings, are covalently bound to aliphatic chains and peptide moieties (Wakamatsu et al., 2003). An organic radical located on the melanic portion is present in all NM pigments (Aime et al., 1997; Enochs et al., 1993; Zecca et al., 1996, 2008a). The amount of lipids covalently bound to NM pigment of the SN is about 20%, while lipid content is higher for NMs from other brain areas (Engelen et al., 2012; Zecca et al., 2000). Dolichols and dolichoic acids (containing 14–22 isoprene units) are the main component of NM lipids, and to a lesser extent cholesterol, ubiquinone-10, α-tocopherol, phospholipids, and lactones (Engelen et al., 2012; Ward et al., 2007, 2009; Zecca et al., 2000, 2008a). In NM pigments, there is a soluble portion composed of dolichols bound to proteins and melanic oligomers, and an insoluble portion with a more extended melanic component (Engelen et al., 2012; Ward et al., 2007, 2009).

The synthesis of NM pigment depends on the cytosolic concentrations of catechols that are not accumulated in synaptic vesicles (Sulzer et al., 2000). Catechols can be oxidized to quinones and semiquinones via iron catalysis, and these can react with aggregated proteins accumulated in cytosol. The polymerization process forms a melanin-protein component, which can also bind high amounts of metals, especially iron. The resulting undegradable material is taken into autophagic vacuoles, and after fusion with lysosomes, it can interact with lipids and other proteins forming the NM-containing organelle (Engelen et al., 2012; Zucca et al., 2014).

The synthesis of NM is itself a protective mechanism that traps excessive cytosolic quinones and semiquinones in an inactive form, thereby blocking their toxicity (Sulzer et al., 2000). Moreover, the dihydroxyindole groups of NM pigment are able to bind several potentially toxic metals forming stable complexes (Zecca and Swartz, 1993; Zecca et al., 1994, 2008a), and in particular, the redox-active ferric ions, mitigating the formation of hydroxyl radicals (Zecca et al., 2008b). NM pigment can initially play a protective role also by sequestering several toxins, such as 1-methyl-4-phenylpyridinium ion (D'Amato et al., 1986) and the herbicide paraquat (Lindquist et al., 1988).

Catecholamine neurons of SN and LC, which degenerate in PD, express the major histocompatibility complex class I (MHC-I), and this is highly concentrated in NM-containing organelles. MHC-I can bind antigenic peptides and expose them on neuronal membrane, so that cytotoxic T cells target these neurons inducing cell death. The finding of MHC-I in pigmented catecholamine neurons of SN and LC provides an explanation for selective cell death of neurons containing NM, suggesting the presence of a novel neurodegenerative process of autoimmune type that is specific for PD (Cebrián et al., 2014). In parkinsonian SN, NM is released from dying neurons and remains in the extracellular space, along with sustained microglial activation (Langston et al., 1999; McGeer et al., 1988). Due to its insolubility, NM pigment could remain for long time in the extracellular milieu of the SN and may become an agent of chronic inflammation, slowly releasing the metals and toxic components encased in its structure. It was found that this NM can activate microglia causing release of tumor necrosis factor-α, interleukin-6, nitric oxide, and hydrogen peroxide. Then, activated microglia and these molecules induce degeneration of dopaminergic neurons, thus fueling a continuous vicious cycle of neuroinflammation and neurodegeneration causing the progression of PD (Wilms et al., 2003; Zecca et al., 2008c; Zhang et al., 2011).

Birds, reptiles, amphibians, and fish

Among non-mammalian vertebrates, birds are the group where the properties and functions of melanins have been so far most intensively investigated. Many of the genes encoding the main components of the mammalian melanogenesis pathway, that is, pro-opiomelanocortin (POMC), five melanocortin receptors (MCRs), and agouti, have been characterized in birds (Takeuchi et al., 2003). While in mammals it is the agouti-signaling protein (ASIP) that antagonizes MC1R signaling on melanocytes, in birds this role may be fulfilled by the agouti-related protein (AGRP), which is involved in the regulation of energy balance in mammals (Boswell and Takeuchi, 2005). Thus, the melanogenesis pathway is remarkably similar in mammals and birds and at least the basic mechanisms seem to have evolved early (Schiöth et al., 2003).

Despite the similarity in the mechanisms of melanin production, the functions of melanins in birds and mammals seem to be different. Feathers colored by melanins have evolved as signals in many species with

which individuals can honestly reveal their genotypic quality to conspecifics and thus maximize their reproductive success or performance during social interactions (reviewed in McGraw, 2008).

Besides the signaling potential of the two main forms of melanins, eumelanin and pheomelanin, in birds (Galván and Solano, 2009), non-signaling functions of these pigments must also be considered. Melanins deposited in tegumentary structures such as feathers and bills increase hardness (Bonser, 1995) and resistance of these structures against abrasion caused by airborne particles (Schreiber et al., 2006), although eumelanin and pheomelanin do not seem to differ in their capacity to increase plumage strength (Pannkuk et al., 2010). Melanins also protect feathers from the damaging effects of feather-degrading bacteria (Burtt and Ichida, 2004), although they may even serve as a substrate for some species of bacteria (Grande et al., 2004). Melanins in plumage may also be involved in thermoregulatory functions in birds, as the dark colors typically conferred by these pigments absorb more radiant energy than light colors, although there is no clear evidence of the actual importance of plumage melanization in covering the thermoregulatory needs of birds (reviewed in Bortolotti, 2006). The capacity of dark colors to absorb radiant energy may however represent a physiological cost, which may have led to the evolution of compensatory mechanisms (Bamford et al., 2010; Negro et al., 2006). Lastly, studies with birds have increased our understanding of the function of melanins from non-classical melanocytes that are found in internal organs. Thus, the presence of melanin in testes have evolved in species of birds with high mtDNA substitution rates, probably an adaptive response related to the protective capacity of melanins against the oxidative stress generated by mitochondrial dysfunction caused by mutations (Galván et al., 2011a). In the same context, studies with birds have suggested that melanins in internal organs such as the brain may function as protective agents against reactive quinones and toxic metals (Galván and Møller, 2011; Galván et al., 2011b).

Melanins are also present in poikilotherms. The most obvious difference in the mechanism of melanin production between homeotherms and poikilotherms is the fact that in the latter the transfer of melanosomes from epidermal melanocytes to the target structures does not occur; instead, dermal melanophores have the ability to disperse and aggregate their melanosomes (Kawasaki et al., 2008). The change in melanization patterns in poikilotherms can thus be a dynamic and rapid process that occurs in response to a diversity of hormonal and environmental stimuli as opposed to homeotherms, in which changes in melanization depend on the turnover rate of the dead keratinized structures where melanosomes are transferred (Fujii, 2000). The melanogenesis pathway of poikilotherms, however, does not seem to greatly differ from that of homeotherms (Cerdá-Reverter et al., 2011). Gallone et al. (2007) described that the liver melanin of amphibians (*Rana esculenta*) is composed of DHI-rich eumelanin showing characteristics very similar to those of *Sepia* melanin, although it contained little pheomelanin. Indeed, up to recently, it was believed that pheomelanin was only synthesized by higher vertebrates (i.e. birds and mammals), but the presence of this pigment has been discovered in the shell of a reptile (the Eastern Hermann's tortoise *Eurotestudo boettgeri*; Roulin et al., 2013) and in the skin of adults and tadpoles of an amphibian (the African dwarf frog *Hymenochirus boettgeri*; Wolnicka-Glubisz et al., 2012).

Given the dependence of poikilotherm vertebrates on the temperature of the environment to conduct their vital processes and the capacity of the dark colors conferred by melanins to absorb radiant energy, it is expected that melanins play a crucial role in the thermoregulation of poikilotherms, as it actually does by facilitating the adaptation of some individuals to cooler environments (Clusella-Trullas et al., 2007). The universal function of (eu)melanin as a protective agent against UVR has also been investigated in poikilotherm vertebrates, particularly in amphibians, where skin melanization in response to increase in UVR has been shown to be crucial for the survival of larvae (e.g. García et al., 2004). Melanization as a response to UVR has been observed also in adults (Adachi et al., 2005) and larval fish (Mueller and Neuhauss, 2014).

Cephalopod ink

Black insoluble eumelanin is the major visible constituent of cephalopod ink, which is produced by the activity of a gland located in the bottom of the ink sac. The ink gland consists of an immature inner portion and a larger mature portion. Pigmentation in ink gland cells is regulated by the glutamate/NO/cGMP pathway (Palumbo et al., 2000), is associated with extensive protein nitration (Fiore et al., 2009), and is an index of the degree of maturation (Derby, 2014).

Although tyrosine metabolism is the central biochemical feature of the ink defense system, the actual significance of this process, with special reference to the biological function of melanin in the ink, is still little understood. The occurrence of large amounts of tyrosinase in the ink (Palumbo, 2003) together with another melanogenic enzyme which catalyzes the rearrangement of dopachrome to 5,6-dihydroxyindole (DHI) (Palumbo et al., 1994) was supposed to ensure efficient conversion of catechol constituents into toxic quinones preventing the attack of predators. DOPA and dopamine are also found in the melanin-free fraction of cephalopod ink (Fiore et al., 2004) and can affect squid olfactory neurons (Di Cristo et al., 2007). It can thus be suggested that the melanin granules in the secreted ink serve as sink/carrier for bioactive substances such as dopamine, preventing dilution after ejection and ensuring efficient interactions with target organs. In addition, sepia tyrosinase was found to be toxic to a variety of cell lines, including PC12

cells (Russo et al., 2003), leading eventually to an irreversible apoptotic process.

Insects

Melanogenesis is a prominent expression of tyrosine metabolism in insects which depends on fine-tuned systems of enzymes (Sugumaran, 2002; Vavricka et al., 2014), including phenoloxidase and dopachrome (decarboxylating) isomerase, and is mainly related to cuticle sclerotization and innate immune response. Sclerotization is a complex process involving the rapid hardening of the soft and pale freshly produced cuticle to prevent dehydration and to afford protection to eggs and animals at the various levels of development, from larvae to adults. Sclerotization is initiated by the action of phenoloxidase on catecholamine sclerotizing precursors, such as *N*-acetyldopamine and *N*-*β*-alanyldopamine, to generate their corresponding quinones which in turn, rapidly form adducts not only with the reactive amino acid side chains of structural proteins, but also with the side chain hydroxyl groups of chitin polymer, which is the major structural component of the cuticle.

Besides its role in cuticle sclerotization, melanogenesis provides an important mechanism in the defense against parasites and pathogens, due to the lack of immunoglobulins and other components of mammalian immune system (Nappi and Christensen, 2005; Shao et al., 2012). The aim of melanogenic reactions is to prevent the uncontrolled growth and the damage caused by the intruder. In response to an alarm signal, insect phenoloxidase in the hemolymph is activated and adheres to the invader inducing melanization by quinone formation. Melanin formation then blocks the parasite and prevents its growth (Clark and Strand, 2013).

Plants, fungi, and bacteria

Plants usually incorporate melanins for cell wall strengthening purposes. In contrast to animal pigments, plant melanins are devoid of nitrogen and their color may range from dark brown to totally black, depending on the nature of the main unit oxidized. Most common precursors include catechol, 1,8-dihydroxynaphthalene (DHN) and a number of acids, such as caffeic, chlorogenic, protocatechuic, and gallic acids (Figure 2), yet their structure is still little understood.

In the sclerotization reactions of plants, polyphenol oxidases are liberated from vesicles in response to injury and, in the presence of oxygen, catalyze the two-electron oxidation of tannins to produce very reactive quinones. Cross-linking by quinones occurs typically through reaction with a nucleophilic group in a protein, such as a thiol or amine, to give a melanoprotein. The result is blackening and hardening at a site of injury, a reaction known as enzymatic browning.

Increasing interest is currently being devoted to fungal melanins (Eisenman and Casadevall, 2012; Jacobson, 2000; Langfelder et al., 2003; Nosanchuk and Casadevall, 2003) due to their biotechnological importance and their central role in the virulence of plant and human pathogenic fungi. In fungi, melanogenesis occurs during specific developmental stages. Melanin may be synthesized in internal vesicles and then transported to the cell wall where it is cross-linked to polysaccharides. Main phenolic precursors of fungal melanins include DHN, which is typical of Ascomycetes, and γ-glutaminyl-4-hydroxybenzene and catechol (Dunn, 1986), characteristic of Basidiomycetes.

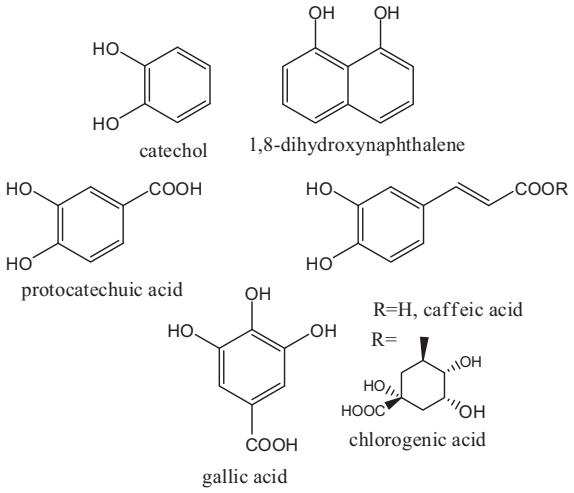

Figure 2. Structures of main melanin precursors in plants, fungi, and microorganisms.

Some fungi can synthesize two types of melanin. The neuropathogenic basidiomycetous Cryptococcus neoformans (Casadevall et al., 2000) can attack human brain and contains a phenol oxidase capable of oxidizing dopamine and norepinephrine to NM-like polymers (Polacheck and Kwon-Chung, 1988) which can increase the virulence of the infection. The same fungus oxidizes homogentisic acid (HGA) to synthesize pyomelanin in the form of an insoluble fluorescent material that can likewise increase the virulence. *Aspergillus fumigatus*, an airborne fungal pathogen in immunosuppressed humans, is also able to produce DHN melanin or pyomelanin starting from L-tyrosine through HGA (Schmaler-Ripcke et al., 2009), while *Aspergillus nidulans* can form DHN melanin and DOPA melanin (Goncalves et al., 2012).

Melanins are also found in bacteria, where they were initially identified in tyrosinase-containing Streptomyces, *Marinomonas mediterranea* (Lopez-Serrano et al., 2004) and *Bacillus thuringiensis* (Patel et al., 1996). This topic has been the subject of much interest also in view of the potential applications and has been extensively reviewed (Dadachova and Casadevall, 2008; Plonka and Grabacka, 2006; Riley, 1997; Solano, 2014). Bacterial melanins usually contain nitrogen, although non-nitrogenous variants from catecholic precursors have been identified. *S. marcescens* (Trias et al., 1989) can produce a brown

pigment by oxidation of 3,4-dihydroxyphenylacetate, as a means of counteracting accumulation of this catabolic intermediate. Some *Pseudomonas* produced a pyomelanin from 4-hydroxyphenylpyruvate. Besides pathogenesis, melanin synthesis in microorganisms is believed to be part of the defense systems against environmental stresses such as UVR and oxidizing agents, and more in general against the consequences of environmental cues. It is relevant in this connection that after the Chernobyl disaster black and highly melanized fungi responded to ionizing radiation with enhanced growth (Dadachova and Casadevall, 2008).

Melanin properties and control of melanogenesis

Biological control of melanogenesis

Biological control of melanogenesis and melanin pigmentation in living organisms derives from the interplay of different factors and mechanisms. For example, melanin-forming enzymes are not functional unless they enter the melanosomes and become activated in the endoplasmic reticulum (Lamoreux et al., 2010). Correct control over this process is necessary for normal pigmentation and to prevent activation of toxic melanogenesis-related pathways outside the melanosome.

Melanin type

Within melanocytes, the receptor for αMSH (melanocortin 1 receptor, MC1R) encoded by the *extension* locus is the major determinant of the type of pigment formed in mouse coat. Activation of MC1R stimulates eumelanogenesis, whereas loss-of-function mutations at *extension* are associated with pheomelanic coats (Robbins et al., 1993; Valverde et al., 1995). Likewise, human MC1R regulates the amount and type of pigment formed in human skin, with the wild-type receptor promoting eumelanogenesis whereas variants with impaired signaling are associated with pheomelanogenesis and a red hair color phenotype (Garcia-Borron et al., 2014).

Activation of the MC1R by its endogenous agonists αMSH and ACTH stimulates tyrosinase activity by transcriptional stimulation of *tyrosinase* gene expression as well as by posttranscriptional mechanisms. The molecular events leading to upregulation of *tyrosinase* gene expression downstream of MC1R are relatively well understood and rely on activation of the cAMP signaling pathway and the microphthalmia transcription factor. MC1R signaling also triggers a cAMP-dependent increase of the melanosomal pH from acidic to near-neutral values, which enhances the catalytic efficiency of tyrosinase (Cheli et al., 2009). Surprisingly, little is known about possible effects of MC1R signaling on the intracellular pool of low molecular weight thiols in mammalian melanocytes. However, it has been shown that cysteine depletion promotes eumelanogenesis in human melanoma cells (del Marmol et al., 1996). Conversely, high levels of cysteine in the culture media of mouse melanocytes, coupled with tyrosinase inhibition with phenylthiourea in the presence of the agouti-signaling protein (ASIP), dramatically increase the pheomelanin/eumelanin ratio up to 200-fold (Hida et al., 2009).

Treatment of normal human melanocytes with αMSH or ACTH also increases expression of the other melanogenic enzymes, the tyrosinase-related proteins TRP1 and TRP2 (Abdel-Malek et al., 1995). Given that the three proteins of the tyrosinase family most likely do not undergo identical fold stimulations, it is conceivable that the ratios of their enzymatic activities would not be the same in resting versus stimulated melanocytes. These variations may have a subtle and still uncharacterized impact on the final structure of the pigment, for instance through the modification of the ratio of carboxylated monomeric units incorporated to eumelanins.

MC1R signaling is regulated by at least two endogenous ligands in addition to αMSH and ACTH. In mice, ASIP blocks the stimulatory effect of αMSH on tyrosinase and decreases *TRP1* and *TRP2* gene expression and total melanin production (Hida et al., 2009; Le Pape et al., 2009). In fact, ASIP behaves as a potent inverse agonist (Siegrist et al., 1997), as it decreases agonist-dependent signaling as well as the basal constitutive activity of the receptor (Sanchez-Mas et al., 2004). The effects of ASIP on cultured normal human melanocytes are similar (Suzuki et al., 1997; Swope et al., 2012), and polymorphisms in human *ASIP* are associated with skin, hair, and eye pigmentation phenotypic changes (Bonilla et al., 2005; Kanetsky et al., 2002). A third MC1R ligand, the small secreted protein β-defensin-3 (BD3), binds MC1R with nanomolar affinity (Candille et al., 2007). Human BD3 behaves as a neutral antagonist unable to activate cAMP formation in normal human melanocytes, but efficiently blocking αMSH-induced cAMP production and activation of tyrosinase (Swope et al., 2012).

The knowledge of the structure and function of MC1R ligands, and of the signaling pathways that they engage to regulate melanin pigmentation and non-pigmentary defensive mechanisms against UVR-induced cellular damage may direct the rational design of small peptides with potential pharmacological applications (Abdel-Malek et al., 2009), and of receptor-independent strategies to achieve physiological responses such as tanning (D'Orazio et al., 2006).

Hair graying

Gray hair condition varies between individuals, worldwide, with various ages of incidence and distinct progression in magnitude (Panhard et al., 2012). The graying of hair is due to a progressive depletion of melanocyte stem cells (MSC) in hair follicles, leading to the defect of the hair pigmentation unit renewal at the telogen to anagen transition during the hair cycle, and to the growth of white hair, eventually (Commo et al., 2004a; Nishimura et al., 2005). It is noteworthy that the exhaustion of bulb

melanocytes is progressive and during the graying of hair, transitional gray hairs show a reduced, albeit not null, melanocyte number in the bulb with reduced melanin content in the hair shaft, as compared to fully pigmented hair (Commo et al., 2004a).

Following the demonstration that hair graying was due to MSC depletion, Arck (Arck et al., 2006) showed a selective oxidative stress sensitivity of hair follicle melanocytes compared to other hair follicle cells, namely keratinocytes and fibroblasts, along with increased mitochondrial DNA deletion in the bulb of gray hair. Genetic mutations in mice also pointed out the particularly high susceptibility of the MSC to oxidative stress-associated DNA damage leading to premature hair graying and furthermore highlighted determinant genes for coat pigmentation maintenance in mice, for example, BRCA1, ATM, Bcl2 (Cao et al., 2003; Inomata et al., 2009; Yamamura et al., 1996). Although of strong interest as mechanistic approach, there is still no evidence whether imbalance in the activity of these genes is involved in natural hair graying in humans.

Of interest, human hair follicle melanocytes (Caucasians, Asians, and Africans) do not express the melanogenic enzyme dopachrome tautomerase (DCT) or TRP2, as opposed to epidermal melanocytes (Commo et al., 2004b). Despite the lack of DCT, not all hairs are gray. Actually, hairs on a given scalp are different and behave differently. Depending on the scalp areas, distinct hair follicle environments would impact differently MSC fate/maintenance, for example, distinct stimuli or distinct stress sources. Moreover, hairs do not have hair cycles of the same duration and do not have the same number of cycles through life, and the number of MSC is various when comparing hair follicle from the same individual and between individuals (similarly, in mice the distribution of melanocytes in hair is different depending on the body area, for example, belly vs back, due to cell migration process at during development).

Besides its function in melanin synthesis, DCT provides a beneficial effect to melanocytes exposed to oxidative stress (Michard et al., 2008a). Likewise, cyto-protective property of DCT was demonstrated in other distinct models (Michard et al., 2008b; Sendoel et al., 2010). Hence, the specific lack of DCT in hair follicle melanocytes likely contributes to their particular susceptibility to deleterious conditions, and the observation that DCT expression could decrease with age in human hair further strengthens a possible relation between DCT expression and hair graying (Commo et al., 2012). Interestingly, in vitro comparison of hair follicle melanocytes isolated from young and old donors suggested a decrease of catalase expression in melanocytes in the elderly (Kauser et al., 2011).

Chemical control of melanogenesis

Both eumelanin and pheomelanin derive from the common precursor dopaquinone formed by oxidation of L-tyrosine by tyrosinase (Figure 3; Raper, 1927; Cooksey et al., 1997). Intramolecular cyclization of dopaquinone then produces cyclodopa which, following redox exchange with dopaquinone itself, gives dopachrome and DOPA, respectively. Dopachrome then rearranges to generate mostly DHI and to a lesser extent DHICA (Edge et al., 2006) which are then further oxidized and

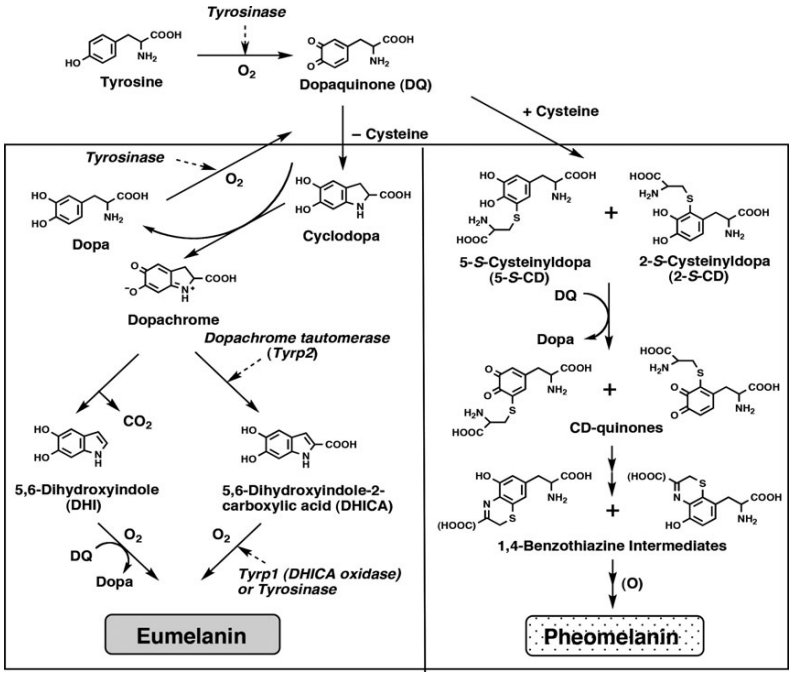

Figure 3. Biosynthetic pathways leading to eumelanin and pheomelanin production (Ito and Wakamatsu, 2008). Note that the activities of tyrosinase, TRP1, and TRP2 are involved in the production of eumelanin, while only tyrosinase (and the amino acid cysteine) is necessary for the production of pheomelanin.

polymerized to produce eumelanin. Sulfhydryl compounds (such as cysteine), if present at sufficient levels, may deviate the normal course of the pathway to give thiol adducts of DOPA, *that is,* 5-*S*-cysteinyldopa (5SCD) along with a minor isomer 2-*S*-cysteinyldopa (2SCD) (Ito and Prota, 1977). Further oxidation of these thiol adducts leads to the formation of 1,4-benzothiazine intermediates which are then converted to pheomelanin (Figure 3).

Tse et al. (1976) showed that the addition of sulfhydryl compounds proceeds very quickly to generate thiol adducts. Reduction to the parent catechols through redox exchange proceeds as quickly as thiol addition (Tse et al., 1976), and therefore, these two reactions are competitive (Figure S1). The redox reaction proceeds with reducing agents (QH_2) such as ascorbic acid, DHI, and 5SCD in biological system. Intermolecular reactions with amine compounds do not proceed as quickly. However, in cases when the amino group is present within the same molecule, the amino group rapidly undergo either an intramolecular addition reaction to give an aminochrome (such as dopachrome) or an intramolecular condensation reaction to give an *o*-quinonimine (such as a cyclization product from cysteinyldopaquinone, see Figure 3, Figure S1). It should be emphasized that all of these reactions are controlled by the intrinsic *chemical* reactivity of *o*-quinones. The high reactivity of *o*-quinones may result in their strong cytotoxicity (Riley, 2003). This subject is beyond the scope of this review, but it should be mentioned that cysteinyl residues of proteins are able to bind to *o*-quinones of biological interest such as dopaquinone and dopamine-quinone (Ito et al., 1988). It is thus possible that sulfhydryl enzymes may be inactivated by *o*-quinones in the course of melanogenesis.

How dopaquinone controls the course of mixed melanogenesis has been discussed in detail (see Ito and Wakamatsu, 2008). Kinetic studies suggested a three-step pathway for mixed melanogenesis (Ito, 2003): The initial stage is the production of cysteinyldopas, which continues as long as the cysteine concentration is above 0.13 μM. The second stage is the oxidation of cysteinyldopas to produce pheomelanin, which continues as long as cysteinyldopas are present at concentrations above 9 μM. The last stage is the production of eumelanin, which begins only after most cysteinyldopas and cysteine are depleted. Therefore, the ratio of eumelanin to pheomelanin is determined by tyrosinase activity and the availability of tyrosine and cysteine in melanosomes (Land et al., 2003). A 'cross-over value' for switching between eumelanogenesis and pheomelanogenesis has been determined in vitro when cysteine concentration reaches 0.8 μM.

Recently, Greco et al. (2011) provided chemical evidence supporting the casing model for mixed melanogenesis, by showing that 5SCD melanin oxidizes DOPA into a black insoluble polymer (eumelanin) which encapsulates the active cysteinyldopa melanin core (pheomelanin). Although this model certainly stands true for the synthesis of NM in the substantia nigra of the brain where dopamine and cysteine act as precursors (Bush et al., 2006; Wakamatsu et al., 2012b), it is less clear whether it can be applied to the mixed melanogenesis in melanosomes where tyrosine (not DOPA) and cysteine act as precursors (Ozeki et al., 1997).

Physical properties of melanins

Supramolecular structure and aggregation

Until very recently no consensus has been reached on the precise nature of melanins' physical structure. The nature of the supramolecular structure of eumelanin has been addressed by several studies (Arzillo et al., 2012; Capozzi et al., 2006; Clancy and Simon, 2001; Ghiani et al., 2008; Kaxiras et al., 2006; Liu and Simon, 2003a,b; Meng and Kaxiras, 2008; Reale et al., 2012; Stark et al., 2005). At the molecular level, cross-linking of eumelanin basic building blocks can occur at a number of sites—and this confers upon the melanin structure a degree of disorder which gives rise to many of its physical and chemical properties. This model of chemical disorder, which does not exclude the possible presence of preferential aggregation and aggregation-dependent functional properties in the eumelanin biopolymer (Panzella et al., 2013), is now generally accepted. As proposed by Meredith and colleagues (Watt et al., 2009), accepting the simple proposition that melanin structure is defined by disorder at all levels controlled by the feedstock and synthetic environment, then the question of its precise structure would become moot. However, based on monomer supply and experimental conditions, the degree of disorder at the various levels may vary, which may entail a certain level of control over melanin properties and functions.

Chemical disorder has implications for higher levels of structure within the system. Watt et al. (2009) recently produced compelling electron microscopy evidence that despite the primary-level disorder, melanins both natural and synthetic can self assemble into stacked structures defined by hetero-aromatic non-covalent interactions with characteristic interplane spacing of ~3.7 Å. This work confirms earlier propositions (both theoretical and partially observed with atomic force microscopy) that indolic oligomers could self assemble in this fashion. The extent of the sheets seems dependent upon the synthetic environment—in natural melanins, the secondary sheets can be many hundreds of nanometers if not microns in size, while in synthetic melanin they are much smaller and limited by the relatively poor solvation environment, suggesting that solvophobic interactions drive sheet stacking and subsequent aggregation. Watt et al. (2009) and Bothma et al. (2008) went on to show that the optical absorption of melanin is related to the monomer composition and mode of coupling as destacking the sheets did not influence the shape of the optical absorption. Armed with this knowledge, they also used destacking methods to create device quality, solution processable melanin thin

films. It is also worth noting that a number of studies point to the similarities between the structural and optical features of eumelanin and sp^2 partially disordered carbon materials, such as carbon black (Gallas et al., 1999; Jastrzebska et al., 2010).

Hydration

The historical model of hydration structure of melanin granules envisages both 'strongly' and 'weakly' bound water fractions (Bridelli and Crippa, 2010). The removal of water possibly produces irreversible structural modifications, affecting eumelanin properties. The dried material exposed again to water does not easily swell and regains only part of the water originally bound (Crippa et al., 1989). Thermogravimetric analyses (TGA) showed that Sigma eumelanin loses weakly bound water below 125°C and strongly absorbed water at about 125°C. Above 250°C, it decomposes with release of carbon dioxide. TGA on Sigma eumelanin powder samples indicate that weight loss up to 140°C, mainly attributable to water, ranges from 11.6 ± 1.0 wt% at 60% RH to 16.8 ± 0.7 at 90% RH (Wünsche et al., 2015).

Paramagnetic properties

The presence of unpaired electrons in melanin is one of the key features of the pigment that determines some of its basic physicochemical properties. Based on results of a thorough study, in which an array of complementary physical methods were employed, Mostert et al. (2012a) have concluded that melanin is not an amorphous semiconductor as conventionally believed. The authors demonstrated that upon absorption of water by dehydrated synthetic eumelanin, free charge carriers were produced. The carriers—extrinsic free radicals (electrons) and hydronium ions (protons)—resulted from the comproportionation reaction of the melanin basic units (Figure 4).

The second study by Mostert et al. (2012b) has shown that the solid-state EPR signal of a synthetic eumelanin is dominated by a carbon-centered radical (g-factor 2.0032) with a significant contribution from a semiquinone radical (g-factor 2.0045) from the comproportionation reaction. Although the actual structure of melanin radicals is unknown, EPR analysis allowed to distinguish signals in melanins that derive from species in which the spin density is localized on carbon atoms, and from species which are oxygen-centered radicals, with the spin density being localized on oxygen atoms (semiquinone radicals). The spin density of these radicals can be changed by the addition of either water or base, which increases the concentration of semiquinone radicals. On the other hand, the water-dependent decrease in the content of the carbon-centered radical was explained by its destruction due to the wet environment. Such a destruction of the carbon-centered radicals, which are normally protected from extrinsic reactants being located in the center of the stacked oligomeric units, could be promoted by a destacking mechanism induced by extra negative charge carried by the semiquinone radicals, which disrupts the π–π stacking arrangements. As a result, the carbon-centered radical becomes more exposed to the aqueous environment.

The spin chemistry of eumelanin is probably best illustrated by a time-resolved EPR (TR EPR) study to monitor the photochemistry of radical pairs from melanin in porcine retinal pigment epithelial cells on the submicrosecond timescale (Wang et al., 2009). Two distinct radical pair species were seen: one of enhanced absorption/emission pattern at early times and one mostly emissive at later times. Interestingly, while the early time signal can be satisfactorily simulated by assuming a population coming exclusively from the singlet excited level, the later time component to the spectrum suggests the triplet mechanism of spin polarization. Excited triplet states play important role in photochemistry of many compounds, including variety of photosensitizers. This is because of relatively long lifetime of triplet excited states, in comparison with lifetimes of singlet excited states, which increases the probability of a chemical reaction mediated by triplet excited states to occur. Based on previous studies, in which time-resolved techniques were used such as laser flash photolysis and photoacoustic spectroscopy, no involvement of melanin excited triplet states has been detected, suggesting that virtually all of the energy by melanin-absorbed photons was safely dissipated into heat. The suggested involvement of a triplet species in the observed electron spin polarization phenomena in eumelanin is intriguing because it is not consistent with the widely accepted view of melanin photodynamics, but its actual significance for light-induced processes in melanins depends on the quantum yield value of such a process. Thus, although the TR EPR signals in porcine RPE cells are small and do not challenge the accepted view that the majority of light energy absorbed by melanin is efficiently dissipated into

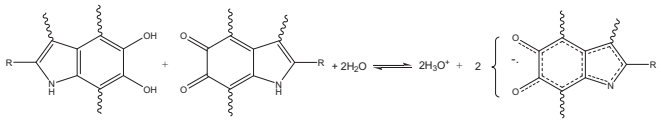

Figure 4. Comproportionation reaction of the basic units within the eumelanin polymer. The scheme shows how water-dependent, pH-driven comproportionation between 5,6-dihydroxyindole and 5,6-indolequinone units generates protons and free radicals (electrons) as free charge carriers.

heat, the role of such minority process in melanin photochemistry and phototoxicity remains to be assessed.

Electrical properties

Eumelanin in the solid state is a hybrid conductor (Mostert et al., 2012a); that is, it can sustain both electronic and ionic currents. The electrical conductivity of melanin films and pelletized powders is strongly dependent upon the state of hydration of the material. Mostert et al. (2012a) showed that for a DOPA-derived synthetic eumelanin, the system shows an almost 'percolation-like' transition at 10–15% by weight water wherein the electrical conductivity increases in a super-exponential manner (Figure 5). The origin of this behavior is a process now referred to as 'chemical self-doping' where the comproportionation equilibrium reaction drives the release of protons at the catechol sites. These protons are transported through the material via the Grotthuss mechanism ('concerted transfer of protons within extended hydrogen-bonded water chains', Kreuer et al., 2004) with the adsorbed water acting as the conducting matrix. In particular, each oxygen atom of the water molecules simultaneously passes and receives a proton through the cleavage and formation of covalent -O-H bonds.

It has been proposed that the mechanism could be generic in hydrophilic conducting bio-macromolecules and that furthermore, the phenomenon could be utilized to create ionic circuitry and even ion-to-electron transducers for use in primary bioelectronic interfaces (d'Ischia et al., 2009; Meredith et al., 2013). A similar process has been recently observed in ionic field-effect transistors utilizing chitosan channels (Meredith et al., 2013).

Melanins have also been observed to be 'photoconductive' in the solid state; that is, their resistance decreases under illumination with UV or visible light (Meredith et al., 2013; Mostert et al., 2012a). The effect has a time constant of order of 10s with pronounced hysteresis. The magnitude of the photocurrent is dependent upon the state of hydration of the material, and there is an order of magnitude difference in the effect above and below the conductivity transition described above (Mostert et al., 2012b). Given the influence of water and the characteristic response times, Mostert et al. have suggested that the photoconductive effect is also related to the comproportionation equilibrium with light acting to drive oxidation of the hydroxyquinone to semiquinone—in the process freeing mobile protons.

Optical properties

Melanins have characteristically broad, monotonic optical absorption (Tran et al., 2006). This was originally attributed to its semiconductivity, but more recently the 'chemical disorder' model has become accepted as an appropriate description (Meredith and Sarna, 2006)—see, however, different views on melanin disorder. In this

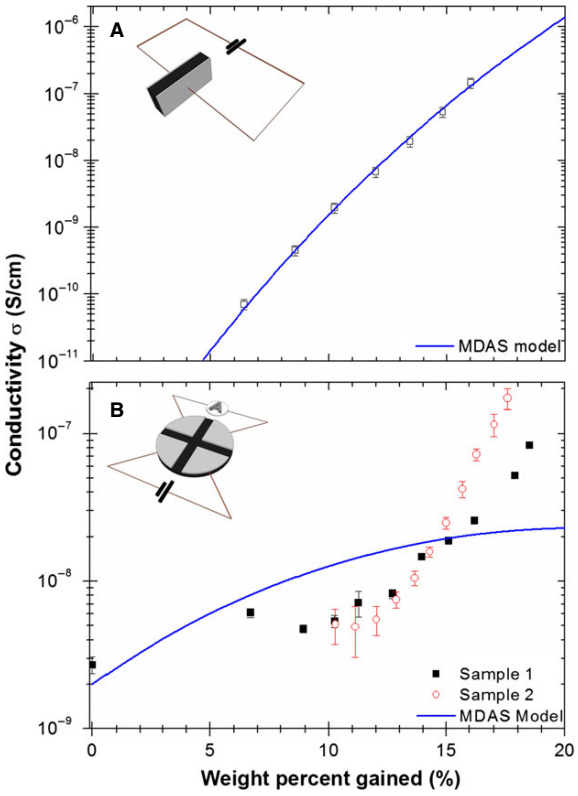

Figure 5. The electrical conductivity of solid-state synthetic eumelanin measured (A) in a simple sandwich configuration and (B) in a surface electrode van der Pauw configuration. The sandwich geometry does not allow the system to come into equilibrium during the measurement and produces an erroneous conductivity isotherm, while the surface electrode configuration does and demonstrates a 'percolation-like' transition at ~10–15% by weight water. The MDAS model is the Mott-Davies amorphous semiconductor model previously (and wrongly) used to describe the behavior (adapted from Mostert et al., 2012b).

model, the featureless absorption is derived from the superimposition of individual inhomogeneously broadened chromophore absorptions resulting from the diversity of chemical species (Meredith et al., 2006). Melanins also demonstrate near-unity conversion of absorbed photons into vibrational energy—photoluminescence quantum yields (PLQYs) of <0.1% (Meredith and Riesz, 2005)—and this is at the core of their role as photoprotectants. Although melanins exhibit variable degrees of photostability (Sarna et al., 2003), in principle eumelanins could be suited for the absorbing component within an optically driven transducing element for bioelectronics. The actual photostability of melanin depends very much on the exact experimental conditions, such as hydration of melanin, pH, presence of redox-active metal ions, oxygen concentration, and the supermolecular structure of the pigment granules. It also depends on the type of melanin with pheomelanin being more photolabile than eumelanin. Indeed, a recently published paper (Ito et al.,

2013) reports that a distinct oxidative photodegradation of melanin occurs even in the human retinal pigment epithelium.

Chemical properties of melanins

A major chemical property of eumelanins is its redox behavior. Multiple oxidation states can be demonstrated by electrochemical reduction with Ti(III) and oxidation with Fe(III) or oxygen. Eumelanins can also catalyze the reduction of Fe (III) by NADH. Redox properties of eumelanins are usually attributed to shuttling of indole units between the catechol and quinone states through semiquinone free radicals. Moreover, eumelanin can either oxidize or reduce metals with single-electron transfer and free radical generation, including ROS, as in Fenton-type reactions (Zecca et al., 1994, 2008a).

The redox properties of a synthetic eumelanin from 5,6-dihydroxyindole (DHI) have been characterized by means of an organic electrochemical transistor (OECT), a powerful tool for biosensing, bioelectronics, and nanomedical applications (Tarabella et al., 2013). Gate current measurements on fine aqueous eumelanin suspensions suggested evolution of the polymer from a far-from-the-equilibrium redox state toward a more stable electronic arrangement promoted by redox exchange with the gate electrode.

As anticipated in previous sections (see Human brain), metal chelation is one of the properties of eumelanin that have important consequences on its biological functions (Chen et al., 2009; Froncisz and Sarna, 1980; Hong and Simon, 2006, 2007; Liu et al., 2004; Meredith and Sarna, 2006; Samokhvalov et al., 2004; Sarna et al., 1980). Eumelanin has a strong binding affinity for Fe(III) and other heavy metal ions such as Cu(II), Sr(II), Cd(II), La(III), Gd(III), Pb(II). In contrast, lighter metal ions including Ca (II), Mg(II), Zn(II), Na(I), K(I) bind less strongly to eumelanin (Chen et al., 2009; Hong and Simon, 2007; Liu et al., 2004; Sono et al., 2012). Studies based on EPR (Froncisz and Sarna, 1980; Sarna et al., 1980), IR absorption (Chen et al., 2009; Hong and Simon, 2006), and Raman spectroscopy (Samokhvalov et al., 2004) revealed that the binding site depends on the type of metal ion, its concentration, and pH. Recently, specific dimers, trimers, and tetramers of DHI have been identified as potential bi-, tri-, and tetradentate metal chelators (d'Ischia et al., 2011; Meng and Kaxiras, 2008).

Eumelanins can also serve as free radical scavengers and antioxidants via H-atom transfer. In vitro studies have shown that DHICA melanin exhibits potent hydroxyl radical-scavenging properties in the Fenton reaction, whereas DHI melanin does not (Jiang et al., 2010; Pezzella et al., 1996). DHICA melanin exhibits more potent free radical scavenger properties than DHI and DOPA melanins in three different assays, the DPPH, ABTS, and NO scavenging assays (Panzella et al., 2013). Photophysical studies on oligomers from DHI and DHICA have shown that the excited state lifetimes increase on passing from DHI monomer to a dimer, but decrease on passing from DHICA to its dimers and trimers, suggesting more efficient UV dissipation mechanisms in the latter case (Corani et al., 2014; Gauden et al., 2008).

These and other observations can be rationalized considering the marked differences in the structural, chemical, and aggregation properties of DHI and DHICA melanins (Figure 6): Whereas the former consists of planar and relatively stacked components, the latter is less efficiently aggregated, is less stabilized by electronic delocalization, and is more prone to react with free radical species. Both antioxidant and energy dissipation properties of DHICA melanin offer a plausible chemical

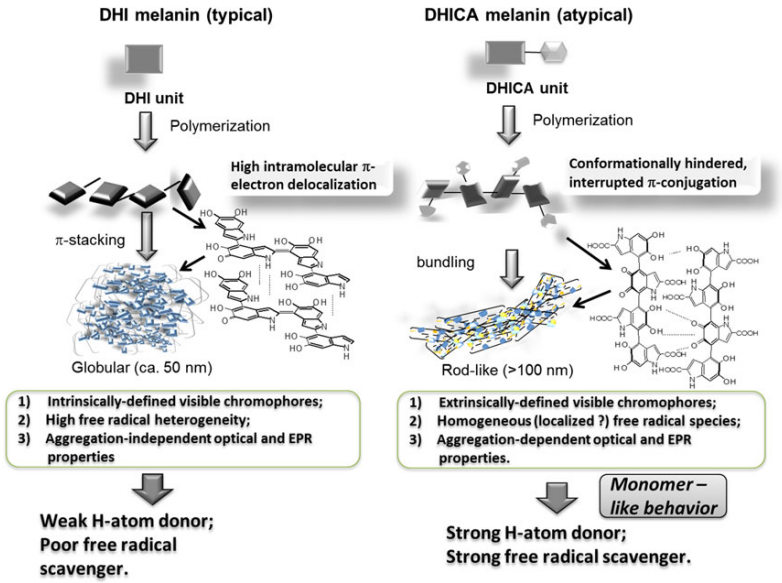

Figure 6. Overall view of main differences between synthetic DHI and DHICA melanins (adapted from Panzella et al., 2013). The scheme shows how differences in the mode of coupling of indole units determine the extent of electron delocalization, absorption and paramagnetic properties, and the mode of aggregation at the supramolecular level.

explanation as to why nature selected DHICA instead of the more pigmentogenic DHI as main eumelanin building block.

As mentioned in previous sections, eumelanins can bind a range of drugs such as methotrexate, chlorpromazine, cocaine (Bridelli et al., 2006); amphetamines; donarubicin, quinidine, disopyramide, and metoprolol (Buszman and Rózańska, 2003). Highly specific melanin-binding sites for iodobenzamides (Moreau et al., 2005) can be exploited to diagnose and stage melanoma using radiolabeled iodine-containing compounds. Thioureylene derivatives such as propylthiouracil can be incorporated as false thioureylene-based melanin precursors into eumelanins during synthesis and have been proposed as melanoma seekers for both diagnosis and therapy of the tumor (Larsson, 1993; Mårs and Larsson, 1995; Napolitano et al., 1996; Palumbo et al., 1997).

Oxidative breakdown of the eumelanin by exposure to strong alkali (bleaching) has been extensively studied because of its practical importance for hair dyeing (Ghiani et al., 2008). The degradation process apparently involves a fast solubilization of the pigment (granules disruption), followed by a slower bleaching, eventually resulting in a pale yellow solution (Borges et al., 2001; Ghiani et al., 2008; Korytowski and Sarna, 1990; Wolfram et al., 1970).

Melanosome architecture and surface properties

An extensive and detailed coverage of melanosome biology is provided in a recent book and various reviews (Borovansky and Riley, 2011; Simon et al., 2008; Solano, 2014). The surface of the intact melanosome plays a critical role in the binding and sequestering of metal cations and determines its functional capacity to mitigate oxidative stress and to participate in photogeneration of ROS (Hong and Simon, 2007; Simon et al., 2008). Imaging studies of red and black human hair melanosomes indicate different morphology, suggesting that the three-dimensional structure of the melanosomes is influenced by its constituent melanin (Liu et al., 2005). More importantly, the photoionization thresholds of pheomelanin and eumelanin are markedly different (326 and 282 nm, respectively) (Samokhvalov et al., 2005), implying that pheomelanin can induce ROS formation upon exposure by UVA light, while eumelanin cannot (Takeuchi et al., 2004; Wenczl et al., 1998). Thus, the morphology of melanosomes with mixed pigment composition, like those commonly found in the skin and eyes, may be of interest in relation to the possible presence of photosensitizing pheomelanin exposed on the organelle surface.

In this connection, the 'casing' model (pheomelanin encased by eumelanin), proposed also for NM pigments of the human brain (Bush et al., 2006, 2009; Ito, 2006; Zecca et al., 2008a), has received indirect support by several other studies (del Marmol et al., 1996; Ozeki et al., 1997; Smit et al., 1997). Because different photoionization thresholds characterize the two pigments, spatially resolved imaging of the ionization thresholds of the melanosome surface can effectively determine which pigments are present on its surface. Such studies have been reported on melanosomes isolated from the stroma of human irides, containing eumelanin: pheomelanin ratios varying from 14.8 to 1.3 (over an order of magnitude change, dark brown to blue-green irides, respectively) (Peles et al., 2009). The imaging analyses of these melanosomes establish that only eumelanin is present on or near their surfaces (Liu et al., 2005; Peles et al., 2009). These data provide direct evidence for a casing model (Figure 7). The finding that pheomelanin is encapsulated by an excellent electron scavenger (eumelanin) in natural systems would suggest that the structure of the melanosome and NM pigments mitigates the adverse photochemical properties of pheomelanin. Scanning electron microscopy and atomic force microscopy studies show that the intact melanosome as well as NM pigments is made up of ~30-nm-diameter spherical substructures (Bush et al., 2006), whereby changes in the relative amounts of eumelanin and pheomelanin would be manifested by changes in the diameter of the core and the thickness of the outer eumelanin coat. Damage to this coating and/or significant reduction in the amount of eumelanin present could jeopardize the protective ability of eumelanin, providing mechanism(s) for the exposure of pheomelanin and consequently contributing to oxidative stress.

Surface properties of melanosomes can also be examined using nano-indentation techniques aimed at probing changes in the 'stickiness' of the surface through the tip used in AFM measurements (Guo et al., 2008). Studies of the changes in the surface properties of human RPE melanosomes as a function of age (Rozanowska et al., 2002) showed that the aerobic reactivity of RPE melanosomes increases with age: Thus, photoinduced oxygen uptake is a factor of 2.4 greater at 80 years of age than at 40 years at age (Boulton, 1991). In addition, RPE melanosomes exhibit significant changes in their photophysical properties with age (e.g. the fluorescence yield

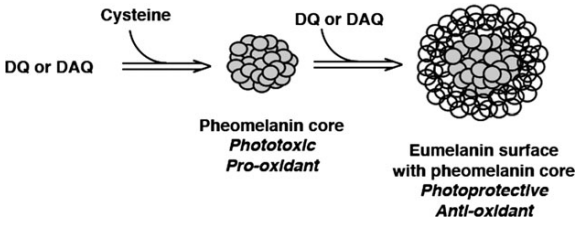

Figure 7. Casing model for mixed melanogenesis (Bush et al., 2006; Ito, 2006; Greco et al., 2011). Note that in the process of mixed melanogenesis, pheomelanic pigment is produced first, followed by the deposit of eumelanic pigment. In the granule with the eumelanin surface, the side was intentionally cut away to reveal the inner pheomelanin core. Eumelanin is believed to act as a photoprotective antioxidant and pheomelanin as a phototoxic pro-oxidant.

increases with age). Nano-indentation experiments combined with spatially resolved imaging of ionization thresholds (Hong et al., 2006) quantitatively established age-related changes in the photophysical and photooxidative properties of RPE melanosomes, likely related to the adhesion of lipofuscin to the melanosome surface. Several conditions have been shown to produce alteration in melanosome shape, for example, pMel inactivation (Hellström et al., 2011).

Applications

Dermocosmetic applications

Dermocosmetic applications of melanins and melanogenesis include mainly the use of the melanin pathway to control skin color (Guerrero, 2012; Solano et al., 2006), the use of the pigment from various sources in skin photoprotection formulations, the use of melanin precursors for hair dyeing, and the development of novel strategies for hair recoloration.

Hair repigmentation

As described previously, hair follicle melanocytes appear deficient in proteins protecting melanocytes from oxidative stress, particularly in the elderly. This particular phenotype likely contributes at least in part to the specific hair follicle melanocyte exhaustion that occurs during hair graying. These and previously reported observations suggest new approaches in anti-hair graying aiming at protecting melanocyte stem cells (MSC) from oxidative stress and DNA damage. Although the use of antioxidants may appear efficient to reach the goal, the demonstration that numerous antioxidants, for example, phenolic compounds, flavonoids, vitamins C and E, act as limiting factors for melanin synthesis through various properties, for example, copper chelators and tyrosinase alternative substrates both acting as tyrosinase inhibitors, reducers of melanin synthesis intermediates leading to reduced melanin polymerization (Briganti et al., 2003; Smit et al., 2009), make this approach neither easy nor obvious to get a satisfactory effect.

Alternatively, the demonstration that the bulb of gray hair (by contrast to the bulb of white hair) still contains active melanocytes (actual synthesis of melanin) albeit reduced in number (Commo et al., 2004a) suggests that the use of melanin synthesis-stimulating compounds may lead to the correction of gray hair. Indeed, this approach would require fighting hair graying at a time where residual bulb melanocytes remain active in gray hairs. Likewise, acting on the genes shown to be involved in melanocyte stem cell maintenance, for example, BRCA1, ATM, and Bcl2, appears hardly realistic to correct hair graying in humans. This remains a most challenging area of applied pigment cell research of considerable scientific, economic, and industrial relevance.

Hair dyeing with melanin precursors

Conventional hair dyes, especially oxidative hair dyes, although widely used, exhibit some inconvenience and liability, such as color fading, hair damage, and complicated handling. A valuable alternative is provided by melanin precursors, which can be converted to natural-looking pigments by air oxidation, and readily penetrate into hair, differently from large-sized preformed melanins. Hair dyeing with DHI and related compounds, for example, diacetoxyindole or DHICA, although commercially appealing, is methodologically challenging because of the difficulty to obtain a natural hue in the final dye, the relatively low penetration and affinity for hair, and, as a main problem, the poor oxidative chemistry with hydrogen peroxide.

A valuable biotechnological approach to cover gray hair with melanin precursors has been developed using *Aspergillus oryzae*, a fungus widely used in Japanese traditional industries, especially brewing of *Sake* (alcohol), and *Shoyu* (soy sauce), and recently shown to contain a strong tyrosinase activity (Koike and Hata, 2010; Nakamura et al., 2012). Pure DOPA (>99%) extracted and purified from a plant was conveniently used for the production of DHI as the melanin precursor using *Aspergillus* tyrosinase. The basic formulation for the dyeing test on Japanese or Chinese gray hair includes melanin precursors (0.1–0.5%) and ammonia or ethanolamine as alkaline components and (Koike and Ebato, 2013). The dyeing ability of the DHI-based formulation is higher at alkaline pH, with a concentration dependence up to approx. 0.5%. The formulation leads to lower levels of hair damage than with traditional oxidative hair dyes and the skin staining level is lower than with direct dye systems (Koike and Ebato, 2013).

Polydopamine-based multifunctional coatings and films

Inspired by the adhesion properties of mussel byssus proteins (Waite, 2008; Waite and Tanzer, 1981), Messersmith and colleagues reported in 2007 a universal eumelanin-like coating material, which was produced by the spontaneous oxidative polymerization of dopamine at pH 8.5 in the presence of oxygen (Lee et al., 2007). The resulting material, commonly referred to as polydopamine (PDA), forms highly adhesive polymeric films which can coat many types of surfaces, including superhydrophobic ones. Several studies addressed the nature and main structural features of PDA (see for example; Hong et al., 2012; Della Vecchia et al., 2013; Liebscher et al., 2013). The current view is that PDA consists of oligomeric scaffolds exhibiting a three-component structure comprising uncyclized amine-containing units and typical eumelanin units, namely DHI units as well as pyrrolecarboxylic acid moieties derived from the oxidative breakdown of indole units (d'Ischia et al., 2014) (Figure 8).

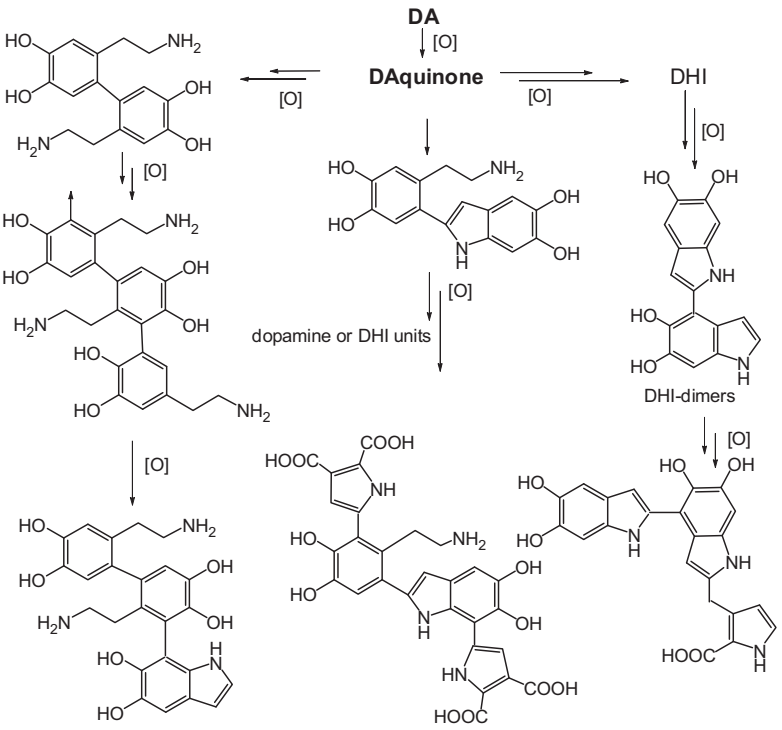

Figure 8. Main reaction pathways involved in polydopamine formation (adapted from Della Vecchia et al., 2013). The model was supported by a subsequent experimental and computational investigation showing that PDA consists of mixtures of oligomers in which indole units with different degrees of (un) saturation and open-chain dopamine units give rise to charge transfer interactions between o-quinoid and catechol units (Liebscher et al., 2013).

PDA adhesion properties depend on the preparation conditions (Bernsmann et al., 2011), and it has been demonstrated that changing dopamine concentration or using Tris buffer markedly affects PDA structure and possibly its efficiency depending on applications.

Because of its robustness, universal adhesion properties, biocompatibility, reversible and pH-switchable permselectivity for both cationic and anionic redox-active probe molecules, PDA-based coating technology is expanding rapidly to include energy applications, sensing, bioengineering, and nanomedicine for nanoparticle functionalization, drug delivery, and interfacing with cells (Dreyer et al., 2012; Kang et al., 2012; Liu et al., 2014). As an example, multilayered polyelectrolyte films made from PDA particles and polyamines like poly-(L-lysine hydrobromide) (PLL) and pure PDA grains in suspension in the cell culture medium were found to affect melanoma cell growth in different manners (Eap et al., 2013). Copolymerization of dopamine with 5-*S*-cysteinyldopamine (CDA) is a useful means of controlling and modifying PDA structure to change electrical properties and impart photocapacitor-like behavior (Ambrico et al., 2014). The analogy in the structure and properties of dopamine-CDA copolymers with those of neuromelanin (Wakamatsu et al., 2003) and the photoresponsive behavior of CDA polymers akin to that of pheomelanins (Napolitano et al., 2014) reinforces the value of melanin biochemistry and biophysics as a source of inspiration for functional biomaterials (Della Vecchia et al., 2015).

Bioelectronics and biosensing

Synthetic eumelanins may feature chemical and morphological diversity which affects a number of key properties of relevance for bioelectronic applications (Ambrico et al., 2015), in particular electrical conduction (Meredith et al., 2013; Mostert et al., 2012a,b). Several groups have published various device architectures with applications such as memory (metal–insulator–semiconductor geometries) (Ambrico et al., 2011, 2012), batteries (Kim et al., 2013), and biomimetic interfaces (Ambrico et al., 2014; Wünsche et al., 2013a). The device fabrication, and consequently the device performance, is affected by parameters such as melanin precursor and oxidizing system/conditions during eumelanin synthesis.

Significant progress has been made in eumelanin thin film fabrication and processing using different methodologies (Abbas et al., 2011; Ambrico et al., 2011; Bettinger et al., 2009; Bloisi et al., 2011a,b; Bothma et al., 2008; Díaz et al., 2005; Kim et al., 2011; Orive et al., 2009; Pezzella et al., 2015; Sangaletti et al., 2009; daSilva et al., 2004; Subianto et al., 2005). Processing of eumelanin films in aqueous NH_3 significantly increases the nitrogen content in the films, as shown by X-ray photoemission spectroscopy analyses (Wünsche et al., 2013a). Eumelanin has proved to gain in terms of performances and range of applications after *ad hoc* mixing with eumelanin-like macromolecules and inorganic structures. Oliveira et al. prepared an intercalated hybrid material by reacting DOPA with a V_2O_5 nH_2O gel preserving the

Table 1. Relationship between melanin properties and the proposed biological roles

Organism/organ//biological context	Role/function	Property	References
Mammalian fur, avian feathers, reptile scales	Camouflage, sociosexual display	Diverse color patterns	Galván and Alonso-Alvarez (2009)
Mammalian and avian testicles	Protection against oxidative stress caused by mutation	Antioxidant, free radical scavenger	Galván et al. (2011a)
Skin, eyes	Photoprotection, visual acuity, antioxidant action.	Light absorption and scattering, excited state deactivation, sequestration of redox-active metal ions, free radical scavenging	Meredith and Sarna (2006)
Substantia nigra, inner ear	Energy transducer	Water-dependent hybrid ionic–electronic semiconductor properties	Mostert et al. (2012a)
Inner ear	Calcium homeostasis regulation, metal reservoir, and buffering system	Metal chelation, drug binding	Hong and Simon (2007)
Inner ear, substantia nigra	Redox buffering and control	Redox behavior	Samokhvalov et al. (2005)
Insect cuticle, avian feathers	Structure strengthening	Chemical reactivity, quinone cross-linking with proteins and polysaccharides	Andersen (2010)
Insects	Innate immune system	Melanin bioadhesion and coating properties	Liu et al. (2014)
	Wound healing	Quinone intermediates of melanogenesis	Ito and Wakamatsu (2008)
Bacteria	Virulence	Shield against host immune response	Shao et al. (2012)
Eye, substantia nigra	Protection	Antioxidant, free radical scavenger	Panzella et al. (2013), Jiang et al. (2010), Zecca et al. (2008b)
	Detoxification	Drug binding and covalent incorporation, metal binding	Zecca et al. (2008a), Bridelli et al. (2006)
Cephalopod ink	Defense against predator	Toxic activity of melanogenic enzymes, catecholamine binding	Cooksey et al. (1997), Solano (2014)
Reptiles, amphibians, cold-blooded animals	Thermoregulation	Energy dissipation into heat	Meredith and Sarna (2006)
Browning of fruits	Response to injury	Catechol oxidation	Korkina (2007)
Fungi	Radioprotection	Free radical quenching, spatial arrangement	d'Ischia et al. (2009)

lamellar structure of V_2O_5 and demonstrating the reduction of V(V) to V(IV) ions with associated stability of the V_2O_5 electrochromic response and conductivity (Oliveira et al., 2000). Recently, a bulk heterojunction of porous silicon and eumelanin was obtained, featuring an increased photocarrier collection efficiency at longer wavelengths with respect to bare porous silicon matrices (Mula et al., 2012). Qu et al. (2010) polymerized DOPA onto Au nanoparticles (AuNP) and demonstrated tumor cell imaging with the hybrid nanoparticles. AuNP were also coated with polydopamine for sensing applications (Li et al., 2011). Fei et al. (2008) prepared polydopamine-coated carbon nanotubes and further functionalized them with AuNP. González Orive et al. (2011) fabricated eumelanin-iron-coated AuNP on HOPG with a strong catalytic activity for hydrogen peroxide electroreduction and hydrogen evolution reaction. Melanin hybrid structures can be fabricated under very mild conditions ('chimie douce') as it has been demonstrated by the in situ formation of the eumelanin-coated TiO_2 nanoparticles through direct reaction of the precursors (Pezzella et al., 2013).

When using eumelanin in devices, particular attention has to be given to the choice of the metal of the electrodes. Indeed, for thin films of synthetic eumelanin included between gold electrodes, under prolonged electrical biasing in humid air, gold dissolution has been observed (Wünsche et al., 2013b). Nano-aggregates of gold and eumelanin generate dendrites, in turn forming conductive pathways between the electrodes. This phenomenon was observed with eumelanins from different sources, as long as they had active phenolic hydroxyl

groups. It was suggested that the dissolution of the Au electrodes is enabled by Cl^- present in eumelanin and that the electrochemical-reducing ability and metal-binding ability of eumelanin were responsible for a strong enhancement of Au dissolution.

In general, eumelanin can act either as electron acceptor or as electron donor, according to a biphasic mechanism reminiscent of the electron transfer processes in redox-conducting films deposited as solid electrodes (Manimala and Horak, 1986; Mostert et al., 2012a). Irreversible oxidation peaks have been observed in measurements on eumelanin synthesized by enzymatic oxidation of L-DOPA and incorporated into a carbon paste electrode (Serpentini et al., 2000) as well as, indirectly, on aqueous eumelanin suspensions, where synthetic eumelanin had been prepared by DHI oxidative polymerization (Tarabella et al., 2013). The metal chelation properties of eumelanin offer interesting possibilities for eumelanin-based metal ion sensing. Huang et al. demonstrated a eumelanin-coated piezoelectric sensor with particularly high sensitivity for Hg(II) (Huang et al., 2007).

Nanotechnology for biomedicine

Melanin nanoparticles are increasingly exploited for biomedical applications, and only few limited examples are listed below. PDA nanoparticles that are <100 nm have been shown to exhibit good biocompatibility to HeLa cells after appropriate surface modification and potent free radical scavenger properties (Ju et al., 2011). Synthetic melanin-like nanoparticles complexed with paramagnetic Fe^{3+} ions are of potential interest as contrast agent for clinical MRI diagnosis because of their higher relaxivity values with respect to existing MRI T_1 contrast agents based on gadolinium (Gd) or manganese (Mn), leading to significant enhancement to MRI contrast (Ju et al., 2013). Melanin nanoparticles have also been proposed as nanocarrier in pH-responsive formulations for colon- and intestine-targeted drug delivery (Araujo et al., 2014).

Melanin-covered nanoparticles prepared by enzymatic polymerization on silica have been shown to provide efficient protection to bone marrow against radiotoxicity during radioimmunotherapy and in some cases external beam radiation therapy, permitting the administration to tumors of significantly higher doses (Schweitzer et al., 2010).

Photoresponsive and photodegradable hydrogel networks composed of commonly used biocompatible synthetic polymers and natural melanin nanoparticles as photothermal sensitizer can find application in clinical studies (Ninh et al., 2014).

Challenges and Perspectives

Table 1 provides a summarizing view of the relationships between melanin occurrence, roles, and properties. A look at melanin-based patents in 2012–2014 (see SI, not including the over 100 polydopamine-based patents) gives an idea of current and future trends in applied melanin research.

Despite considerable advances, however, several challenges must be met before melanin-based technology becomes a mature field. There is a need to expand the present set of structure–property–function relationships and to develop novel rational strategies to tailor melanins for specific applications. More efforts should be devoted moreover toward a deeper understanding of the conductivity properties of melanins to optimize their response for applications in organic electronics and bioelectronics. It is also critical that new experimental approaches are devised to improve processability of melanins for preparation of thin films and nanostructures. These and other goals can be realistically achieved through a closer cooperation between researchers involved in academic, industrial, and clinical settings, which should encourage an increasing number of companies to invest on melanin research for innovative and sustainable solutions for human health and technology.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Intrinsic *chemical* reactivities of *ortho*-quinones.