Melanin: Nature's 4th Bioorganic Polymer

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ABSTRACT

The pigments known as the melanins are widely recognized for their responsibility in the coloration of human skin, eyes, hair, and minimising the harmful effects of solar ultraviolet radiation. But specialists are aware that the melanins are present in all living kingdoms, barring viruses, and have functionality that extends beyond neutralizing ionizing radiation. The ubiquitous presence of melanin in almost all human organs, recognized in recent years, as well as the presence of melanin in organisms that are evolutionarily distant from each other, indicate the fundamental importance of this class of material for all life forms. In this review, we argue for the need to accept melanin as the fourth primordial class of biological polymers, along with nucleic acids, proteins and polysaccharides. We consistently compare the properties of these canonical biological polymers with the properties of melanin and highlight key features that fundamentally distinguish melanins, their function and its mysteries.

KEYWORDS: melanin, bioorganic polymers, supramolecular material

SECTION I. GENERAL INTRODUCTION

Melanins are a class of natural organic pigments found among all groups of living organisms [1]. Despite some cases of different general synthetic origin, which speaks in favor of convergent appearance of melanins in far-related biosystems, these pigments can be united in a phenomenological group because of important common structural and functional features, not usually observed in other biopolymers. Among them are an intense optical absorption in visible and ultraviolet ranges giving, with a structural organization built of C-C bonded quinoid moieties, combined into both linear and branching chains with relatively high concentrations of persistent radicals [2].

Initial classification of the melanins was suggested by Nicolaus and included three types: nitrogen-containing eumelanin (EM), nitrogen- and sulphur-containing pheomelanin (PM), and allomelanins lacking both nitrogen and sulphur [3]. The source of nitrogen in EM and PM, which are the only melanins found in animals and fungi [1], is the tyrosine amino acid utilized in the initial phase of their *in vivo* production. As for the allomelanins, they are produced by bacteria and plants and are derived from acetate or malonyl-CoA precursors via additional oxidation of synthesized phenolic compounds [1,4–6]. In this classification, a special position is occupied by bacterial pyomelanin [7,8], which, although it is a derivative of tyrosine like EM and PM, being built of benzoquinoneacetate units does not contain nitrogen, since it loses it during synthesis as a result of the transamination reaction [7].

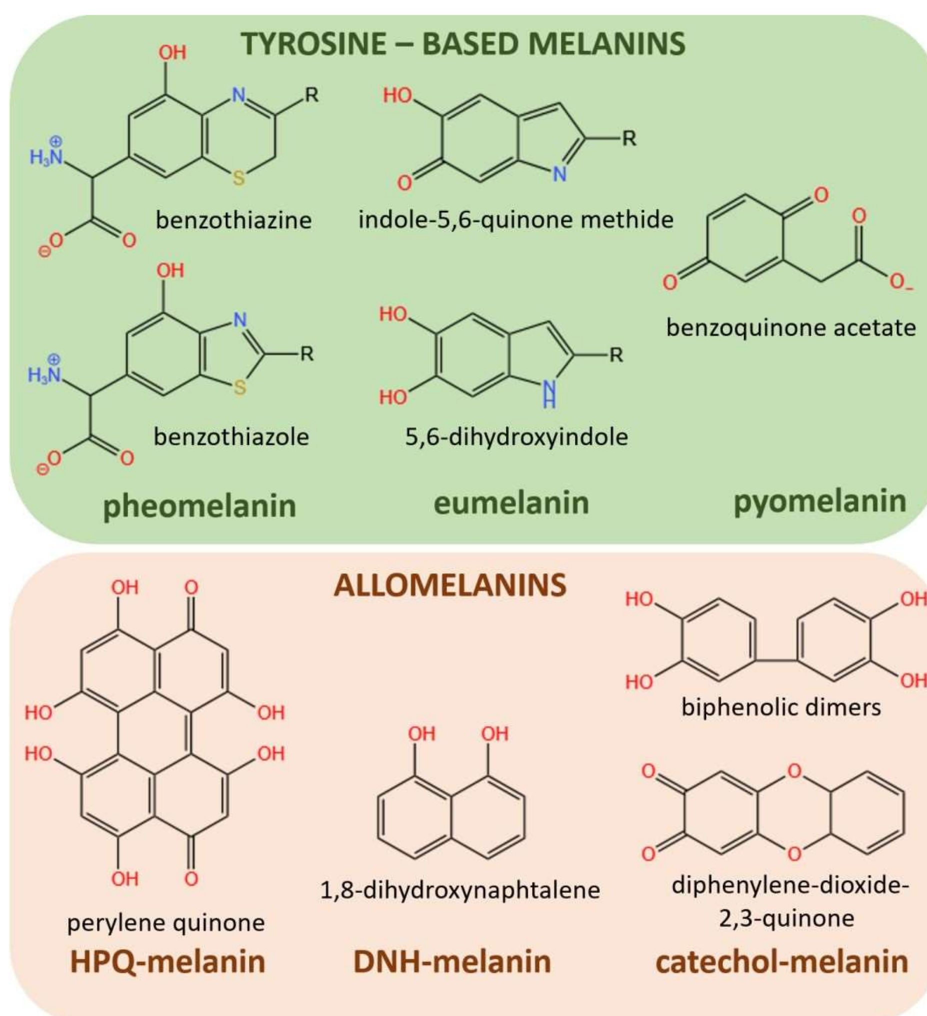

Fig. 1. Current diversity of melanins as demonstrated by their characteristic moieties [1,4,9]. The upper row includes typical monomers of melanin subfamilies synthesized from the tyrosine amino acid. For the pheo- and eumelanins -R can be either -H or -COOH. Typical natural *Animalia* melanins usually combine both pheo- and eumelanins [10]. They also can be found in *Fungi* as well as in the other biological kingdoms. One specific combination of eu- and pheomelanin manifest itself as core-shell structured neuromelanin, which can be found in the brain basal structures of the *substantia nigra pars compacta* and *locus coeruleus* of a few *Vertebrata* species including human beings [11]. Pyomelanin is widespread in *Bacteria*. Allomelanins include hexahydroxyperylenequinone (HPQ) melanin [12], dihydroxynaphthalene (DNH) melanin, part of a diverse class of catechol melanins. Allomelanins are absent in *Animalia* and *Fungi*, but are typical among *Plantae* and some *Protista*. We inform the reader that the presented structures do not reflect the full diversity of the corresponding melanin moieties, but demonstrate the key basic forms. As will be shown below using the example of eumelanin, there is a wide variety of tautomers and redox states corresponding quinone/hydroquinone monomer forms.

We depict some of the characteristic monomer moieties of melanin materials in **fig.1.** The detailed aspects of *in vivo* and *in vitro* synthetic pathways leading to the formation of corresponding structures are out of the scope of the current text, but can be read in detailed reviews elsewhere [4,7,9,10,13,14]. However, to set the context for the rest of the work, we will briefly describe the synthesis of EM and PM, since they are characteristic to animals and fungi and are the most available and studied types of material.

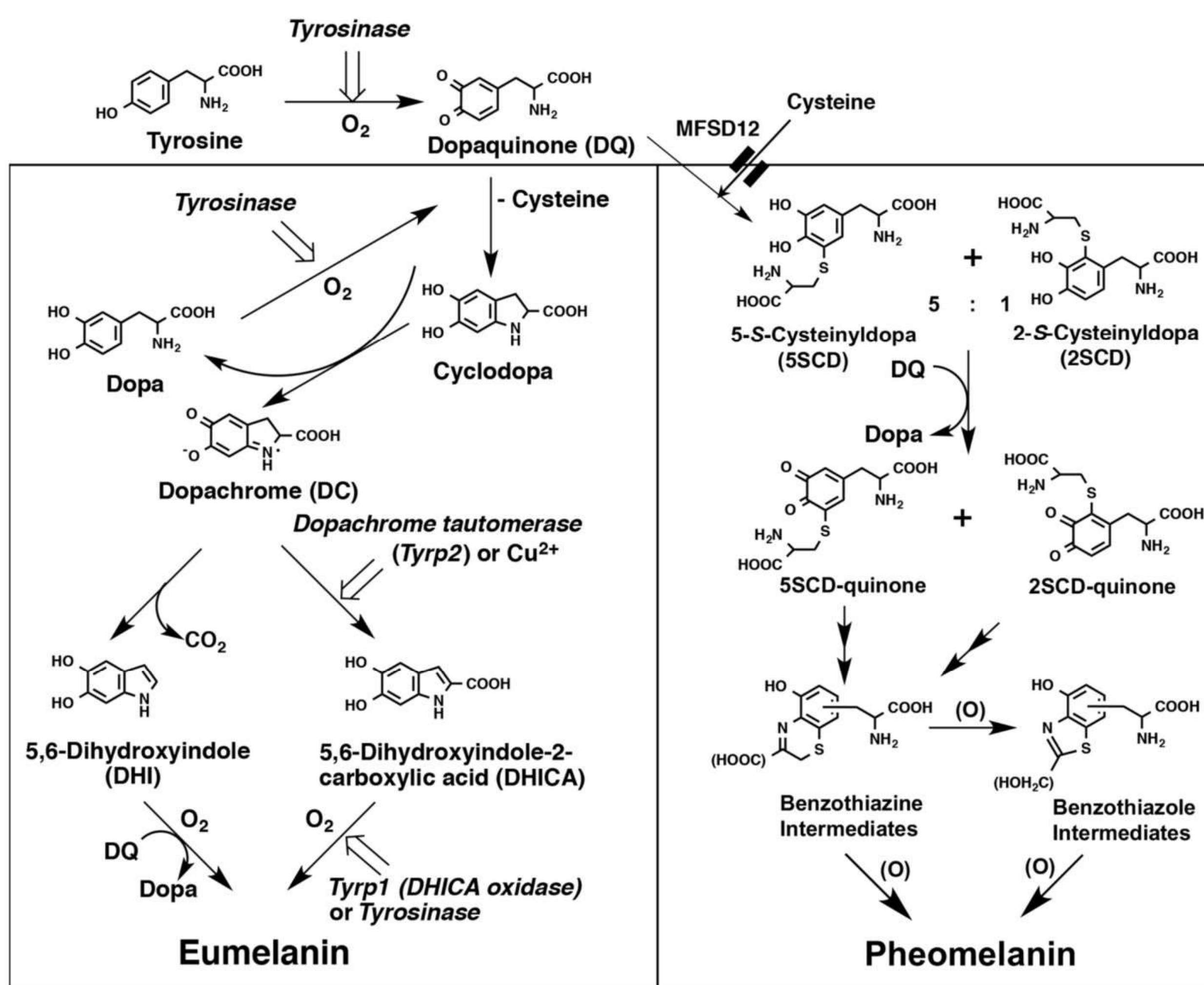

Fig. 2. Synthetic schemes of EU and PM production *in vivo*. Note that for PM synthesis, there is less participation of enzymes and leads to a greater diversity of possible structural moieties. The figure is adapted from K. Wakamatsu and S. Ito, Recent Advances in Characterization of Melanin Pigments in Biological Samples // *International Journal of Molecular Sciences*, 24, 9, 8305 (2023).[13]

Synthesis of both EM and PM *in vivo* utilizes common tyrosinase enzyme machinery [4,9,14,15] (**fig. 2**). Moreover, animal melanins often represent a combination of EM and PM taken in different proportions [10]. Probably, the most known and specific of these EM/PM hybrids is neuromelanin presented in the basal ganglia of some mammals [16]. It has a typical core-shell structure with PM nucleus and EM outer layer [17–19]. It worth noting that unlike PM the synthesis of natural EM demands also activity of other enzymes: dopachrome isomerase or tyrosinase-related protein-2 (Tyrp2 **in fig. 2**) and tyrosinase-related protein-1 or 5,6-dihydroxyindole-2-carboxylic acid (DHICA) oxidase (Tyrp1 **in fig. 2**). Activity of these proteins is particularly important to support the relatively high concentrations of DHICA-based moieties in the final *in vivo* obtained EM product.

The branching of corresponding synthesis pathways between EM and PM starts after the dopaquinone phase. In presence of cysteine amino acid, dopaquinone instead of undergoing cyclisation into cyclodopa incurs into an aromatic nucleophilic substitution reaction with cystein thiol group leading to the formation of 5-S-cysteinyldopa. Other positions of substitution are also possible. Further cyclization, oxidation, group rearrangements and partial decarboxylation of this molecule lead to a veritable zoo of pheomelanin monomers, based on various benzothiazole and benzothiazine moieties. Depending on the synthesis conditions (cysteine concentration, pH, etc.), the predominance of pheomelanin or eumelanin units can vary significantly in natural melanins [10].

The tyrosine-based PM and EM have been more intensely studied and, in many cases, are of greater interest to physiologists [20,21]. The reason for the interest is their ubiquity. For example, these melanins are found across the vertebrates, virtually in all organs where they have a purported wide range of functions (**fig. 3**). Given the melanins abundance and wide range of potential functionalities, they have remained an enduring philosophical challenge as to its structure-property-function relationship (*vide infra*) [2]. In its turn, physical properties of EM are by far and away the most investigated [2]. This is due to this material type being the most widespread and accessible via extraction from natural sources (e.g. cuttlefish inks, bovine eyes, hairs etc.) and through chemical synthesis [2,4,22].

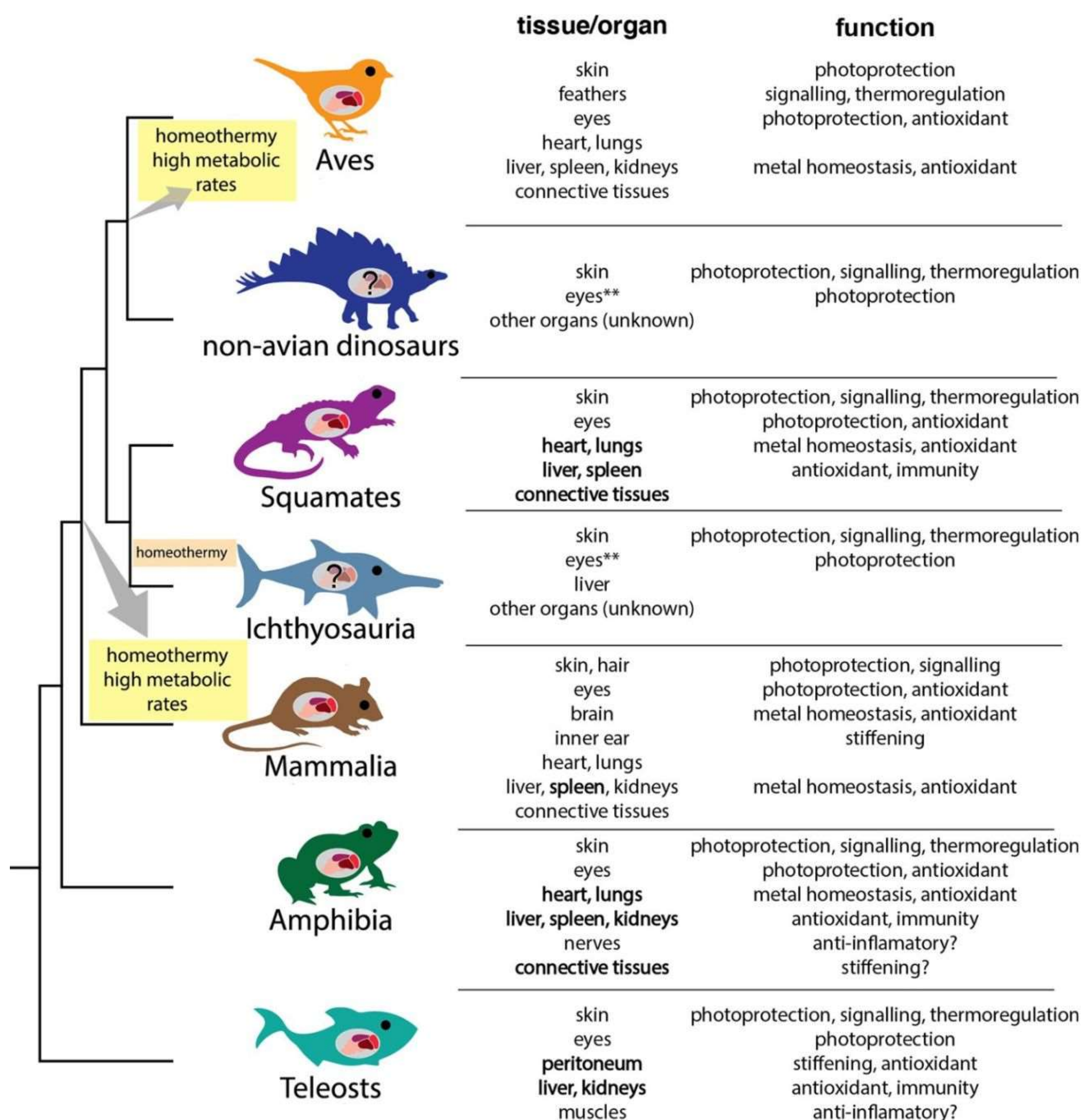

Fig. 3. The diversity of EM and PM found in vertebrae. Summarized above by McNamara et al. [23], where they summarize recent literature to map out the extent of melanin in the vertebrates. The symbol "?" denotes hypothesized function of melanin. We recommend to the reader McNamara article as well as the associated references to appreciate the scale and extent of melanin in nature. Reprinted (adapted) from McNamara *et al.*, Decoding the Evolution of Melanin in Vertebrates // *Trends in Ecology & Evolution*, 36, 430-443, Copyright (2021), with permission from Elsevier.

It is natural that there is an interest in the EM and PM due to their presence in nature and apparent wide functionality, at least from a naturalist or medical point of view. But, there are additional biochemical reasons for interest in these materials, as they are associated or implicated in diseased states such as melanoma [24–26], Alzheimer's [27,28] and Parkinson's [28–32]. EM is also being studied as a medical material to help reduce inflammation and accelerate repair in damaged tissue (e.g. see [33]).

Since most of the published studies of melanins' physical properties have been performed on EM (e.g. see [2]), in this work we mostly focus on this material. Note that not all statements made for the EM case can be automatically transferred on the other melanins. However, the main structural and physico-chemical features differentiating the melanins from the other bioorganic oligomers and polymers are shared among all types of melanin.

What may not be readily apparent to the uninitiated, is that EM has unique material properties that sets it apart from other common materials for the materials scientist, chemist and condensed matter physicist. These properties include broad-band optical absorption [34,35], a persistent free radical signal [36–38] as measured by electron paramagnetic resonance (EPR), protection against harmful ionizing radiation [39], almost 100% nonradiative conversion of light energy [34], metal ion chelation [40,41], moisture-dependent conductivity [42–47], photoconductivity [42,48,49], and hydration-dependent electrical switching behavior [50–52] to name a few.

These properties have been applied in a variety of applications, thanks in large part to materials scientists developing methods to form smooth and homogenous thin films for device applications [22,53–55]. This has yielded a wide array of EM-based devices and applications applied in biomedicine, organic electronics, and bioelectronics [56,57]. Examples include electrochemical transistors [58–60], energy storage [61–64], memory devices [65], optoelectronic skins [66], phototransistors [67], and pH sensors [53,68–71].

However, despite decades of research on EM and its importance, its structure-property-function relationship is still not well understood [72]. As indicated in **(fig. 3)** and numerous other authors (e.g. [2,4,4,14,22,23,73–76]), there are numerous proposed functions for melanins, partially dependent on where it is located within the body. This has led to much speculation as to what the “true” function of melanins is, especially given the usual understanding of natural molecules having a well-defined function, based upon the molecular properties and structure.

This wide array of potential functions should not be too big of a surprise, given that the properties of melanins are also not very well understood. As should have been inferred from above, there are a wide range of properties, which have not been fully explored [2,4,14,22]. To give just one example from the current authors’ experience, EM was considered for several decades to be an amorphous semiconductor [2,22,51,77,78]. However, in the past decade an alternative model has been proposed, that is instead a mixed ion-electron conductor [22,42]. Even though this latter model has a lot of explanatory power, there are still many researchers publishing surprising results indicating some form of semiconductivity (e.g. [22,52,79,80]). Questions such as what should be considered the definitive “native EM material” state are important in determining what should be considered in unraveling the charge transport mechanism of EM [4]. In short, the charge transport understanding of EM is still an outstanding question.

The range and flexible tuning of the properties of EM is a consequence of its chemical heterogeneous, disordered structure [22]. Even here though, there are outstanding questions in regards to its morphology, which we will discuss below.

Overall, it should be clear to the reader there are several outstanding issues in regards to our understanding of melanins, even some of a fundamental nature. Given this situation, and the multifaceted nature of melanin, we firmly believe that a multi-disciplinary approach is the most fruitful way to “crack” the melanin problem. Such approaches have been responsible for recent advances in our understanding of EM, e.g. [42] where traditional physics methods were paired with physical chemistry methods to elucidate the current charge transport model for melanin.

Therefore, the aim of this article is to set up a framework for a multidisciplinary approach to melanin studies. Specifically, we wish to bring together the two fields of biochemistry and condensed matter physics, a melding that has not been attempted before for melanin research. We believe if the importance of melanin can be highlighted, both due to its ubiquity, medical

importance and material properties by contrasting it with more familiar bio polymers, researchers in these fields will find common cause to evaluate melanin in greater detail.

To set up this multidisciplinary framework, we will highlight key properties and contrast it to the common biopolymers of nucleic acids, proteins and polysaccharides to give biochemists a framework for understanding the melanins. While doing so, we will discuss outstanding material properties that a condensed matter physicist would find unusual among biopolymers.

SECTION II. MELANIN AND OTHER BIOORGANIC MATERIALS

A. Components of eumelanin chain and the general material robustness

As a consequence of Raper-Mason synthetic pathway [81–83] pictured on the left side of fig. 2, EM is composed from 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) and their oxidized and semi-oxidized derivatives and tautomers. These are shown in **fig. 4**. The ratio between oxidised and reduced moieties is shifted towards oxidized forms I-III [84]. Fairly simple synthetic techniques leading to EM also exist (see [4]), but yields a material with a marked decrease of DHICA-based components, inducing important possible changes in final material secondary and tertiary structure.

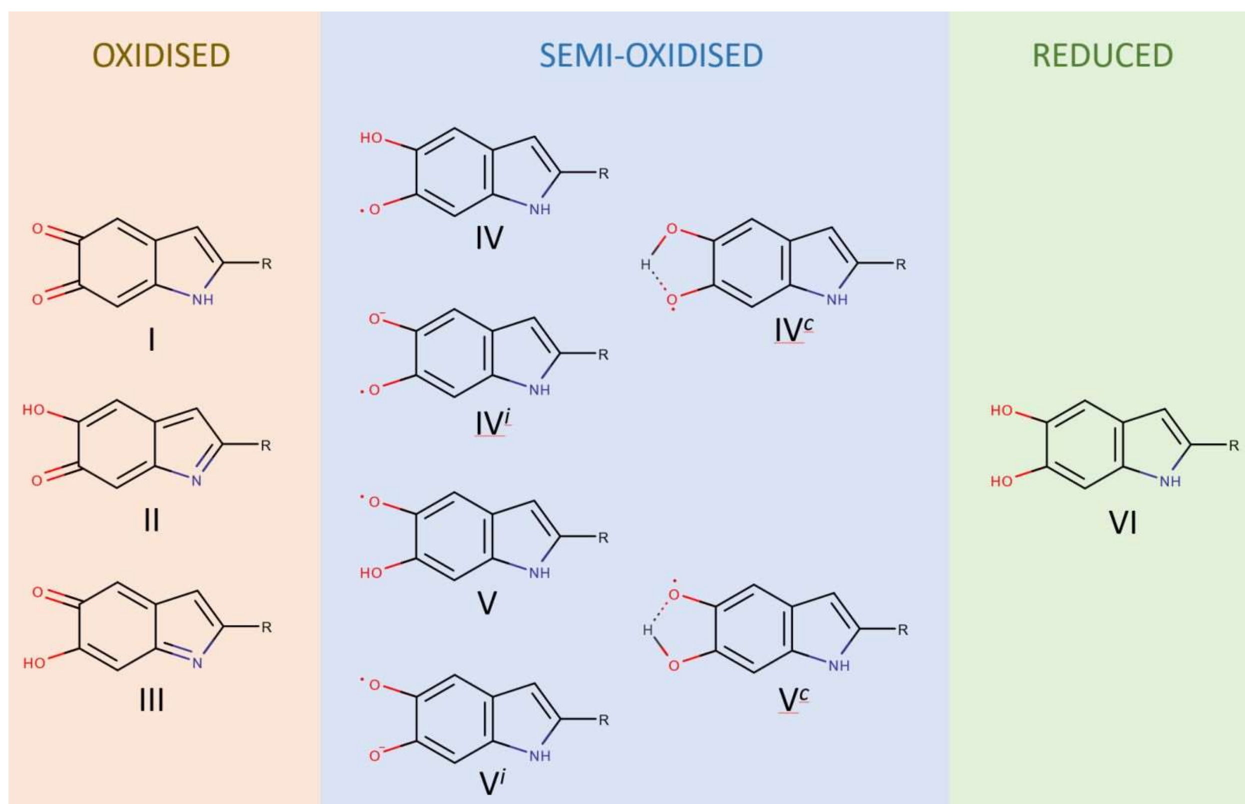

Fig. 4. The various monomer units responsible for the synthesis and structure of EM. For when the R group is an H, the fully reduced unit (VI) is a 5,6-dihydroxyindole (DHI) and for when R is a COOH, the fully reduced unit is a 5,6-dihydroxyindole-2-carboxylic acid (DHICA). The left column shows the fully oxidised forms: I - quinone, II - quinone methide, III - quinone imine. The central column depicts semi-oxidised radical forms: IV & V – semiquinones, IV^i & V^i – deprotonate semiquinones, IV^c& V^c – semiquinones in cycled form with an internal hydrogen bond [85]. The right

column depicts the fully reduced form - VI - hydroquinone/catechol. Among oxidized forms the quinone and the quinone methide should be regarded as thermodynamically favorable [84]. According to the results of study [86,87], the localization of semiquinone on oxygen at carbon atom 6 (IV^i structure) gives a slight energetic advantage and is more probable.

Much of the chemical behaviour of EM can be understood in light of the fact that it is based upon 5,6-dihydroxyindole derivatives [2,84]. These compounds are highly reactive [88–90] being *in-vivo* in a reduced state relative to the mean cytosolic redox level. They react and form the macromolecular system, a fairly inert material, via radical polymerization supported by molecular oxygen as a final acceptor of corresponding electrons. Such intensive synthetic free radical chemistry is highly unusual for an everyday biomaterial [91].

The radical polymerisation mechanism is completely different from that what is common for proteins, nucleic acids and polysaccharides, i.e. conventional biological polymers (BOP) synthesis. In the latter cases there is no redox-component in the condensation between amino acids, nucleotides or monosaccharides: there is just an elimination of water and the corresponding formation of a relatively polarized and, consequently, hydrolysable C-N or C-O bonds. That makes condensation and the backward hydrolysis easily performable under physiological conditions. However, in the case of the EM condensation, there is a redox-component related to oxidation of the DHI/DHICA unit by molecular oxygen and corresponding yielding of two water molecules. Both in natural and in laboratory conditions, the electron acceptor during the sequential oxidation of tyrosine to EM is the oxygen molecule. In natural conditions, the tyrosinase enzyme utilizes copper ions as cofactors of the oxidation process. These enzymes share a classical binuclear copper cluster presented among proteins responsible for the joint 4-electron reduction of the oxygen molecule [92]. It features the similarity of EM-related biochemical machinery with the other high-energy chemical processes like the terminal phase of electron transfer in mitochondria, the main biological power stations, in cytochrome c oxidase enzyme [93]. It puts EM chains far from the “click chemistry” approach wide-spread among other BOP and BOP-like systems. Indeed, to perform a backward process, the energies that will be required will rival that of photosynthesis when water is oxidized into oxygen using visible light photons.

The C-C bond between EM and PM monomers is much less polarized and cannot be hydrolysed without introduction of a redox-component, which should become apparent below when discussing its redox chemistry. The strength of C-C bonds between monomers is one of the factors, perhaps the main one, that makes EM (and PM) a most stable bioorganic material, capable of being preserved in the dark in animal fossils for millions of years [94–96]. Neither proteins nor DNA can survive for such a long time. However, as will be shown below, this chemical robustness is limited. For example, since water greatly affects the properties of material, attempts to dry it using heat are common. It should be noted that in this case there is a risk of decarboxylation (the corresponding transition of DHICA to DHI) [97,98]. There are also other reactions involving the dynamic changing of stable free radicals (*vida infra*). In terms of its dynamic reactivity in response to environmental changes, EM (as well as PM) will outperform many bioorganic materials. In short, it should be clear that relatively low-energy enzymatic hydrolysis reactions catalyzed by nucleases or proteases are inapplicable to EM (and PM) formation (Table 1), making EM (and PM) a relatively inert material *in-vivo*.

To summarize, from its "birth" via tyrosine oxidation EM is an active participant of redox processes comprising ionising UV-visual range quanta and reactive oxygen species. This makes *in vivo* melanin chemistry much closer to high-energy chemical phenomenology, like in photosynthesis and oxidative phosphorylation, than to general biochemistry of biological polymers.

B. Stable Free Radicals in Melanins

Another unusual chemical property of the melanins, both EM & PM, is a persistent stable free radical, which indicates another fundamental difference between these materials and other BOP. It is usually considered dogma that radicals are antithetical to biological systems since radicals cause damage to the surrounding cellular molecular systems. Thus, having a common biomaterial, present everywhere within and without an organism, possessing a stable and long lived radical is sure to attract interest. The origin of these radicals are believed to be in part due to unterminated radicals left over from synthesis, and in part due to the generation of radicals from the underlying quinone chemistry of the monomer units [99–101]. Their concentration according to some of the published estimates may reach more than 10^{20} radicals per gram of material [84,102]. In general case concentration of radicals in PM seems to be higher than in EM [103,104]. These large concentrations lead to the fact that in addition to interactions that are well known for the other biological polymers, i.e. hydrogen bonds, aromatic stacking, Coulomb interaction of separated charges, in melanins there are exchange and magnetic dipole interactions caused by unpaired electron spins sensing each other in proximity.

Currently, it is an outstanding question as to how exactly these radicals are spread throughout the melanin matrix. Do they form in clusters, or is it homogenous? There are a number of issues where the concerned reader may find a wide discussion and summary of the present level of understanding in a recent EM electron paramagnetic resonance (EPR) spectroscopy review [100]. However, we will highlight some key points in regards to the radicals in melanin.

The appearance of some radicals in EM is a consequence of one electron redox reactions, as one adds (removes) an electron from the oxidized (reduced) monomers, specifically the quinone (catechol) seen in fig. 4. These reactions though tend to manifest themselves in the form of a comproportionation reaction (**fig. 5**).

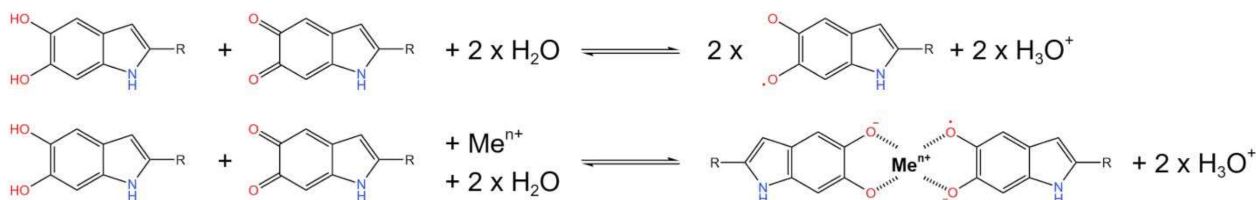

Fig. 5. Top) The comproportionation reaction in EM. In earlier literature (e.g. [40,105]) (bottom), the reaction scheme was used to explain the increase in semiquinone radical concentration in the presence of diamagnetic metal ions. The comproportionation has also been invoked to explain the effect of water on the solid state conductivity and muon spin relaxation response [42]. This reaction responds most clearly in aqueous suspensions of EM with increasing pH [102,106].

The semiquinone radical is not the only species present within the polymer, as at least another species is present, referred to as a carbon centered radical (CCR) due to the g-value

("chemical shift" in NMR parlance, see below) of the species. It is still an outstanding question as to the exact nature of this radical species and whether there are even more, minor radical species present within EM [100].

Given that radicals, or unpaired spin ½ particles are present, EM has been extensively studied with electron paramagnetic resonance (EPR). For a recent comprehensive review, see [100]. When investigating these two types of radical, they appear to react differently to environmental changes such as humidity, pH, temperature and numerous physical parameters related to the EPR technique. Under conditions of high pH values, semiquinone-type radicals (SQR) dominate, exhibiting a g-factor value near 2.0045-2.0050 and Arrhenius-like temperature behavior. These same radicals increase when EM chelates diamagnetic metal ions, which is a result due to a shift in the comproportionation reaction (fig. 6). SQR radicals also increase when irradiated with UV-light, inducing photoacidic behavior (and corresponding deprotonation) of various quinol moieties [106,107].

Unlike the SQR, the CCR temperature behavior shows Curie-Weiss behaviour and has a g-factor value near 2.0036. It is the main source of the EPR signal in EM at low and neutral pH values and in the dry state. Despite numerous attempts to model, its chemical nature remains unclear and is an ongoing topic of research [100]. However, what is relatively unambiguous is that CCR is remarkably stable and less reactive than the SQR.

C. Photochemistry

The main attribute that is usually associated with melanin is its role as a photo protectant against harmful UV radiation. This is believed to be due to its exotic UV-Vis spectrum, an example for EM is seen in **fig. 6**. What is remarkable of this spectrum is its featureslessness. Any chemist worth their salt would question why it is that an organic chromophore within the body exhibits no peak in the UV-vis range. Setting this issue to the side, it should be apparent that its ability to absorb energy across a wide band, and especially strongly at high energies, is why melanin present in the skin is thought to act as a photo protectant.

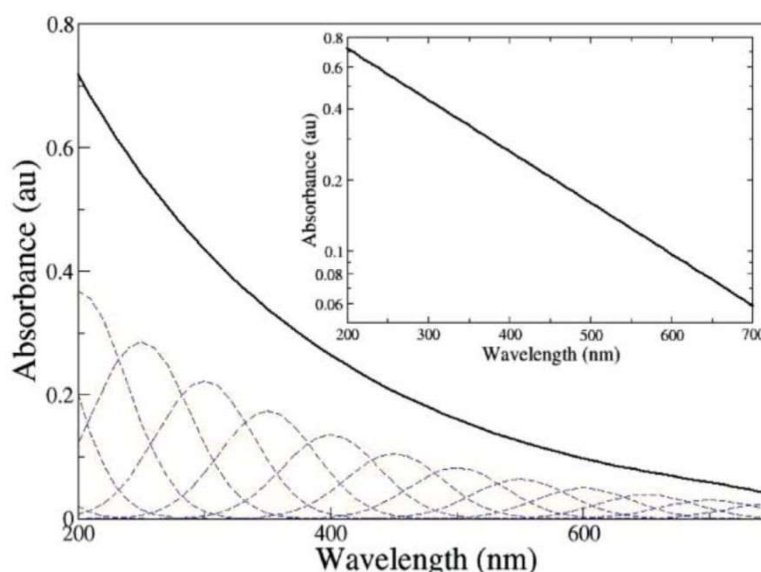

Fig. 6. An example of the UV-Vis spectrum of eumelanin in solution. Inset shows a log linear plot, demonstrating that the spectrum is of exponential nature. Below are computationally determined absorbance profiles for simple melanin monomers and dimers, which sum to yield an exponential curve. Reproduced from P. Meredith *et al.*, Towards structure–property–function relationships for eumelanin // *Soft Matter*, 2, 1, 37-44 (2006)[108] with permission from the Royal Society of Chemistry.

Closely associated with the photo protection is its emission spectrum (**fig. 7**). The presence of this emission spectrum indicates that there are a number of different chromophores in the material [34,109,110]. With this in mind, and computational work, the current accepted view is that melanin is made of a number of different chromophore species, each with their own absorbance and emission. It is fortuitous that these different units' absorbances overlap such as to yield the broad band spectrum [108]. This view has been articulated as the chemical disorder model [108,111]. Essentially, the disorder of this biological polymer is what yields an important function, contrary to that which is usually understood about biological systems.

One consequence of the disorder and the supramolecular structure (discussed below), is that the broad band absorbance is coupled to a very low quantum yield of emission of <0.1% [34]. Essentially, melanin is able to take high energy radiation and dissipate it as heat. This is to contrast it to nucleic acids, which are usually shielded in biological cells from high-energy photons and interactions in different ways with various repairing machinery as the keystone. As for proteins, to operate with energies of 1 eV and even higher at the nanoscale, e.g. in biological photoantennas or in mitochondrial oxidative phosphorylation chains, they contain non-amino acid fragments. Those can be *d*-metal single ions or ion clusters, pi-conjugated prosthetic chemical groups like heme, retinal, flavin, chlorophyll etc. In proteins the need for these structures is caused by the difficulty of dissipating or utilizing energy of the order of 1 eV by amino acids without homolytic bond cleavage and corresponding generation of hot radicals. Some radical or excitonic state is always formed when a biosystem absorbs UV-vis photon or catalyzes redox reactions with comparable energy. However, a decrease in the parasitic and dangerous activity of the forming radicals can be achieved either by delocalizing their electron and spin density along the conjugated pi system or by fast and localized recombination into safe products. In this sense, melanin can be considered as an extended quinoid prosthetic group, such as flavin or menadione. Strong delocalization of electronic density increases thermodynamic stability of the radical and makes melanin, probably, the coldest biological radicaloid.

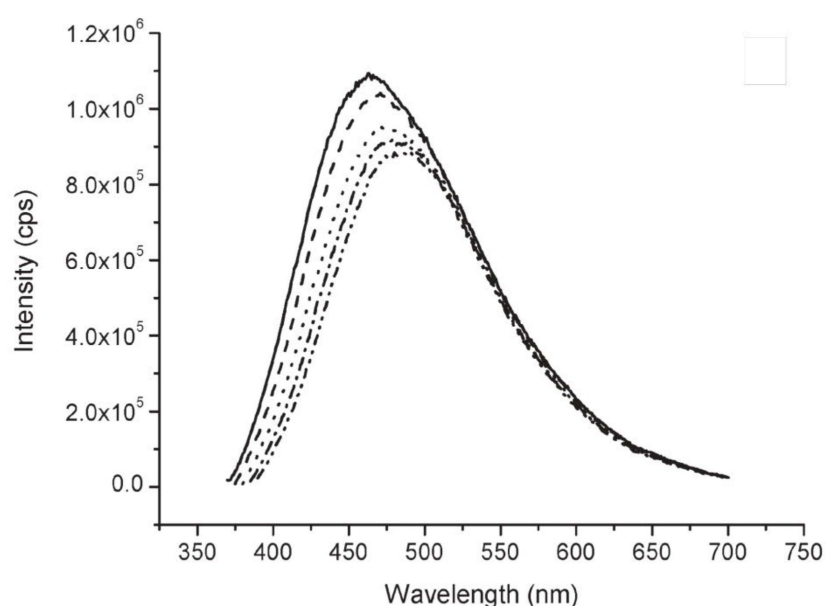

Fig. 7. An example of the UV-Vis emission spectrum of eumelanin in solution. Different lines indicate different excitation wavelengths employed. Reproduced from P. Meredith *et al.*, Towards structure–property–function relationships for eumelanin // *Soft Matter*, 2, 1, 37-44 (2006)[108] with permission from the Royal Society of Chemistry.

However, it should be noted the current disorder model should not be considered a complete explanation, as one would like to know the underlying chromophores responsible for the overall spectrum. Recent work by Wang *et al.* have made significant experimental progress in this direction by first isolating the elusive indolequinone moiety (**fig 4, structure I**) via steric stabilization, see **fig. 8** [86]. A key result is the absorbance spectrum of the quinone, which is able to cover the full optical absorbance range of EM, unlike the reduced, DHI form (**fig. 9**). It is easy to infer, alongside the disorder concept above, that if there are a number of quinones in different configurations and local chemical environments, that a smoothing would occur to yield the EM spectrum. This result strongly suggests that EM is a poly indole quinone system in the main.

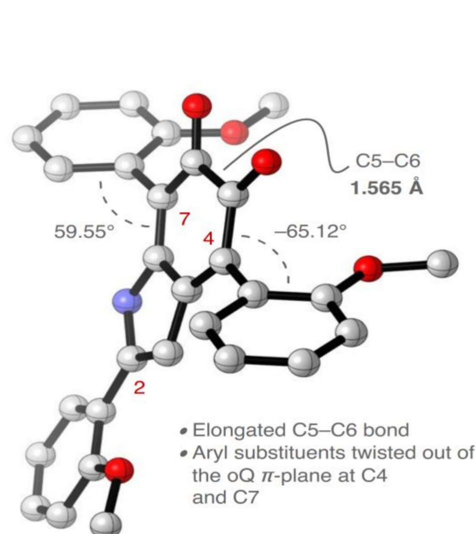

Fig. 8. A representation of the sterically stabilised indolequinone by Wang et al., where aryl groups were bonded at key points to the monomer, yet minimizing electronic interaction with the plane of electrons on the quinone. Note that oQ stands for ortho-Quinone. Adapted from Wang et al., Indole-5,6-quinones display hallmark properties of eumelanin // *Nature Chemistry*, 15, 787-793 (2023)[86], with permission from Springer Nature.

Even though EM is capable of deactivating the excited state efficiently, photo irradiation still can generate radicals [102,106,107,112–116]. Naturally, how can the material both be photoprotective and generate radicals? Recent sets of work have indicated that there are essentially two absorber subsets/pathways [117]. Photo kinetic analysis reveals that the chromophores in EM follow common excited-state decay pathways that include rapid excited-state relaxation as well as radical photogeneration via ionization and charge separation [118]. This suggests that the ubiquitous ultrafast excited-state deactivation seen in EM comes about through the recombination of charges or neutral radicals that are created promptly by photoexcitation [118]. An important consequence of the recent photodynamic work is that one cannot consider EM to contain non-interacting monomer chromophore units. This would be consistent with the stacked oligomer model, which will be explored below.

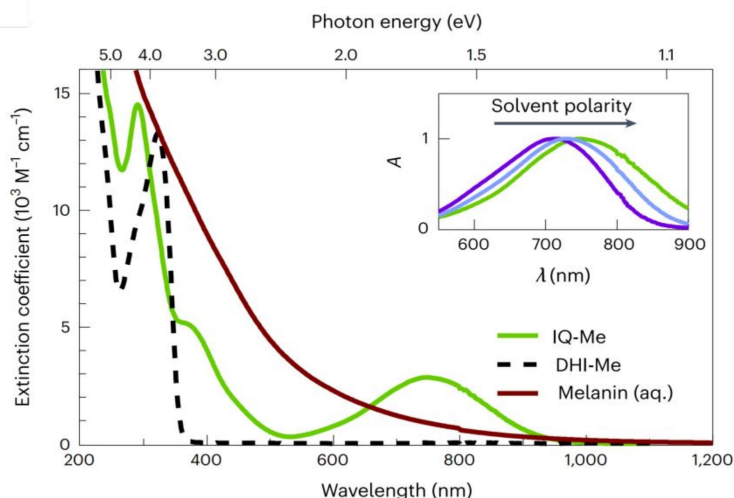

Fig. 9. An example of the UV-Vis absorbance spectrum of the stabilised ortho quinone (green), a stabilized DHI molecule (dashed) and melanin in solution (brown). The inset shows the shift of the quinone spectrum due to solvent polarity. Adapted from Wang et al., Indole-5,6-quinones display hallmark properties of eumelanin // *Nature Chemistry*, 15, 787-793 (2023)[86], with permission from Springer Nature.

In line with EM's paradoxical photo properties, EM, and melanins more generally, is responsible for some of the brightest colours in nature, such as colours seen on bird feathers. These colours are produced in large part to the structural arrangement of melanin particles that, in combination with other materials, scatter light across multiple wavelengths [119]. These effects have been replicated in the laboratory, by the use of nanoparticles of EM deposited on substrates [120].

D. Metal Ion Chelation

As mentioned above, melanin is known to chelate metal ions. What makes it interesting is the breath of ions, quantity and strength of binding that melanin is capable of [40,41]. It is also well known that generally, chelation of transition metal ions lead to an increase in the radical concentration. The explanation is as stated above, that there is a general tendency to bind to the catechols of the melanin monomer moieties, especially radical semiquinones. However, we must note that there are multiple binding sites, but these do depend on the periodic group of the ion and its valence [41].

However, paramagnetic metal ions (Cu^{2+}, Gd^{3+}, etc.) reduce the apparent signal intensity of the radicals detectable by EPR technique, even though they bind to the catechol moiety. It has been considered for a long time that the nature of the latter effect originates from Leigh-type interactions [121,122] which considers dipolar broadening of the narrow radical EPR signal due to interaction with a fast fluctuating metal ion magnetic moment. This model though, at least for Cu^{2+}, has been questioned as it was originally thought to be chemically driven by redox reactions [38], which has been recently inferred via X-ray photoelectric spectroscopy (XPS) [59].

Overall, it should be clear that there are still outstanding issues when it comes to the underlying redox chemistry. The radical nature is not fully understood, and the interactions with some biologically common metal ions still need to be fully investigated.

To give a broader context we should mention that BOPs include a large number of different nucleophilic and basic groups like hydroxyls, amines, thiols etc. Some chelation capabilities may arise also from strongly delocalized electron densities, supplied by aromatic nitrogen bases of nucleotides, by aromatic amino acids, or pi-conjugated prosthetic groups. All these chemical moieties make BOPs effective chelators of metal cations.

In some cases, chelation can be quite ion-selective, functionally important and associated with the catalysis of particular electron or proton transfer reactions. Thus, almost the entire nucleotide machinery is tied to the chelation of magnesium ions [123]. The same ion is a natural component of chlorophyll pigment playing a key role in photosynthesis. Chelation of iron and copper ions determines many stages of the cellular respiratory processes [124]. The important roles of zinc (*zinc finger* protein structural motif [125]), molybdenum (for biological nitrogen fixation [126]) and manganese (especially in oxidative stress regulation [127]) chelated ions are widely known.

One of the often discussed physiological functions of melanin is the buffer/depot system for different potentially toxic compounds including d-elements ions like copper and iron [128]. It is worth noting that in biological systems there are developed systems for stabilizing the concentration of metal ions with potential toxicity. For example, for non-heme iron, various ferritins are used that can accumulate up to 4000 iron atoms per multidomain complex [129]. In addition, there are various systems for the control and disposal of organic xenobiotics, including relatively well-known p450 cytochrome family [130] and less clear catabolism pathways driven by microbiome [131]. The mechanism of melanin's participation in these processes is unclear, but its presence is beyond doubt.

E. Eumelanin high-level structure

Melanins cannot form crystals the size of which would allow the structure to be studied with atomic resolution. Therefore, our ideas about its spatial packaging are based upon models. They arose from various indirect data. Quite naturally, at present there is no single established model of supramolecular EM or PM structure. On the one hand, the available data on from X-ray, neutron diffraction [132–138] as well as transmission [139,140] and tunneling [138] electron microscopy, speak in favor of interplanar stacking as a key ordered length scale within the structure of melanins. This presumes flat oligomer stacking structures of a sp^2 hybridized bonding arrangement to form the sheets, which is believed to be 4-5 units in length [2,138,141,142]. It has been suggested by numerous authors that these oligomeric sheets may form porphyrin or heme-like cyclic tetramers structure[140,143–151] (**fig. 10**). We note there has not been unequivocally demonstrated experimentally though there has been some inferences made [152]. What should also be noted is that these supposed structures are only viable/applicable to DHI based monomer systems, and not DHICA monomer systems.

This leads to a natural question which is to ask what the relative DHI/DHICA ratios are, as this can affect the morphology and stacking of the system as well, [101,153] see **fig. 11a**. DHICA content in natural EMs is often more than 50% compared to synthetic ones, where for the latter the DHICA content strongly depends on the synthesis method [4]. What can result is for materials with high DHICA content, the stacking can become very porous, allowing for greater radical scavenging properties [101]. In contrast, a DHI rich system can stack very tightly, such that all water can be excluded from the structure, at least theoretically on highly reduced systems [145]. Though

recent wide angle x-ray scattering data may have an interesting implication on the hydration effect of stacking (*vida infra*). Currently, a standard way to assess the DHI/DHICA monomer ratio is by means of X-ray photoelectron spectroscopy (XPS) or high performance liquid chromatography (HPLC) on digested products [4,154–158].

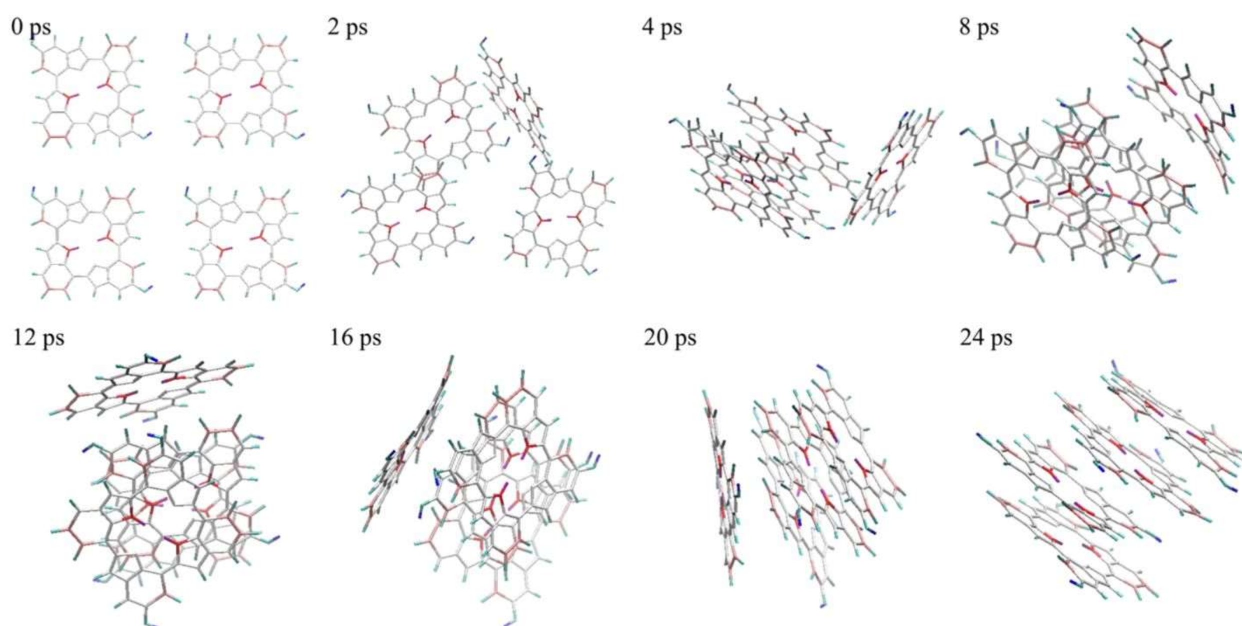

Fig. 10. Snapshots of the self-assembly process of four eumelanin protomolecular tetramers. The four tetramers are separated from each other in the initial configuration but quickly stack together to form a secondary structure. Reprinted (adapted) with permission from Chen et al. Self-Assembly of Tetramers of 5,6-Dihydroxyindole Explains the Primary Physical Properties of Eumelanin: Experiment, Simulation, and Design // *ACS Nano*, 7, 1524-1532 (2013) [140]. Copyright 2013 American Chemical Society.

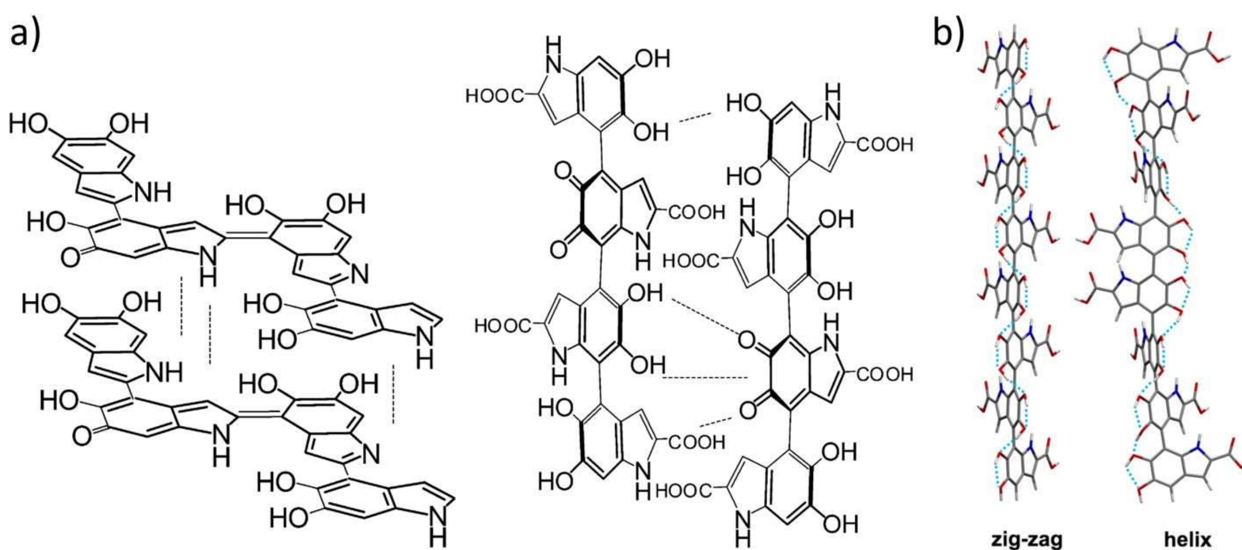

Fig. 11. a) Different DHI and DHICA monomer chains will have a major effect on the stacking of EM oligomers, which is illustrated here for contrast. DHI heavy systems will be more tightly stacked as fewer COOH units and steric effects will be reduced. Reprinted (adapted) with permission from d'Ischia et al. Polydopamine and Eumelanin: From Structure-Property Relationships to a Unified Tailoring Strategy // *Accounts of Chemical Research*, 47, 3541-3550 (2014)[73]. Copyright 2014 American Chemical Society. **b)** Optimized computational geometries of DHICA octamers in zigzag and helix conformations, with dotted lines highlighting the chain of hydrogen bonds. Reprinted (adapted) with

permission from Matta et al. Relation between Local Structure, Electric Dipole, and Charge Carrier Dynamics in DHICA Melanin: A Model for Biocompatible Semiconductors // *Journal of Physical Chemistry Letters*, 11, 1045-1051 (2020)[159]. Copyright 2020 American Chemical Society.

Modeling the properties of short chains of DHICA monomers speaks in favor of their formation of a helical (and therefore chiral) secondary structure [153,159] (**Fig. 11b**). This structural organisation can make EM more like other bioorganic polymers and oligomers, for which helicity is the normal. An important limitation of examples of modeling the structure of DHICA oligomers is that it is not known how such helices will interact with each other. It is very likely that in this case it will not be possible to maintain a clear helical organization, although chirality at the supramolecular level can be maintained. As for the scattering evidence previously mentioned, on such small structures, the length of the chains according to MALDI analysis appears to be short, only 3-6 monomers in length [160,161]. An NMR study [162] suggests that between 1.6 and 2.7 protons are lost from carbon atoms per monomer as 5,6-dihydroxyindole oxidises in EM. Therefore, 1.6 - 2.7 covalent bonds per monomer should be formed to obtain the material. For natural eumelanin the corresponding number was found to be 2.5. It means that some branching of monomer chains is quite possible, though this evidence is a far cry from indicating helical organization. Finally, as for the porphyrin structures, these structures are speculative.

EM high-level organization based on heme-like secondary structure approach, is usefully summarized in (**fig. 12**). It is widely reported that EM aggregates at short length scales in a π-π stacking arrangement [73,132,133,139,163]. However, recent hydration dependent wide angle x-ray scattering data indicates that the stacking distance shortens to below 3.4 Å to ~3.2 Å, beyond the shortest possible π-π stacking distance [132]. This has led to suggestions of “pancake bonding” within EM. But again, this latter model is by no means proven and intriguing questions of how hydration yield this structure are open.

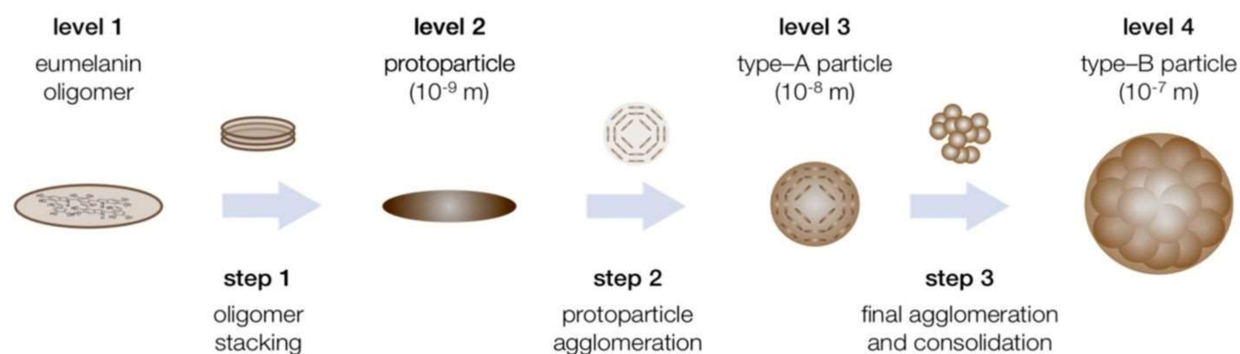

Fig. 12. The current multilevel morphological structure model for EM as depicted by Büngeler et al. [164]. At the shortest length scale, the basic structural unit of EM is connected by covalent chemical bonds in an oligomer molecule, consisting of four or five monomers. Further levels of organization are built through the formation of hydrogen bonds and π-π stacking. Stacking of oligomers lead to the formation of protoparticles about 1 nanometer in size [133]. The next level of supramolecular organization is particles with a characteristic size parameter of about 10 nm. In turn, they are packaged into structures with dimensions of about 100 nm. The size of even larger aggregates—melanosomes—can reach several tens of microns. Each of these levels of melanin organization, including the highest, the melanosome, has important functional significance in living systems [1]. Figure adapted from Büngeler et al., The Supramolecular Buildup of Eumelanin: Structures, Mechanisms, Controllability // International Journal of Molecular Sciences, 18, 9, 1901 (2017)[164].

From the above, we wish to highlight one key point. Melanins are essentially a sp^2 hybridized systems, which means that the monomer units comprising material have no inherent chirality. This is one reason why one can make an easy synthetic analogue of EM in the laboratory with a race mixture as a starting material. This is in contrast to proteins, polysaccharides and nucleic acids, where chirality is inherent (Table 1).

F. Oligomeric Stacking to Supramolecular Organization

Multilevel packaging of primary structure is characteristic of all types of bioorganic materials. Just like in other major BOP materials, there is also an apparent complex regulatory machinery present in vivo to arrange melanin into various supramolecular architectures [1], which add additional weight to the argument that melanin should be thought of as a major BOP. The extent to which contaminant proteins, including tyrosinase, affect the supramolecular organization of natural melanin is not entirely clear. In general, natural melanins are characterized by greater structural order than synthetic ones. [132,165]. Note that, according to various estimates, the mass fraction of protein in melanosomes can reach 50 and even 70%.

However, one can still talk about specific, melanin organization characteristics that cuts across various sources of the material. As can be inferred from section **II.D** above, the oligomer units of, for example EM, are believed to undergo interplanar stacking (**fig. 11**), mainly based upon X-ray/neutron scattering data and real space imagery such as TEM [132,138–142,165,166]. Up until recently, it was believed that the interplanar stacking of ∼3.4–3.9 Å under vacuum, was commensurate with a π–π stacking arrangement. However, given eumelanin's well known hygroscopic response, which affects both molecular [85,167,168], structural [169], magnetic [42,170–173] and electrical properties [42,45,46,52,172,174–176], the current authors undertook recently to test this basic, well entrenched stacking model by performing a hydration dependent wide angle X-ray experiment, with results shown in **fig. 13**.

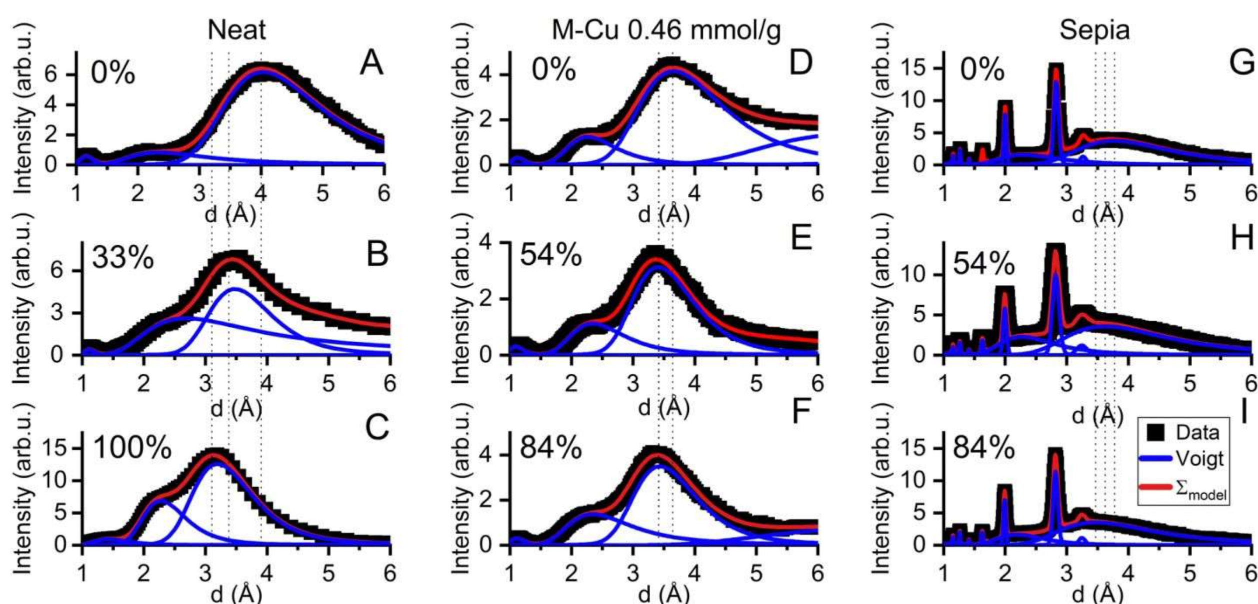

Fig. 13. Wide-angle x-ray scattering (WAXS) data for a synthetic EM (A–C), Cu-chelated synthetic EM (D–F) and a natural EM from *Sepia officinalis* (G–I). Sections A, D, G correspond to dry conditions; sections B, E, H to intermediate

hydration conditions; sections C, F, I correspond to high hydration conditions. The relative humidities are given in the upper left corner of each section. Black squares correspond to the experimental data, blue lines are modeled data and red lines depict the total envelope. The dashed lines through each column of sections correspond to key Bragg peak positions for different hydration levels that are traditionally associated with interplanar distances. Reproduced from Abramov et al., Signatures of Pancake Bonding in Hydrated Eumalenin // *PCCP*, 25, 16212-16216 (2023)[132] with permission from the PCCP Owner Societies.

The position of the biggest peak (interplanar stacking distance) is clearly sensitive to moisture content, but in a counter intuitive way. In the case of a neat synthetic EM the shift is the strongest with the position shifting from 4.031 Å (dry) to 3.194 Å (very wet) yielding a delta of 0.837 Å. For the Cu-chelated and natural EM (*sepia officinalis*) the interplanar deltas are smaller with values of 0.248 Å and 0.298 Å respectively. In short, instead of swelling, which was expected due to meso and macroscale hydration dependent measurements [132,169], on this short length scale, shrinking is observed. Furthermore, the interplanar distances achieved are too small to be explained by pi-pi stacking (e.g. neat, wet EM at 3.194 Å) [177]. This observation has significant implications for magnetic properties, which imply its radical chemistry, as well as charge transport, all of which we will explore in the manuscript below.

Suffice for now is that a working hypothesis [132] to explain the observations is that EM is not a traditional closed-shell pi-pi stacked system, but an open-shell pancake bonded system [178–182]. Instead of offset planar stacking, face-on stacking is to be expected, with radicals from the various oligomers coming stacking closely together to form a multi-site bond. This model has the great virtue of explaining contradictory data sets from the literature that would be of particular interest to physicists.

Specifically, previous works have demonstrated that the EPR radical signal decreases with hydration, implying a loss of radical concentration **(fig. 14A)** [42,173,183]. In contrast, if a local magnetic probe is used, such as muon spin resonance (muSR), the radical concentration increases **(fig. 14B)** [42]. This contradiction can be alleviated with pancake bonding, since pancake bonding screens radicals on a macroscopic level (EPR), but not necessarily on a local level (muSR) [132,184].

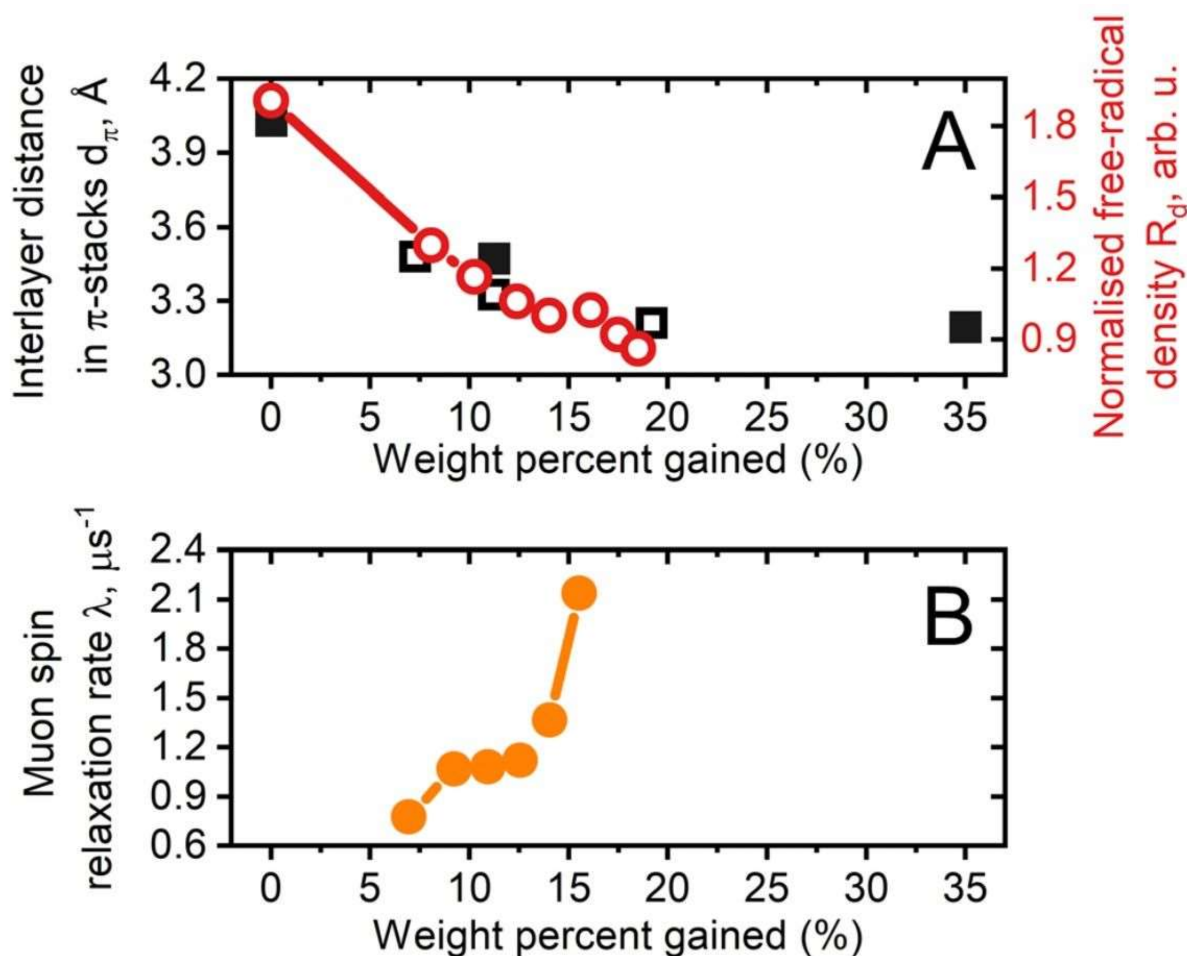

Fig. 14. A - EPR signal strength as a function of water content of a EM sample (red) and interlaying stacking distance of EM as a function of hydration (black). The results suggest that the manifestation of pancake bonding is leading to screening of the EPR signal. B - muSR relaxation rate, which is proportional to free radical concentration. It suggests that using a local probe enables the observation of the true state of affairs when inferring radical concentration changes. Reproduced from Abramov et al., Signatures of Pancake Bonding in Hydrated Eumalenin // *PCCP*, 25, 16212-16216 (2023)[132] with permission from the PCCP Owner Societies.

Overall, the pancake bonding model is a very attractive model for understanding EM stacking behavior. Understanding of this behavior is not just theoretical, since these measurements were done at room temperature, on wet materials. As such, we should anticipate something similar within nature and the body. Indeed, one can start speculating that this bonding setup is one reason for the stable nature of melanins, accounting for its radical nature. However, full confirmation of such a model is required. Pancake bonded systems exhibit interesting magnetic properties, such as diamagnetism, ferromagnetism or antiferromagnetism[178–182,185]. Here the expertise of condensed matter physicists will be needed, as such work is usually conducted within solid state physics. We believe this is one area where biochemistry and physics can synergistically work together.

We have taken the time to discuss this pancake bonding model, as we wish to properly set up a compare and contrast with stacking behavior seen in the major BOP systems. The key obligate condition for pancake bonding to arise in a material is pi-stacking between open electron shells. Among classical BOPs pi-stacking interaction is key for all nucleic acids and for some proteins containing aromatic prosthetic groups and amino acids. In the general case an open electron shell (electron radical) in nucleic acids and proteins is a problem since it brings structural

and functional instability, physiological dysfunction and redox stress. However, there are some examples in which pancake bonding is possible, which we now discuss.

Low-molecular quinoid moieties are relatively diverse in biosystems [186]. Many of them are related to electron transfer [187–189]. For electron charge transfer in biosystems they are preferred to act as shuttles, owing to their ability to capture, in their relatively stable quinhydrone/quinol form, two electrons simultaneously. This is unlike hemes or iron-sulfur clusters. To the best of our knowledge these quinoid systems do not form multilayered stacks within organisms. However, they may form pairs of molecules, for example, in mitochondrial or bacterial oxidative phosphorylation chains as a part of natural electron and proton membrane transfer catalysis [190,191]. But again, in this case one cannot speak about multilayered structures as is typical for melanins.

Another class of materials can be considered are the humic acids. According to XRD and TEM studies, pi-stacked substructures are widely presented in humic acids [192,193]. But humic acids should be regarded as bio-originating, but not physiological materials. It is likely that to some extent humic acids have an intermediate structural position between the melanins and polysaccharides (from which they are synthesized). Indeed, within a polymer matrix the humic acids can form quinone and semiquinone moieties with stable radicals [194], exhibiting properties similar to melanin EPR studies [100]. Yet, these monomers are usually connected via ether bonds without pi-conjugation. High-temperature carbonization due to, for example, forest fires, may change these bonds into melanin-like.

G. Hydrogen Bonding in Melanin

Hydrogen bonds are a fundamental type of interaction of bioorganic materials at the intramolecular and intermolecular levels, as well as the key type of interaction with their primary medium — water [195]. Within the framework of this article, we cannot broadly cover the features of hydrogen bonds characteristic of biological materials of various structures and in various functional states, but we will briefly note several key points that highlight hydrogen bonds formed both by EM itself and the water associated with it.

The classical hydrogen bond (CHB) has two minimum potentials. The gradual shortening of the distance between two atoms interacting through a hydrogen bond leads to a transition from a two-minimum potential to a one-minimum potential (single-well hydrogen bonds (SWHB)) with an intermediate state called the low-barrier hydrogen bond (LBHB) [196–200], where the height of the barrier between the minima is comparable to the energy kT. For hydrogen bonds formed by oxygen atoms, the approximated distances d defining the boundaries of SWHB, LBHB and CHB are, respectively, $d < 2.4$ Å, 2.4 Å $\leq d < 2.55$ Å, 2.55 Å $\leq d$. For comparison, the distances observed in hexagonal ice is $d = 2.76$ Å.

For a long time, it was believed that only CHB is characteristic of bioorganic systems. However, since the 1990s, thanks to a qualitative methodological leap in protein structural measurements, studies emerged highlighting the fundamental importance of LBHB for the allosteric regulation of enzymatic processes and catalysis of proton transfer in the active site [199–202]. These results coincided and coordinated with major advances in understanding the importance of proton quantum tunneling for protein catalysis [203–205]. It is important to note that LBHB and SWHB can change the pK value by several units (make a chemical moiety more acidic).

The formation of SWHB leads to efficient proton delocalization between two acceptor atoms, directly observed by neutron diffraction [206]. It is assumed that the formation of strong hydrogen bond bridges of the LBHB/SWHB type in biologically closely related systems like phosphoric acid radically increases proton conductivity compared to that in pure water [207].

What we currently know about hydrogen bonds in EM is rather meager, indirect, and stems from the results of vibrational spectroscopy and X-ray powder diffraction at different humidities. According to inelastic neutron scattering data (acquired at temperatures below 20 K) the water in EM powder even at the highest levels of hydration predominantly forms high-density amorphous ice (HDA) [167]. Though in and of itself, the method of freezing may be important. The aforementioned XRD study performed at room temperatures (**fig. 13**) revealed a spectral feature that is associated with SWHB. In addition, low-temperature THz-data show that water collective dynamics in EM remain active at relatively low temperatures if compared with other protein systems [45,208]. These studies taken together indicate that EM (and by extension PM) may be regarded as a chaotropic material capable of suppressing the long-range, regular structural networks within water in favor of dense, irregular organization with strong H-bonds supporting greater local proton mobility.

Furthermore, mid-infrared data shows that EM hydration increases the concentration of the loosely connected water molecules (O-H stretching bands near 3600 cm^{-1} and 3450 cm^{-1}) much stronger than that of the tightly connected (O-H stretching bands near 2890, 3070 and 3250 cm^{-1}) [85]. However, even those 3-5 layers of water [167], which the system achieves at 100% relative humidity (at room temperature) do not form a bulk water fraction.

EM's tendency to form HDA at low temperatures and SWHB at room temperatures appears self-consistent. However, this arrangement differs remarkably from the other studied bioorganic systems, where it is more typical to see the formation of low-density amorphous ice. We should also emphasize that known protein structures only include LBHB and SWHB as a small fraction of all hydrogen bonds. In contrast to EM, the situation seems to be the opposite: SWHB dominates.

SECTION III. Conclusion

We have made a number of observations about the melanins, particularly the most studied EM, and compared and contrasted the details with other BOP materials found in nature. Melanins are ubiquitous in the biosphere including in animals, in all organs and lower life forms. It demonstrates a wide array of potential functions that include metal ion scavenging, redox scavenging, photo protection and coloration. It is implicated in various diseases including Alzheimers, Parkinsons and melanoma, all of which highlight their importance, just like protein deficiencies lead to diseases. What is also significant in contrast to other BOP is that melanins are capable of high energy chemistry and physics unlike other biomaterials, yet is robust. We have highlighted the differences and similarities to the other major BOPs in the **table. 1** for ease of reference.

Melanins have no long-range order, but short-range order and that these unique structural, magnetic and chemical elements yield an integrated material that is poorly understood relative to its peers. In conclusion, melanins should be considered nature's 4th,

fundamental biological polymer class, which is ripe for further exploration to biochemists and physicists.

Table 1. Key comparative characteristics of the four main classes of biological polymer/oligomeric materials. Unique properties of eumelanin are highlighted in red.

	nucleic acids / oligonucleotides	**proteins / peptides**	**polysaccharides / oligosaccharides**	**eu- and pheo-melanin**
Ability to hydrolysis	Yes	Yes	Yes	No
The role of hydrogen bonds for structural organization	Very important	Very important	Very important	Importance is unclear
The role of *π-π* interactions for structural organization	Very important	Important in some cases	Absent	Very important
The role of radical-radical interactions for structural organization	Absent	Absent	Absent	Important
The role of d-metals' ions for structural organization	Absent	In some cases, d-metals are presented and important	In some cases, d-metals are presented and important	Important
Helicity	Presented, important	In some cases, presented and important	In some cases, presented and important	Importance is unclear. Absent in the main model
Chirality centers in monomers	Presented	Presented	Presented	Absent
Crystallizable	Yes	Yes	Yes	No?
Branching of chains	No	Very rare	Yes	Yes
Ability to form aqueous gels	Yes	Yes	Yes	No
Hydration-induced ionization	Yes	Yes	No	Yes
Hydration-induced synthesis of radicals	No	No	No	Yes
Cyclic oligomerization	Very widespread (bacterial chromosomes, plasmid DNA)	Not very widespread	Not very widespread (cyclodextrins)	The main type of oligomerization according to current model

Data availability statement

Data sharing does not apply to this article as no datasets were generated or analyzed during the current study.

CONFLICT OF INTERESTS

There are no conflicts to declare.

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