Published in final edited form as:

J Phys Chem B. 2007 July 19; 111(28): 7938–7947. doi:10.1021/jp071439h.

Current Understanding of the Binding Sites, Capacity, Affinity, and Biological Significance of Metals in Melanin

Lian Hong and John D. Simon

Department of Chemistry, Duke University, Durham, NC 27708

Abstract

Metal chelation is often invoked as one of the main biological functions of melanin. In order to understand the interaction between metals and melanin, extensive studies have been carried out to determine the nature of the metal binding sites, binding capacity and affinity. These data are central to efforts aimed at elucidating the role metal binding plays in determining the physical, structural, biological, and photochemical properties of melanin. This article examines the current state of understanding of this field.

1. INTRODUCTION

Melanin has a high affinity for metal ions, and pigmented tissues contain significant amount of metal elements *in vivo*.^{1–8} Because metals are precisely regulated in the body, the involvement of melanin in the metabolism of metals is of great importance and has recently attracted much attention.^{1,9–11} Specifically, there has been an extensive effort to characterize the binding capacity and affinity of many metals to melanin and to describe the chemical nature of its metal binding sites and the effects of its coordinated metal content on its biological properties, e.g., melanin's ability to act as an effective antioxidant.

As a ubiquitous biological pigment, melanin is generally classified into types depending on the molecular precursor from which the pigment is made.^{12–15} Eumelanin (black melanin) is mainly composed of oligomers of 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA), while pheomelanin (orange-red melanin) is derived from benzothiazine units.¹⁴ Neuromelanin (NM) differs from these two classes of melanin and is hypothesized to be a complex of dihydroxyindole and benzothiazine units, possibly along with other unknown groups.^{16,17} The functional groups associated with these molecular building blocks are central to the metal coordinating abilities exhibited by melanins.^{4,7,18–20}

In situ, with the exception of NM, melanin is generally deposited in melanosomes, specialized organelles in pigment generating cells. Similar to most cellular organelles, melanosomes are membrane-bound structures containing a variety of molecular species, including lipids, proteins, and melanin. The contents of these components vary with the sources and origins of melanosomes.^{8,21–23} The morphology of melanosomes, which can provide information on the integrity of melanosomes as well, has been extensively studied by imaging with scanning electron microscopy (SEM), transmission electron microscopy (TEM), and atomic force microscopy (AFM).^{8,23–27} Those images showed that melanosomes containing eumelanin are generally round or elliptical granules. NM also appears as a set of membrane-bound granules *in situ*, but its biosynthesis does not take place within defined melanosomes. The NM pigment is usually associated with the age pigment lipofuscin.^{28–30}

*corresponding authors: jsimon@duke.edu.

While there has been extensive work reported on the morphology of melanosomes and NM organelles, little information on the chemical and physical structures of the organelle at the molecular level has been obtained. The amorphous, opaque, and insoluble nature of melanin largely precludes an accurate determination of the chemical composition and structure of this pigment. Moreover, little is known about the organization of melanin inside this organelle. There are several methods used to extract melanosomes from their natural environment. However, because of the lack of information about the molecular structure of the pigment and its organization, it is difficult to determine whether these extraction processes preserve the natural pigments. As a result, it is difficult to compare studies to one another. This is certainly the case with respect to the binding of metals, where studies on different samples of melanosomes (either of different origin, or common origin but different extraction techniques) often result in contradictory conclusions. The goal of this Feature Article is to articulate the wide range of major results, sometimes conflicting, about the interactions of metals with melanin and/or melanosomes. We also focus on the impact of these measurements on our understanding of the biological function of melanin and the melanosome.

2. BIOLOGICAL IMPLICATIONS OF METAL BINDING TO MELANIN

Before discussing detailed studies, it is important to highlight two themes that pervade this literature: the ability of melanin to serve as a reservoir for metal ions – enabling storage, release and exchange – and the ability of melanin to strongly bind and sequester reactive metals thereby mitigating their possible role in inducing oxidative stress.^{1,9–11} We will refer back to these functional roles as we explore the physical measurements that have been reported.

Natural melanins are associated with a number of metal ions and have the capacity to accumulate metals.^{1,2,6,7,9,11,31–33} It is intriguing that a strikingly high concentration of Ca (II) and/or Zn(II) is observed in pigmented tissues.^{1,7–9,11} While the capacity of melanin to bind Ca(II) or Zn(II) is high, as much as ~ 1.5–1.6 mmol/g³⁴ (because the molecular weight of melanin is unknown, concentrations will be expressed in terms of mass (g) of pigment), its affinity for these two metal ions is only moderate.^{1,10,35} This combination of high binding capacity and medium binding affinity to Ca(II) and Zn(II) enables melanin to accumulate these two metal cations and then, under certain conditions, to release them. Thus, the first biological role ascribed to metal binding to melanin is one of serving as a reservoir for Ca(II) and Zn(II).^{9,11}

In contrast, heavier metal cations, e.g., Fe(III) and Cu(II), bind tightly to melanin.^{1,4,10} It is well known that Fe(II) and Cu(I) can cause damage in biological systems by catalyzing Fenton reactions. By binding iron and copper, melanosomes sequester these metals, protecting them from reduction by cellular components and subsequent induction of oxidative stress.^{1–8,36–38} Thus, a second important biological function ascribed to metal binding to melanin is its ability to sequester reactive metals.

It is fascinating to consider then that melanin can act both as a metal reservoir and a metal sink, the behavior depending on the specific metal. One of the goals of this article is to present the current understanding of how the pigment differentiates the metals, and whether the presence of specific metals influences the ability of melanin to perform this dual function.

3. OCCURRENCE OF METALS IN NATURAL MELANINS

The distribution of metals within the melanosomes could simply be a reflection of its surrounding environment. Two studies show that *Sepia* (cuttlefish) melanin, for example, contains mainly Ca, Mg, Na, and K, which are generally the most abundant metal elements in both the organism and in sea water.^{7,39} The amounts of some metals, such as Cu and Mn, are higher than in the biological environment (seawater), suggesting the ability of melanin to

accumulate these metals. However, these two studies report different concentrations of bound metals for the same biological system: the total metal content (mostly Ca, Mg, Na, and K) determined by Liu et al. (1.6 mmol/g),⁷ was on the order of four times greater than that reported by Sarzanini et al.³⁹ While one might expect some variation in the distribution among these metals from sample to sample, the total metal content of Ca, Mg, Na, and K is expected to be the same, *vide infra*, because these metals share a common binding site and so the sum of their concentrations reflect the total concentration of available binding sites in *Sepia* eumelanin.^{34,40}

This finding highlights the importance of the methods of isolation and preparation of the melanin samples, the difficulty in obtaining reproducible samples, and the lack of an accepted set of standardized procedures to obtain *in vitro* samples that preserve, as best as can be achieved, the inherent properties of the natural *in vivo* organelles. Specifically, the variation in metal abundance in *Sepia* melanin obtained by Sarzanini et al. and Liu et al. can be attributed to the procedures employed to purify the melanin granules. In this particular case, Sarzanini et al. employed sonication of the material, a step that was not undertaken in the study by Liu et al. Sonication of melanin for an extended period releases material from the intact granule, and chemical degradation analysis reveals that this dissolved material contains a higher DHICA fraction than that of the intact granule.⁴¹ Because it is the carboxylic acid group associated with the DHICA moieties that coordinates many metals, especially Ca, Mg, Na, and K, *vide infra*, sonication results in a reduced capacity of the remaining granule to coordinate the alkali and alkaline cations,^{34,40} accounting for the discrepancy in the published results.

The metal (Ca, Mg, Zn, Fe, and Cu) content of enzymatically extracted melanosomes from human black hair and red hair has been reported⁸. The major metal present is Ca, with concentrations of 1.0 mmol/g and 0.4 mmol/g of melanin in black hair and red hair melanosomes, respectively. While the red hair melanosomes have less Ca than the black hair sample, they contain more Mg and Fe. The difference in Fe content is hypothesized to reflect the role that sulfur, unique to and abundant in the red hair melanosomes, plays in coordinating iron. There are several earlier studies characterizing metal content in hair melanosomes.^{42, 43} Unfortunately, these studies used acid-base extraction to isolate the melanosomes, a technique that has been shown to modify metal content and alter the molecular structure of melanin and the morphology of the melanosomes⁸. Results obtained from acid-base extracted material are unreliable, and likely incorrect, and such studies will not be discussed herein.

The metal content in melanosomes from the choroid, iris, and retinal pigment epithelium (RPE) in the bovine eye have also recently been reported.⁶ The RPE melanosomes were isolated using sucrose gradient centrifugation, and the choroid and iris melanosomes were isolated using enzymatic extractions.⁶ SEM and TEM analyses confirm that these extraction procedures do not alter the morphology of the melanosomes, and while it is routinely assumed that the chemical composition and properties are also unaffected, rigorous proof of this assumption has yet to be provided. The total amount of detectable metals in RPE melanosomes (~ 1–2% by mass) was less than that in choroid or iris melanosomes (3–6% by mass). Zn and Ca concentrations in mature bovine RPE melanosomes were greater than those found in the same tissue in newborns, indicating that RPE melanosomes accumulate these metal cations with age. In this particular study, the choroid and iris melanosomes were exposed to sodium phosphate buffer, which also contained trace Ca. Thus the reported Ca content may differ from the melanosomes in their natural state.

Chemical analyses of bovine ocular melanosomes indicate that choroid melanosomes contain more DHICA than either the iris or RPE melanosomes, and that the DHICA content in both choroid and iris melanosomes decreases with age. This difference in composition may be related to melanin's biological functions, and it will impact the metal binding properties.⁶

Eibl et al. used the combination of electron dispersion x-ray analysis and transmission electron microscopy to quantify metals in melanosomes from humans, monkeys, and rats.⁴⁴ The total amount of metals detected (except for Al, which is the material of the grid) in melanosomes was about 2% (by mass) in the monkey and rat samples, and much less, ~0.2% (by mass), in the human ocular samples. The amount of Ca, Mg, and Na was higher in choroid melanosomes than in RPE melanosomes, suggesting a higher content of DHICA in choroid melanosomes than in RPE melanosomes, consistent with the discussion of bovine ocular samples.

The metals in neuromelanin have attracted great attention owing to the potential link between iron-neuromelanin-induced oxidative stress and the selective degeneration of pigmented neurons in the *substantia nigra* (SN) of the brains of patients with Parkinson's disease.^{30,36,45–47} Zecca et al. suggested that NM is the major iron storage site in the SN,⁴⁸ and they have quantified metals in extracted human NM with various methods.^{33,48–50} This group has also shown that in extracted neuromelanin from patients without any brain disorders, the concentration of iron is between 10,000 and 30,000 $\mu\text{g/g}$, making it the most abundant metal element in the pigment. Shimer et al. showed that for NM in the unprocessed SN, the iron concentration is about 7000 $\mu\text{g/g}$, suggesting that iron accumulates in the pigment during the isolation process.⁵⁰ While iron is the most abundant metal in NM, it is present at lower concentrations, and often in trace amounts, in most melanosomes (ocular, *Sepia*, hair) that have been analyzed. This difference likely is caused by the difference in melanogenesis pathways for NM compared to these other melanins, and Fe(III) has been invoked as an active participant in the biosynthesis of NM.⁴⁶ With the exception of NM, human melanosomes have Ca as their most abundant metal, which may stem from a functional role of melanin in regulating calcium homeostasis.^{9,51}

4. CHARACTERIZATION OF METAL BINDING TO MELANIN

In this section we examine physical measurements that have advanced our understanding of the details of metal binding to melanin. We focus on experimental determination of the binding sites, and binding affinity and capacity. Most of the work reported in the literature focuses on eumelanin and so we restrict our discussion to this pigment.

General Principles

To set the stage for this discussion, it is important to stress from the outset that the molecular structure of melanin and the organization of the pigment within the melanosomes is unknown. So in addressing the biophysics of metal binding to intact melanosomes, it is not possible to begin the discussion with knowledge of existing binding motifs within the structure. However, it is possible to propose structures and local environments for the metals in melanin drawing on the knowledge of metal binding sites in other biological systems and analyses of melanosomes, by techniques such as chemical degradation, infrared spectroscopy (IR), x-ray photoelectron spectroscopy (XPS), and solid state NMR.

Chemical studies of eumelanin indicate that this pigment contains DHI, DHICA, pyrrole, and dopa in different oxidized forms,¹⁴ as shown in Scheme 1. With these monomers, eumelanin should contain carboxyl, amine, hydroxyl groups (phenolic), quinone and semiquinone groups, all of which can serve as potential sites for metal accommodation. The presence of these functional groups has been confirmed by XPS and IR analysis.^{6,40} For example, Figure 1 shows the C(1s) XPS spectrum of bovine choroid melanosomes, revealing peaks that can be assigned to carbon that is bonded as follows: C-H, C-O, C=O and COO. IR analyses of melanomas clearly reveal the presence of OH and NH groups. Each of these functional groups or combinations of them can potentially serve as a metal binding site. In addition, different oxidized states of these groups, e.g. quinone or semiquinone, could also act as metal binding sites, as has been discussed in the work by Szpoganicz et al.⁵²

For carboxyl, hydroxyl, amine, and semiquinone groups, the binding of metals is likely to be pH-dependent. Knowing the pK_a values of these functional groups would be helpful in developing models for metal binding. Unfortunately, pK_a values of these functional groups in eumelanin are difficult to measure due to the heterogeneity of the pigment. The pK_a 's of the monomeric units DHICA and DHI have been examined and determined to be 4.2 for DHICA, and ~9.8 and ~13.2 for the two hydroxyl groups in both DHI and DHICA⁵³. In the case of *Sepia* eumelanin, the pK_a of the carboxylic acid group (thought to be derived from the DHICA monomer) is reported to be 3.1.⁵⁴

Binding Sites

Alkali metals—Little effort has been made to identify binding sites in melanin for the alkali cations. Considering their chemical properties and the pK_a 's of the functional groups in melanin, it is reasonable to conclude they bind to carboxyl groups under neutral or mildly acidic conditions, and that the binding should be fairly weak and ionic in nature.

Alkaline earth metals—The coordination of Ca to *Sepia* eumelanin has been examined using infrared spectroscopy.⁴⁰ The IR spectrum of Ca-loaded granules reveals that the COOH stretching vibration at 1710 cm^{-1} decreases with increasing metal concentration, indicating loss of the proton in the presence of metal. The pH of the solution also decreases with increasing Ca concentration, indicating that Ca binds to carboxylate groups. Exchange studies further reveal that Mg and Ca occupy a common binding site. This exchange process is exemplified in Figure 2, where Ca-saturated *Sepia* granules are exposed to different solution concentrations of Mg, then isolated and analyzed for metal content. With increased exposure to Mg, Ca is released and Mg is taken up by the granule. The exchange is *stoichiometric* and the saturation concentration is the same for the two metals.

Zinc—Szpoganicz et al. obtained binding constants of Zn(II) to various functional groups of synthetic DHI-melanin and determined the distribution of Zn(II) bound to these different functional groups as a function of pH (see Figure 3).⁵² The results indicate that in synthetic DHI-melanin Zn(II) binds to a mixed imine-catechol group at neutral to mildly acidic pH. In contrast, IR analysis suggests that in *Sepia* melanin Zn(II) binds predominantly to carboxyl groups at pH ~4, with only a minor contribution arising from NH and OH groups.⁴⁰ This difference likely arises from the quite different structures of these two samples. Because synthetic DHI-melanin contains a negligible concentration of carboxyl groups, its structure and binding properties do not necessarily correspond to those of *Sepia* melanin, which contains ~1.6 mmol/g of accessible carboxyl groups.⁷ This highlights the caution that must be exercised in comparing natural and synthetic samples.

For *Sepia* melanin, Ca(II), Mg(II), and Zn(II) share the same binding sites.³⁴ However, Fe(III) and Cu(II) do not coordinate to the carboxylate groups (see below) and interestingly can accumulate in the melanosome without influencing the organelle's ability to bind Ca(II), Mg(II) and Zn(II) (see Figure 4).³⁴ The data presented in Figure 4 show that when Fe(III)-saturated *Sepia* granules are exposed to Ca(II), Mg(II), or Zn(II), these metals accumulate to saturation without any appreciable loss of the coordinated Fe(III). These results reveal two important points: (1) Fe(III) does not occupy the same binding site as Ca(II), Mg(II) and Zn(II) and (2) the presence of Fe(III) in the granule does not block access to the Ca(II), Mg(II) and Zn(II) binding sites. These data clearly indicate a sophisticated organization of the pigment within the melanosomes.

Iron—Sarna et al. reported that both electron paramagnetic resonance (EPR) spectra and Mossbauer spectra of Fe(III)-melanin prepared at neutral pH were indistinguishable from those in acidic solution, which they interpreted to mean that there is only one type of binding site for

iron in melanin.²⁰ Based on this observation and considering the strong affinity of catechol-like molecules for Fe(III), they further suggested that iron is likely complexed with ortho-phenolic groups (present in both DHI and DHICA) in melanin. Bridelli et al., studying the complexation of Fe(III) with human neuromelanin and synthetic melanin with IR spectroscopy, observed an enhancement of the 1245 cm⁻¹ peak (phenolic C-OH) of neuromelanin after treatment with EDTA and concluded that neuromelanin binds iron to phenolic OH groups.⁵⁵ Samokhvalov et al. examined the binding of Fe(III) to melanin with Raman spectroscopy,⁵⁶ finding that the melanin Raman bands at 580 cm⁻¹ and 1470 cm⁻¹ (Fe-OR stretching and ring deformation modes, respectively) increased with increasing concentration of Fe(III) in melanin (see Figure 5), further confirming that iron binding sites in melanin are composed of catechol-like subunits.

Gerlach et al. found similar OH involvement of iron binding in neuromelanin using Mossbauer spectroscopy.⁵⁷ However, they proposed that a ferritin-like protein linked with melanin is responsible for iron binding. Owing to the lack of information about the chemical structure of melanin, it is currently difficult to determine whether the linked protein or melanin itself is responsible for iron binding. On the other hand, if we compare the capacity of melanin for iron (~ 1 mmol/g)³⁴ and the amount of protein typically found in melanin (5–10% by weight), we conclude that the proteins/peptides in melanin could only be responsible for a small fraction of the iron binding. Based on the published work to date, it is reasonable to conclude that iron binds to the aromatic hydroxyl groups of melanin.

An important outstanding issue related to the binding of iron is the oxidation state of the metal when coordinated to melanin. The reduction potential of Fe(III) and the oxidation potential of eumelanin are 0.77 V and 0.2 V,^{58,59} respectively, suggesting that melanin can thermodynamically reduce Fe(III) to Fe(II). In fact, such a reduction of iron by melanin has been demonstrated.³⁷ However, it needs to be pointed out that to detect Fe(II) in the reported study, 1,10-phenanthroline monohydrate (OP) was added to the reaction mixture. This compound preferentially binds to Fe(II) and so its presence alone may have shifted the natural equilibrium between Fe(III) and Fe(II) bound to melanin. Clarifying the relative amount of Fe(II) and the relative binding affinity of the two oxidation states of iron to melanin is of importance because Fe(II) participates in a series of redox reactions (e.g., Fenton chemistry) whereby reactive free radicals are generated and can induce oxidative stress. This potential reactivity may underlie the observation the melanin exhibits protective properties only when the Fe(III)/melanin ratio is within a certain range.^{36,37}

Sofic et al. quantified iron content in the SN of the brains of Parkinson patients, revealing an increase in Fe(III) and a decrease of the Fe(II)/Fe(III) ratio compared to normal controls.⁶⁰ In spite of these differences, a significant amount of Fe(II) and Fe(III) is found in the SN (Fe(II)/Fe(III) ~ 1.1).⁶⁰ It would be of interest to study the chemical aspects of iron binding to NM in order to determine what role the pigment plays in the Fe(II)/Fe(III) distribution, and then explore whether the differences between diseased and normal tissue arise from chemical changes in the pigment. In a recent work, Yoshida et al. examined iron bound to NM using X-ray absorption near-edge structure (XANES).⁶¹ Their work indicated that the iron bound to NM is shifted from Fe(II) to Fe(III) during the neurodegeneration process. This conclusion is consistent with the findings of the relative content of these two oxidation states of iron in the SN for normal and diseased tissue reported by Sofic et al.⁶⁰

Melanin has been shown to exhibit protective properties by chelating Fe(II).³⁷ But melanin-Fe(III) also has been shown to promote the production of free radicals when the Fe(III) concentration is high.³⁷ These data may indicate that the melanin acts as an antioxidant through its ability to chelate Fe(II) along with normal concentrations of Fe(III) found in the brain. But when excess Fe(III) is present, melanin becomes a pro-oxidant, perhaps by liberating Fe(II)

through displacement reactions by Fe(III). Such processes will induce oxidative stress, whereby cellular components are damaged and could ultimately lead to neuron death. However, the literature is not consistent in such findings. An earlier work by Jellinger et al. reports that most of the iron bound to NM is in the Fe(III) oxidation state, with only a minor amount of Fe (II) being present in brains of both Parkinson patients and normal controls.⁶² At this point, more work is required, including a quantitative determination of the relative binding affinities for melanin of the two oxidation states of iron.

Copper—IR spectroscopy has been used to examine Cu(II) binding to *Sepia* eumelanin (see Figure 6), and for the pH range from 3 to 4, it was concluded that the functional group responsible for Cu(II) binding is the hydroxyl group.⁴⁰ This conclusion is inconsistent with previous suggestions by Fronciz and Sarna, who used EPR to study the Cu(II) binding behavior in synthetic dopa and catechol melanin, and in natural bovine choroid melanosomes.^{63,64} The EPR analysis showed that Cu(II) binding to these melanins varied with pH as follows: for pH 2–7, Cu(II) binds to carboxylate and/or amine groups, and for pH 7–11, Cu(II) binds to hydroxyl (phenolic) groups.

These conclusions, however, are based on the pK_a values of the different functional groups in the absence of metal. Binding of Cu(II) to these functional groups should change their apparent pK_a values. Thus, it cannot be ruled out that in eumelanin, an o-OH (catechol) group in which one of the OH groups is deprotonated may bind to Cu(II); this possibility is in fact supported by the pH changes observed for melanin suspensions upon Cu(II) binding.⁴⁰ Moreover, it has also been shown that at pH 4.5, the methylation of melanin by CH_2N_2 and $(CH_3)_2SO_4$ blocks almost all the sites for Cu(II) binding, while acetylation or ethylation by acetyl anhydride and thionyl chloride can only block about 25% of the binding sites.²⁰ Because the former two reagents block both phenolic and carboxylate groups while the latter two only block the carboxylate group, this result suggests that at pH 4.5, Cu(II) is mostly bound to phenolic groups, consistent with the IR spectroscopic analysis.

Metal Binding Affinity_[A1]—Potts and Au studied the binding affinity of melanin to 23 metals by examining the amount of metals taken up by bovine ocular melanosomes when a certain mass of melanosomes was suspended in a metal solution of known concentration (a metal to pigment ratio of 1.2 mmol/g).¹⁸ The percentage of metal cations remaining in solution was used to estimate the metal binding affinity. Implicit in this analysis are two assumptions, the validity of which is not clear. First, the number of binding sites is taken to be a constant, independent of the metal being considered. As described above, some metals share the same binding sites (and in that case this approach would be valid), but others have unique binding locations. To obtain accurate relative binding affinities would then require the data be normalized to the actual number of binding sites available. Second, it is assumed that at this exposure level metal binding to melanin does not reach saturation so that the remaining metal concentration in solution reflects an equilibrium between bound and unbound metal, and not simply an excess of metal following saturation of the melanosome.

While these assumptions were not discussed or addressed, the results of the study are of interest and do reveal that choroid and synthetic melanin showed a higher ability to take up metals than iris and ciliary body melanin. The data also demonstrate that alkali metals have little or no affinity to melanin. For some specific metals focused on in the previous section, the relative binding affinities are in the order $Ca(II) < Zn(II) < Fe(III) < Cu(II)$. Because melanin binds both $Ca(II)$ and $Zn(II)$ with a capacity higher than 1.2 mmol/g^{7,34} (a number not known when the study by Potts and Au was reported), this ordering suggests that $Zn(II)$ binds to melanin more strongly than $Ca(II)$ does. It is now known that melanin's capacity for Fe(III) and Cu(II) is on the order of the metal:melanin ratio used in these experiments,³⁴ and so the data on the affinities

of iron and copper in this study require confirmation from experiments where the capacity of the system is taken into account.

Larsson and Tjalve have examined the relative binding affinity of various metal ions by examining the binding competition between paraquat and melanin.³² In contrast to Potts's results, melanin was found to have considerable affinity for alkali metals, an affinity which increased with atomic weight. But this phenomenon was only apparent for the competing reaction between 10 mM metals and paraquat. For the competition of paraquat with 0.36 mM metal cations, the alkali metal only slightly affected the binding of paraquat to melanin. The origin of the different conclusions between the studies of Larsson and Tjalve, and Pott and Au, can be attributed to the higher metal to melanin ratio used in the latter study (10 mM metal vs. 1.4 mg/ml of melanin in the Pott study). Larsson and Tjalve also reported_[A2] that alkaline earth metal ions showed a much higher affinity than alkali metals and this affinity also increased with atomic weight. Heavy metals, such as Cu(II), Pb(II), La(III) and Gd(III), bind to melanin more strongly than the other metals studied. Importantly, synthetic melanin, which contains little protein, exhibited metal affinities similar to those of natural melanin, indicating that proteins in melanin have only minor effects on metal binding.

In a separate study, Bowness et al. found that the concentration of Zn(II) in melanin was reduced almost to zero after treatment with 0.1 M HCl, while the Cu(II) concentration decreased only slightly.¹ Considering that the pK_a of COOH (~ 3.1, the functional group that binds Zn(II)) and of OH (9 and 13, the functional group that binds Cu(II)), these pH-dependent observations support the conclusion that Cu(II) binds to melanin much more strongly than Zn(II).

Information on the binding affinity of Mg(II), Ca(II) and Zn(II) to *Sepia* melanin was obtained by analyzing the correlations between the pH changes that occur upon binding and the amount of metal taken up by the granules.⁴⁰ Exposed to the same initial pH and metal concentration, EDTA-treated granules can take up more Zn(II) than Ca(II) and Mg(II). These results suggest that the binding affinity varied in the order Mg(II) < Ca(II) < Zn(II), consistent with previous studies.

In addition to these qualitative analyses, several quantitative analysis of metal binding affinity to melanin have been carried out. Ben-Shachar and Youdim studied the binding constants of Fe(III) to synthetic dopamine melanin.⁴⁷ Melanin (3 µg) was suspended in a solution containing one of 20 different concentrations of FeCl₃ from 0.5 to 400 nM at pH 6.5. A Scatchard analysis produced two binding constants for iron to melanin: $7.6 \times 10^7 \text{ M}^{-1}$ and $5 \times 10^6 \text{ M}^{-1}$, with the maximum concentration of binding sites 1.1 nmol/mg and 17.6 nmol/mg, respectively. However, the upper range of iron concentrations was not sufficient to probe the entire range of iron binding to melanin, and other binding sites may have been missed in this research. This may also explain why in this study a capacity of ~ 20 µmol/g was observed, as compared to other work suggesting a 1.2 mmol/g capacity.^{7,34}

Lyden et al. studied the binding of radioactive Mn(II) to bovine ocular melanin, human hair melanin and synthetic dopamine melanin.⁴ Bovine ocular melanin had a higher Mn(II) capacity (1.3 mmol/g) than hair melanin and synthetic dopamine melanin (~ 0.2 mmol/g). But the affinities of the three types of melanins to Mn(II) were similar and four binding constants were reported: 10^7 M^{-1} , 10^6 M^{-1} , 10^4 M^{-1} , and 10^3 M^{-1} . These data showed that Mn(II) binds to melanin with a wide range of affinities. These results suggest that the accumulation of Mn(II) is probably due to the high affinity of melanin to Mn(II). However, the sites with high affinity only represent a small fraction of the available sites. Excess Mn(II) results in only loose binding and may cause a release of Mn(II) under certain conditions, which could further induce redox reactions and cellular damage.

Szpoganicz et al. studied the binding of Zn(II) and Cu(II) to synthetic DHI-melanin and estimated the effect of quinine imine on metal binding.⁵² The binding constants of Cu(II) and Zn(II) to different possible binding sites were calculated, e.g., the binding constants of Cu(II) to the first catechol (deprotonated) and the mixed catecholimine group were $10^{19.6}$ and $10^{20.9} \text{ M}^{-1}$, respectively. Generally, Cu(II) binding constants are about 2 to 4 orders of magnitude higher than Zn(II) binding constants, which is consistent with previous studies comparing Zn(II) and Cu(II) binding to melanin. Based on these binding constants and the concentration of the sites in melanin, the bound species distributions were calculated and the curves were plotted in Figure 7. Zn(II) was more prone to bind to imine sites while Cu(II) bound preferentially to catechol groups. At neutral pH, metal complexed with mixed catecholimine groups was the favorite for both samples.

Salceda et al. examined the coordination of Ca(II) in frog RPE melanosomes.³⁵ By examining the initial rate of Ca uptake by melanin, they obtained an apparent Michaelis constant K_M of 0.5 mM. Although this number is not directly related to binding constants, it does show that the strength of the Ca(II) binding to melanin is only moderate. In a recent study, the binding constants of Ca to *Sepia* melanin is determined to be $3.3 (\pm 0.2) \times 10^3 \text{ M}^{-1}$.⁵⁴

In general, the binding affinity increases in the order alkali metal < alkaline earth metal < Zn(II) < Cu(II), Fe(III), and Mn(II). It is important to point out that melanin's binding affinity to metals will likely vary with the type and origin of the melanin because the relative abundance of function groups that serve as the binding sites varies among different types of melanosomes_[A3].

Metal Binding Capacity

In addressing the total capacity of melanosomes to coordinate specific metals, one needs to be cognizant that there are various binding sites that can be associated with the various functional groups, such as OH, NH and COOH, and their combinations. It is possible that more than one type of site coordinates the same metal. In our discussion below, we use the term binding capacity to refer to the amount of metal that saturates the most favored, lowest energy binding site. If there are two sites with similar binding constants, the situation becomes quite complicated as the lowest energy site may not be saturated before there is appreciable binding to the second site. If there is a large energy difference between different binding sites, then coordination to the second site occurs after the lowest energy site is saturated. Thus it is important to consider the affinity of the binding site(s) when determining capacity. Studies suggest that a metal to melanin ratio of no more than 5 to 10 mmol metal to 1 gram of melanin is a good working range to determine the capacity of the lowest energy binding site.

The binding capacity of *Sepia* eumelanin to Mg(II), Ca(II), Zn(II), Fe(III) and Cu(II) was determined using inductively coupled plasma mass spectrometry.^{7,34} As discussed above, Mg(II), Ca(II) and Zn(II) (along with K(I) and Na(I)) bind to COOH groups. The total natural content of these latter five metals in *Sepia* is 1.6 mmol per gram of melanin. Consistent with the fact that these metals can be exchanged with one another, the saturation level of Mg(II), Ca(II) and Zn(II) in otherwise di- or multi-valent metal-free melanin is about 1.4–1.5 mmol/g. The binding capacity of Fe(III) and Cu(II) is lower, 1.2 and 1.1 mmol/g, respectively.

The metal binding capacity of bovine choroid and iris melanosomes has also been examined.⁶ For Mg(II), Ca(II) and Zn(II), choroid melanosomes have a higher binding capacity (1.3 mmol/g for the newborn and 0.9 mmol/g for the adult) than corresponding iris samples (0.9 mmol/g for the newborn and 0.7 mmol/g for the adult). It is useful to note that the binding capacity for Mg(II), Ca(II), and Zn(II) can be used as a measure of the relative amount of DHICA in melanin, a ratio that affects both its degree of polymerization and its antioxidant

ability.^{65,66} The decreased binding capacity with age suggests that the content of carboxyl groups or DHICA decreases with age, making melanin less efficient as an antioxidant.

In general, the binding capacity will likely depend on the type of melanin, considering that different types of melanosomes have been shown to have different DHICA to DHI ratios.^{8, 21,23} Thus in discussing capacity it is important to note the origin of the sample.

5. EFFECTS OF METAL BINDING ON THE PROPERTIES OF MELANIN

In this section we consider the functional significance of metal binding. We first consider whether the accumulation of metals in melanin results in morphological changes in the melanosomes. This is then followed by a discussion of the effect of metals on the aerobic reactivity of melanosomes – specifically addressing how metals influence melanin's ability to act as an antioxidant. The last section examines the specific case of Ca(II) binding and the role melanosomes may play based on the capacity and affinity of melanin for this biologically important cation.

Morphology

Melanosomes are generally spherical or ovoid shaped.^{8,23–27} The size and shape of the organelles are surprisingly uniform, implying that biogenesis of the melanosomes is controlled. Because natural melanin is associated with a large metal ion content, it is interesting to examine whether metal cations may influence the structuring of melanin within the melanosome, either by templating melanogenesis or altering the natural organization as metals accumulate in the mature melanosome.

While it is not possible to examine the effects of metal ions on melanogenesis in any systematic fashion, it is possible to study how the addition of metals affects the overall morphology of the melanin granules. For example, Liu et al. used SEM to examine the morphology of *Sepia* melanin loaded with various metal ions.⁴¹ The shape of granules showed no dependence on metal content (amount or type), revealing little involvement of metals in either maintaining or altering the morphology of melanin granules (see Figure 8). But it was observed that the size of granules gradually decreased after treatment with EDTA, which extracts di- and multi-valent metal cations.

Since the major metals in natural *sepia* melanin are Mg(II) and Ca(II), Liu et al. suggested that the removal of these two divalent cations increases the solubility of melanin.⁴¹ Sonication of melanin releases material from the intact granule (see above), and chemical degradation analysis reveals that this dissolved material contains a higher DHICA fraction than that of the intact granule.⁴¹ This result clearly indicates that DHICA-rich material can be released to the surrounding solution, in this case in response to a mechanical perturbation. But it is also the carboxylic acid group associated with the DHICA moieties that coordinates Ca(II) and Mg(II), and so it is reasonable to postulate that removal of these metals could also destabilize the DHICA-rich material near the surface of the melanosomes. Such destabilization could alter the equilibrium between DHICA's being associated with the intact melanosomes or dissolved in the surrounding solution. This work suggests that the interaction of metals with melanin could affect the structural integrity of the granules in solution.

Aerobic reactivity

An important biological function of melanin is to act as an antioxidant, primarily demonstrated by quenching free radicals. In addressing this property of melanin, it is important to consider whether the pigment is located in a tissue that can be exposed to ultraviolet light (e.g., skin) or not (e.g., brain). The reason for this differentiation is that melanin also is known to produce free radicals when irradiated with UV light, and in these cases it is important to approach the

problem from a point of view of understanding the balance between its thermal abilities to quench reactive oxygen species and its photochemical abilities to generate them. In both cases (presence and absence of light), however, the effect of the accumulation of metals such as Fe(III) and Cu(II) on these properties is of great interest.

Understanding how the aerobic reactivity of melanosomes changes with the concentration of such metals could provide new insights into our understanding of the pigment-related diseases. For example, are there relationships between (1) the accumulation of Fe(III) by neuromelanin with age and the selective degeneration of pigmented neurons in Parkinson's Disease, and (2) the accumulation of Fe(III) in the melanosomes of human RPE cells, the decrease in the amount of melanin in the RPE with age, the age-dependent increase in photoaerobic activity (oxidative stress) of these cells, and the onset and progression of age-related macular degeneration?

Korytowski et al. studied the reactive species produced upon irradiation of melanin with UV and visible light.⁶⁷ They observed that in the presence of EDTA, the production of reactive oxygen species increases for melanin complexed with iron. To explain their results, they proposed that melanin first reduces Fe(III) to Fe(II), followed by the extraction of Fe(II) by EDTA. Both EDTA-Fe(II) and melanin-Fe(II) can catalyze Fenton reactions, thereby increasing the production of free radicals. This study once again highlights the importance of elucidating the distribution of oxidation states of the bound iron.

In a related work, Pilas et al. studied the reactions of melanin complexed with iron in the presence of EDTA in more detail.³⁷ In particular, they examined the effect of melanin and EDTA on the production of hydroxyl free radicals from the Fenton reaction. In the presence of hydrogen peroxide and Fe(II), melanin showed the ability to decrease the production of free radicals through its uptake and sequestering of Fe(II) from the solution. But for Fe(III), melanin exhibited a biphasic response in the production of hydroxyl radical. The production rate decreased for low concentrations of Fe(III), but increased after reaching a certain threshold concentration of the metal. The rate of production of hydroxyl radicals increased for melanin complexes to either oxidation state of iron in the presence of a strong iron chelator (e.g., EDTA). Based on these observations, the authors proposed that the melanin likely reduces Fe(III) to Fe(II), thereby increasing the rate of reaction of Fe(III) with hydrogen peroxide. At low concentrations, Fe(II) is coordinated to the pigment. But once the pigment nears saturation in iron, displacement of Fe(II) by Fe(III) could occur, producing solvated Fe(II), which would result in Fenton chemistry and production of hydroxyl radicals.

In a recent work, Zareba et al. investigated the effect of a synthetic neuromelanin on the yield of hydroxyl free radicals produced by Fenton reactions.³⁶ Their data showed similar results: melanin with a low iron concentration resulted in a low level of hydroxylation of salicylate to di-hydroxybenzoate (DHB), a measure of free hydroxyl radical production. But when the Fe(III)/melanin ratio reached 45,000 ppm (0.8 mmol/g), the production of DHB increased with increasing Fe(III) concentration (see Figure 9). In the presence of EDTA, which extracts iron from melanin, the formation of DHB increased, consistent with the above studies by Pilas et al. and Korytowski et al.

The effect of metal binding on the aerobic reactivity of melanin has also been studied by quantifying the ability of melanin to nick supercoiled DNA.³⁴ EDTA-washed melanin (mostly free of di- or multiple-valent metals) and melanin containing one of five different metals were examined. In the absence of light, EDTA-washed melanin caused more DNA damage than Mg, Ca, or Zn(II)-enriched melanins, suggesting that coordination of metal cations to carboxyl groups mitigates the aerobic reactivity of the melanin in the absence of irradiation. Cu(II) and Fe(III)-loaded melanin generally caused more DNA damage than Mg(II), Ca(II), or Zn(II)-loaded melanin. The damaging effects of Fe(III)-loaded melanin

increased in the presence of EDTA, consistent with previous results. In contrast to the Fe(III)-loaded sample, the damaging effects of Cu(II)-loaded melanin were greatly decreased by EDTA, which may be due to the different oxidation potential of Cu(II)-melanin or Cu(II)-EDTA as compared to Fe(III)-melanin or Fe(III)-EDTA.

Farmer et al. showed that the binding of Zn(II) and Cu(II) to melanin can increase oxidative stress through generation of superoxide and hydroxyl radicals.⁶⁸ Their results further showed that introduction of Zn(II) or Cu(II) into incubated melanoma cells induces more cell death than in normal pigmented cells because melanoma cells contain a high content of cytosolic melanin fragments.⁶⁸ In contrast to this result in cells, addition of Zn(II) to *Sepia* melanin has little effect on aerobic reactivity.³⁴ At this point, the reason for the difference in the reactivity of the cellular and *Sepia* cases is not known. One possibility is that the melanins are structurally different. It has been shown that Zn(II) binds to DHI-melanin mainly at the mixed imine-catechol groups, while the primary site for Zn(II) in *Sepia* melanin is the carboxyl group.^{7, 34,40,52} This different binding behavior observed for different types of melanin may also lead to the variation in aerobic reactivity.

The antioxidant function of melanin arises from its ability to sequester reactive metals, but it is important to recognize that there is a limit to the capacity of the pigment to perform this function, and once exceeded, the metal-bound melanin can become pro-oxidant, a disadvantage which will be further exacerbated in the presence of metal chelators such as EDTA.

Calcium Homeostasis By Melanin

Calcium regulation in melanocytes affects numerous biological pathways including protecting the redox balance in the cell and regulating the supply of the substrate L-tyrosine for melanogenesis.^{69–71} Melanin has been implicated in maintaining calcium homeostasis in the cell and is known to be involved with calcium ion regulation in the inner ear.^{51,72,73} Maintaining calcium homeostasis in epidermal melanocytes is especially important because these cells are constantly exposed to UV radiation, which can induce oxidative stress through the photo-generation of reactive oxygen species. Calcium involvement in maintaining the redox balance in melanocytes can play a biological role in regulating the cell's response to oxidative stress. For example, thioredoxin reductase is expressed in dark skin in concentrations five times higher than in fair skin.⁷⁰ Since calcium levels can modulate the activity of this enzyme, the control of calcium levels is crucial for regulating the redox balance and preventing DNA damage in cells.

The association constant for Ca(II) binding to *Sepia* melanin was determined to be $3.3 (\pm 0.2) \times 10^3 \text{ M}^{-1}$.⁵⁴ This binding constant (on the order of 10^3 M^{-1}) supports the postulated role of melanin in acting as an intracellular store for calcium and in helping to regulate calcium homeostasis within melanocytes. This degree of binding affinity for Ca(II) is lower by at least 100-fold than several common intracellular calcium transport proteins, such as calmodulin ($K_B \sim 10^6\text{--}10^7 \text{ M}^{-1}$), calbindin ($K_B \sim 10^7 \text{ M}^{-1}$), parvalbumin ($K_B \sim 10^8 \text{ M}^{-1}$), annexins ($K_B \sim 10^6 \text{ M}^{-1}$), and calpain ($K_B \sim 10^5 \text{ M}^{-1}$).^{74,75} A binding constant of $K_B = 10^3 \text{ M}^{-1}$ is desirable for buffering calcium flux since almost all intracellular calcium binding proteins directly involved in signaling are inactive_[A4] until calcium concentrations reach $10^{-6} - 10^{-8} \text{ M}$, and since the extracellular calcium concentration is usually $> 10^{-3} \text{ M}$. Furthermore, a proteolytic cell death cascade is triggered if intracellular Ca(II) concentrations exceed 10^{-5} M in most cells. A good example of a naturally-occurring calcium buffer system is the role of the protein calsequestrin ($K_B \sim 10^3 \text{ M}^{-1}$) in the sarcoplasmic reticulum of skeletal muscle cells. The binding constant and capacity determined for eumelanin suggest that this form of melanin regulates calcium homeostasis in melanocytes by sequestering and buffering intracellular Ca(II) concentrations.

6. FUTURE DIRECTIONS

The binding capacity, relative affinity, and sites of eumelanin's binding to metals are now becoming well established. The effects of metal binding, especially of iron, on melanin's aerobic reactivity have also been studied, with a large range of useful data obtained. However, the exact binding constants for most metals to melanin are not known due to difficulty in obtaining the concentrations of binding sites in melanin as well as the concentration of melanin itself. Thus, it is now necessary to elucidate the molecular structure of melanin and develop methods to quantify the functional groups and their geometry that comprise the metal binding sites. It would also be interesting to investigate the transport of metal cations in or on the surface of the granules and determine how these metals are ultimately trafficked into the interior of the melanosome. Furthermore, it is not known if the metals are stored in the vicinity of one another or homogeneously dispersed within the organelle. This information will be important in understanding not only the metal reservoir and sink functions of melanin, but also how metals may be released in cases when there is degeneration [A5] of melanosomes within tissue. Knowledge of the interaction of metals and melanin may ultimately be applied to the treatment of pigment-related diseases, a goal that might be achievable through adjusting the reactivity of melanin via metal binding.

Acknowledgements

We acknowledge our collaborators: Dr. Yan Liu, Professor Kazumasa Wakamatsu, Professor Shosuke Ito, Professor Tadeusz Sarna, Dr. Alexander Samokhvalov, Dr. Valerie Kempf, and William D. Bush. Our work in this area has been supported by the National Institutes of Health, MFEL Program administered by the AFOSR, and Duke University.

References

1. Bowness JM, Morton RA, Shakir MH, Stubbs AL. *Biochem J* 1952;51:521. [PubMed: 13018116]
2. Horcicko J, Borovansky J, Duchon J, Prochazkova BH-S. *Z Physiol Chem* 1973;354:203. [PubMed: 4803255]
3. Szekeres L. *Arch Dermatol Forsch* 1975;252:297. [PubMed: 1164039]
4. Lyden A, Larsson BS, Lindquist NG. *Acta Pharmacol Tox* 1984;55:133.
5. Okazaki M, Kuwata K, Miki Y, Shiga S, Shiga T. *Arch Biochem Biophys* 1985;242:197. [PubMed: 2996430]
6. Hong L, Simon JD. *Photochem Photobiol* 2005;81:517. [PubMed: 15790301]
7. Liu Y, Hong L, Kempf VR, Wakamatsu K, Ito S, Simon JD. *Pigm Cell Res* 2004;17:262.
8. Liu Y, Hong L, Wakamatsu K, Ito S, Adhyaru B, Cheng CY, Bowers CR, Simon JD. *Photochem Photobiol* 2005;81:135. [PubMed: 15504086]
9. Panessa BJZJA. *Exp Eye Res* 1981;32:593. [PubMed: 6972313]
10. Youdim MBH, Riederer P. *J Neural Transm-Gen Sect* 1993:57.
11. Borovansky J. *Sbornik Lekarsky* 1994;95:309. [PubMed: 8867702]
12. Crippa, R.; Horak, V.; Prota, G.; Svoronos, P.; Wolfram, L. *Chemistry of Melanins*. In: Brossi, A., editor. *The Alkaloids*. 36. Academic Press Inc; 1989. p. 253
13. Ito S. *J Invest Dermatol* 1993;100:166. [PubMed: 7679136]
14. Ito, S. *Advances in Chemical Analysis of Melanins*. In: Nordlund, JJBRE.; Hearing, VJ.; King, RA.; Ortonne, JP., editors. *The Pigmentary System*. Oxford University Press; New York: 1998. p. 439
15. Riley PA. *Int J Biochem Cell Biol* 1997;29:1235. [PubMed: 9451820]
16. Odh G, Carstam R, Paulson J, Wittbjør A, Rosengren E, Rorsman H. *J Neurochem* 1994;62:2030. [PubMed: 8158151]
17. Wakamatsu K, Fujikawa K, Zucca FA, Zecca L, Ito S. *J Neurochem* 2003;86:1015. [PubMed: 12887698]
18. Potts AM, Au PC. *Exp Eye Res* 1976;22:487. [PubMed: 1278259]
19. Sarna T, Hyde JS, Swartz H. *Science* 1976;192:1132. [PubMed: 179142]

20. Sarna, T.; korytowski, W.; Pasenkiewicz-Gierula, M.; Gudowska, E. Ion-Exchange Studies in Melanins. Proceeding of 11th International Pigment Cell Conference; 1980; Sendai.
21. Ozeki H, Wakamatsu K, Ito S, Ishiguro I. Anal Biochem 1997;248:149. [PubMed: 9177734]
22. Ward WC, Simon JD. Pigm Cell Res 2007;20:61.
23. Liu Y, Hong L, Wakamatsu K, Ito S, Adhyaru BB, Cheng CY, Bowers CR, Simon JD. Photochem Photobiol 2005;81:510. [PubMed: 15701042]
24. Duchon J, Borovansky J, Hach P. Pigment Cell 1973;1:165.
25. Hu DN, McCormick SA, Orlow SJ, Rosembat S, Lin AY, Wo K. Invest Ophthalmol Vis Sci 1995;36:931. [PubMed: 7706042]
26. Clancy CMR, Simon JD. Biochemistry 2001;40:13353. [PubMed: 11683645]
27. Hong L, Garguilo J, Anzaldi L, Edwards GS, Robert J Nemanich, Simon JD, Simon JD. Photochem Photobiol 2006;82:1475. [PubMed: 16696595]
28. Moses H, Ganote CE, Beaver DL. Anat Rec 1966;155:167. [PubMed: 4960421]
29. Hirose K. Z Zellforsch Mik Ana 1968;88:187.
30. Enochs WS, Sarna T, Zecca L, Riley PA, Swartz HM. J Neural Transm-Park Dis Dement Sect 1994;7:83. [PubMed: 7710667]
31. Bruenger FW, Stover BJ, Atherton DR. Radiat Res 1967;32:1. [PubMed: 6053863]
32. Larsson B, Tjalve H. Acta Physiol Scand 1978;104:479. [PubMed: 726939]
33. Zecca L, Swartz HM. J Neural Transm-Park Dis Dement Sect 1993;5:203. [PubMed: 8396395]
34. Hong L, Liu Y, Simon JD. Photochem Photobiol 2004;80:477. [PubMed: 15623333]
35. Salceda R, Sanchez-Chavez G. Cell Calcium 2000;27:223. [PubMed: 10858668]
36. Zareba M, Bober A, Korytowski W, Zecca L, Sarna T. Biochim Biophys Acta 1995;1271:343. [PubMed: 7605801]
37. Pilas B, Sarna T, Kalyanaraman B, Swartz HM. Free Radic Biol Med 1988;4:285. [PubMed: 2834276]
38. Zecca L, Shima T, Stroppolo A, Goj C, Battiston GA, Gerbasi R, Sarna T, Swartz HM. Neuroscience 1996;73:407. [PubMed: 8783258]
39. Sarzanini C, Mentasti E, Abollino O, Fasano M, Aime S. Mar Chem 1992;39:243.
40. Hong L, Simon JD. Photochem Photobiol 2006;82:1265. [PubMed: 16696594]
41. Liu Y, Simon JD. Pigm Cell Res 2005;18:42.
42. Horcicko J, Borovansky J, Duchon J. Dermatol Monatsschr 1973;159:206. [PubMed: 4704940]
43. Bolt AG. Life Sci 1967;6:1277. [PubMed: 6039840]
44. Eibl O, Schultheiss S, Blitgen-Heinecke P, Schraermeyer U. Micron 2006;37:262. [PubMed: 16364648]
45. Double KL. J Neural Transm 2006;113:751. [PubMed: 16755379]
46. Sulzer D, Bogulavsky J, Larsen KE, Behr G, Karatekin E, Kleinman MH, Turro N, Krantz D, Edwards RH, Greene LA, Zecca L. Proc Natl Acad Sci U S A 2000;97:11869. [PubMed: 11050221]
47. Benschachar D, Youdim MBH. Prog Neuro-Psychopharmacol Biol Psychiatry 1993;17:139.
48. Zecca L, Gallorini M, Schunemann V, Trautwein AX, Gerlach M, Riederer P, Vezzoni P, Tampellini D. J Neurochem 2001;76:1766. [PubMed: 11259494]
49. Zecca L, Pietra R, Goj C, Mecacci C, Radice D, Sabbioni E. J Neurochem 1994;62:1097. [PubMed: 8113797]
50. Shima T, Sarna T, Swartz HM, Stroppolo A, Gerbasi R, Zecca L. Free Radic Biol Med 1997;23:110. [PubMed: 9165303]
51. Hoogduijn MJ, Smit NP, van der Laarse A, van Nieuwpoort AF, Wood JM, Thody AJ. Pigm Cell Res 2003;16:127.
52. Szpoganicz B, Gidanian S, Kong P, Farmer P. J Inorg Biochem 2002;89:45. [PubMed: 11931962]
53. Charkoudian LK, Franz KJ. Inorg Chem 2006;45:3657. [PubMed: 16634598]
54. Bush WD, Simon JD. Pigm Cell Res. 2007accepted
55. Bridelli MG, Tampellini D, Zecca L. FEBS Lett 1999;457:18. [PubMed: 10486555]
56. Samokhvalov A, Liu Y, Simon JD. Photochem Photobiol 2004;80:84. [PubMed: 15339213]

57. Gerlach M, Trautwein AX, Zecca L, Youdim MBH, Riederer P. *J Neurochem* 1995;65:923. [PubMed: 7616255]
58. Lide, DR. *Handbook of Chemistry and Physics*. 72. CRC Press; 1992.
59. Samokhvalov A, Hong L, Liu Y, Garguilo J, Nemanich RJ, Edwards GS, Simon JD. *Photochem Photobiol* 2005;81:145. [PubMed: 15458368]
60. Sofic E, Riederer P, Heinsen H, Beckmann H, Reynolds GP, Hebenstreit G, Youdim MBH. *J Neural Transm* 1988;74:199. [PubMed: 3210014]
61. Yoshida S, Ektessabi A, Fujisawa S. *J SYNCHROTRON RADIAT* 2001;8:998. [PubMed: 11513007]
62. Jellinger K, Kienzl E, Rumpelmair G, Riederer P, Stachelberger H, Benshachar D, Youdim MBH. *J Neurochem* 1992;59:1168. [PubMed: 1494904]
63. Froncisz W, Sarna T, Hyde JS. *Arch Biochem Biophys* 1980;202:289. [PubMed: 6249219]
64. Sarna T, Froncisz W, Hyde JS. *Arch Biochem Biophys* 1980;202:304. [PubMed: 6249220]
65. Sarangarajan R, Apte SP. *Ophthal Res* 2005;37:136.
66. Blarzino C, Mosca L, Foppoli C, Coccia R, De Marco C, Rosei MA. *Free Radic Biol Med* 1999;26:446. [PubMed: 9895237]
67. Korytowski W, Pilas B, Sarna T, Kalyanaraman B. *Photochem Photobiol* 1987;45:185. [PubMed: 3031709]
68. Farmer PJ, Gidanian S, Shahandeh B, Di Bilio AJ, Tohidian N, Meyskens FL. *Pigm Cell Res* 2003;16:273.
69. Wood JM, Jimbow K, Boissy RE, Slominski A, Plonka PM, Slawinski J, Wortsman J, Tosk J. *Experimental Dermatology* 1999;8:153. [PubMed: 10232409]
70. Schallreuter KU, Wood JM. *J Photochem Photobiol B* 2001;64:179. [PubMed: 11744405]
71. Schallreuter KU, Wood JM. *Biochem Biophys Res Commun* 1999;262:423. [PubMed: 10462491]
72. Meyerzum Gottesberge AM. *Pigm Cell Res* 1988;1:238.
73. Gill SS, Salt AN. *Hearing Res* 1997;113:191.
74. Frausto da Silva, JJR.; Williams, RJP. *The Biological Chemistry of the Elements*. New York, N.Y: Oxford University Press; 1991.
75. Carafoli, E.; Guiseppe, I.; Rosen, BP. Calcium transport across biological membranes. In: Sigel, H., editor. *Metal Ions in Biological Systems*. 17. Marcel Dekker; New York, N.Y: 1984. p. 129

Figure 1.

The high-resolution X-ray photoelectron spectrum of C(1s) of melanosomes isolated from mature bovine choroids. The spectrum reveals the presence of C-H, C-NH₂, C-O, C₂-NH, C=O, and COO functional groups. These groups are derived from the oligomerization of DHI and DHICA. This figure is reprinted with permission from Hong et al.⁶

Figure 2.

The concentrations of bound Ca(II) and Mg(II) to *Sepia* melanin is plotted as a function of the Ca(II) concentration in solution. The levels indicated at 0 M Ca(II) reflect those naturally present in the pigment. Natural melanosomes were then suspended in solutions of varying Ca (II) concentration, allowed to come to equilibrium, and then the resulting concentrations of bound Ca(II) and Mg(II) in dried melanin granules were determined using ICP-MS. These data clearly show that Ca(II) displaces Mg(II) from the pigment in a 1:1 stoichiometric ratio, also confirming that the two metals share a common binding site. This figure is reprinted with permission from Liu *et al.*⁷

Figure 3.

Species distribution curves of a DHI-melanin solution with the presence of Zn(II) ions, in which QI represents quinone imine and Cat represents catechol. The curve shows that the coordination of Zn(II) depends on pH and that under neutral or mildly acidic conditions, Zn(II) favors binding to quinone imine groups. This figure is reprinted from *Journal of Inorganic Biochemistry*, 89, Szpiganicz *et al.*, Metal binding by melanins: Studies of colloidal dihydroxyindole-melanin, and its complexation by Cu(II) and Zn(II) ions, 45–53, (2002), with permission from Elsevier.⁵²

Figure 4.

The effect of binding Mg(II), Ca(II), and Zn(II) on the concentration of bound Fe(III) starting with Fe(III)-saturated melanin. The initial concentrations of Mg(II), Ca(II), and Zn(II) were a small fraction of their saturation value. After exposure of the Fe(III)-saturated melanosome to either 10 mM Mg(II), Ca(II) and Zn(II) solution at pH 4, the concentration of bound iron was slightly reduced, but the concentrations of Mg(II), Ca(II), and Zn(II) were those expected for saturated absorption of these metals. These data indicate that even when saturated with Fe(III), the melanosomes can still coordinate the metals Ca(II), Mg(II) and Zn(II) to their individual saturation values. Therefore these three metals do not share the same binding site as Fe(III) and the presence of Fe(III) does not obstruct the channels needed to coordinate these metals in the pigment. This figure is reprinted with permission from Hong *et al.*³⁴

Figure 5. Raman spectra of *Sepia* eumelanin loaded with varying amounts of Fe(III) in the region 350–700 cm^{-1} . The concentrations of Fe(III) in the dried melanin are 170 ppm (black), 8230 ppm (dark gray), and 86000 ppm (light gray). The intensities of 580 cm^{-1} (Fe-OR stretching) and 1470 cm^{-1} peaks (data not shown, Fe-OR ring deformation modes) increase with increasing Fe(III) content, further confirming that the Fe(III)-melanin binding site is composed of catechol-like structural units. This figure is reprinted with permission from Samokhvalov *et al.*⁵⁶

Figure 6.

IR spectrum of Cu(II)-enriched melanin as a function of the solution concentration of Cu(II). The bottom curves show the difference between the spectra for the 0.25 mM and 2 mM solutions (the gray curve) and the difference between 0.25 mM and 10 mM (the black curve). The data shows a decrease of the 3400 cm⁻¹ (OH) peak intensity, increases of the 1710 cm⁻¹ (COOH) and 1250 cm⁻¹ peak intensities and no difference for the 3200 cm⁻¹ (NH) peak upon Cu(II) binding when the solution concentration ≤ 2 mM. These data implicate the phenolic groups as comprising the binding site. This figure is reprinted with permission from Hong *et al.*⁴⁰

Figure 7.

Species distribution curves of a DHI-melanin solution with the presence of Cu(II) ions as a function of pH. QI: quinone imine, Cat: catechol. The curves show that at neutral or mildly acidic conditions, Cu(II) favors binding to catechol and quinone imine groups. This figure is reprinted from *Journal of Inorganic Biochemistry*, 89, Szpiganicz *et al.*, Metal binding by melanins: Studies of colloidal dihydroxyindole-melanin, and its complexation by Cu(II) and Zn(II) ions, 45–53, (2002), with permission from Elsevier.⁵²

Figure 8. SEM images of melanin samples with different metal contents. A) Intact *Sepia*, B) EDTA washed, C) Fe-saturated, D) Cu-saturated, E) Zn-saturated, and F) Ca-saturated melanin granules. The data reveal that coordination of metals has a negligible effect on the morphology of the melanin granule. This figure is reprinted with permission from Liu *et al.*⁴¹

$$\text{DHB} / \mu\text{M}$$
$$\text{Iron / melanin ratio} / \text{mol/mol}$$
Figure 9.

The production of DHB (products of the hydroxylation of salicylate) as a function of Fe/melanin ratio. The data clearly reveal a threshold value for coordination of iron, above which the production of DHB increases with increasing iron concentration. The mol/mol indicated as the unit along the x-axis is mol of Fe per mol of monomer corresponding to the mass of the melanin. This figure is reprinted from *Biochimica et Biophysica Acta*, 1271, Zareba *et al.*, The effect of a synthetic neuromelanin on yield of free hydroxyl radicals generated in model systems, 345–348 (1995), with permission from Elsevier.³⁶

Scheme 1.

Molecular structures of possible monomers of eumelanin: a) DHI, b) DHICA, c) pyrrole and d) dopa.