Applications of Photoemission Electron Microscopy to Melanin and Melanosomes

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy in the Department of Chemistry
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ABSTRACT

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Abstract

Melanin is a biological pigment that is ubiquitous in nature and generally produced within melanosomes, specialized organelles. Typically, melanin is categorized into two distinct classes, based on color and molecular precursor: eumelanin (brown-black) and pheomelanin (yellow-red). Whereas much is known regarding the molecular precursors to the two pigments, an understanding of their resulting molecular structure remains elusive. Despite this lack of knowledge, several functions are attributed to the pigments, including photoprotection and photosensitization. Epidemiological data for skin and ocular cancers have observed an increased incidence for increased relative concentrations of pheomelanin. Furthermore, eumelanin is generally identified as photoprotective and antioxidant, whereas pheomelanin is generally identified as photoreactive and pro-oxidant. This thesis describes the photophysical properties of the naturally-occuring melanin pigments and presents new insights into their roles within the context of skin and ocular cancers.

Photoemission electron microscopy provides a unique opportunity to probe the complex photoproperties of melanins contained within intact melanosomes isolated from tissues of bovine and human eyes. Photoionization threshold potentials characteristic of eumelanin and pheomelanin have been determined and are used to investigate the molecular architecture of the

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pigments within the melanosome. Furthermore, a novel approach to

photoemission electron microscopy is used to obtain the first direct

measurements of the absorption coefficients from intact melanosomes.

Human iridal stroma melanosomes are comprised of both eumelanin and

pheomelanin in various ratios according to iris color; dark brown and blue-green

iris melanosomes are characterized by a eumelanin:pheomelanin ratio of 14.8

and 1.3, respectively. Despite the significant difference in the overall pigment

composition, a common eumelanin surface photoionization threshold is obtained

for both melanosomes. This data indicates that within the melanosome, the

phototoxic pheomelanin pigment is encased by eumelanin. This structure

mitigates the adverse photochemical properties of pheomelanin. However,

damage to the eumelanic exterior and or significant reduction in the amount of

eumelanin present could compromise the protective ability of eumelanin,

providing mechanisms for exposure of pheomelanin and consequently

contributing to oxidative stress.

The absorption spectra of intact melanosomes of varying melanin

compositions were determined over the spectral range from 244 to 310 nm. The

absorption spectra of eumelanic melanosomes are similar regardless of monomer

composition or embryonic origin. Furthermore, the absorption spectra of

melanosomes containing a mixture of pigments were similar to those containing

pure eumelanin, arguing that the absorption properties of the melanosome are maintained regardless of increased pheomelanin composition. Therefore, the correlation between epidemiological data and the eumelanin:pheomelanin ratio is not predicted to be a reflection of the melanosome's decreased ability to attenuate biologically relevant wavelengths, but instead is predicted to be a reflection of the different photoreactivities of the melanin pigments contained within.

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List of Abbreviations

4-AHP	4-amino-3-hydroxyphenylalanine
AFM	atomic force microscopy
BZ	6-(2- amino-2-carboxyethyl)-4-hydroxy-benzothiazole
CCD	charge-coupled device
DHBTCA	dihydro-1,4-benzothiazine-3-carboxylic acid
DHI	5,6-dihydroxyindole
DHICA	5,6-dihydroxyindole-2-carboxylic acid
EPR	electron paramagnetic resonance
FEL	free electron laser
IPE	iridal pigment epithelium
PBS	phosphate buffered saline
PBSA	Dulbecco's phosphate buffered saline without calcium and magnesium
PDCA	pyrrole-2,3-dicarboxylic acid
PEEM	photoemission electron microscopy
PTCA	pyrrole-2,3,5-tricarboxylic acid
ROS	reactive oxygen species
RPE	retinal pigment epithelium
SEM	scanning electron microscopy
TEM	transmission electron microscopy

TM	total melanin
UHV	ultra high vacuum
UV	ultraviolet

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possibly could.

1.   Description and Functional Significance of Melanin and Melanosomes[1]

1.1   Introduction to Melanin and Melanosomes

Melanin is a generic term used to describe the ubiquitous natural and synthetic phenolic-quinone pigments. The natural melanins are found in: the skin, hair, eye, brain and inner ears of man and other mammals; the feathers of birds; the ink sacs of cuttlefish; and in some fungi. The historical progression of the observation and subsequent study of melanin reaches back over several centuries. Initial experimental studies aimed to understand ethnic pigmentation were reported by Santorio Santorius in 1614, which attributed the color of black skin to the presence of bile (Santorius, 1614). In the early 18th century, studies by Alexis Littre failed to find bile in skin but instead reported the presence of an insoluble black pigment adhered to the reticular membrane (Littre, 1720). Melanin had been discovered, but it wasn't until the turn of 20th century that a more molecular picture of pigments and their formation was obtained.

In 1896, shortly after the discovery of tyrosinase in fungi (Bourquelot and Bertrand, 1895), Bertrand established that tyrosinase converted tyrosine into a black pigment that appeared similar to mammalian melanin (Bertrand, 1896). Experiments

later conducted on mammalian tissues, however, reported melanin formation through the enzymatic oxidation of dopa, rather than tyrosine, by an enzyme termed dopa-oxidase (Bloch and Schaaf, 1925), although, at that point in time, dopa had never been observed in mammalian skin and the enzyme had not been isolated. In 1927, Henry Raper isolated dopa as the primary product of the reaction of tyrosinase with tyrosine and through careful chemical studies subsequently established the primary chemical steps for the enzymatic oxidation of tyrosine to mammalian melanin (Raper, 1927). In doing so, Raper isolated derivatives of 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA), which are the two indoles now considered to be the fundamental building blocks of the black pigment eumelanin. Raper's molecular scheme was extended in 1948 by Howard Mason to introduce a "polymer" model of melanin, involving self-condensation of indole quinones (Mason, 1948).

During the 1960s, studies of various natural pigments resulted in the classification of melanin into two major types – the brown black eumelanin (eu – a Greek prefix for "good, well") and the yellow red phaeomelanin (phaeo – a Greek prefix meaning "dusky", now commonly written pheomelanin). An important chemical difference between these classes was revealed in 1966 when Guiseppe Prota, Mario Piattelli and Rodolfo Nicolaus proposed that pheomelanin resulted from the specific reaction of cysteine with the quinones produced by tyrosine oxidation, a reaction that does not occur in the synthesis of eumelanin (Prota et al., 1966). Between 1967 and 1968,

Prota, Nicolaus, and collaborators isolated melanin from red feathers, and established

the centrality of 5-*S*-cysteinyldopa as a precursor to pheomelanin (Minale et al., 1967;

Prota and Nicolaus, 1967a, 1967b; Nicolaus, 1968).

The current understanding of the initial steps of the melanogenesis of the

melanin pigments, commonly called the Raper-Mason scheme, is shown in **Figure 1** (Ito

and Wakamatsu, 2008). As indicated, the two major types of melanin reflect different

molecular precursors in the melanogenesis. Pigment formation is initiated by the

enzymatic oxidation of tyrosine to dopaquinone, which is subsequently the branch point

between the formation of eumelanin and pheomelanin. In the absence of cysteine,

dopaquinone undergoes an intramolecular cyclization, leading to the formation of

eumelanin through the DHI and DHICA precursors. However, in the presence of

cysteine, dopaquinone progress through a series of reactions to form a variety of

cysteinyldopa adducts, which eventually form pheomelanin through benzothiane units.

Though these two melanogeneses are chemically distinct, both are often present in the

same pigment-generating cell, the melanocyte (Prota et al., 1998; Ito and Wakamatsu,

2003; Wakamatsu et al., 2006; Wakamatsu et al., 2007). While pure eumelanin is found

throughout nature, pure pheomelanin is not observed and rarely makes up a significant

composition of the total melanin present.

Figure 1: Abbreviated depiction of the early steps of melanogenesis. Initiation of melanogenesis is the oxidation of tyrosine by tyrosinase. Dopaquinone is the branch point between the formation of eumelanin and pheomelanin.

In situ, melanocytes synthesize melanin within specialized organelles termed

melanosomes. Note, that like most cellular organelles, melanosomes are membrane-

bound structures that also contain lipids, enzymes, metals and proteins (Prota, 1992).

The melanocytes are categorized into two distinct types: cutaneous, found in the skin

and hair, and extracutaneous, found in the eye and inner ear, among others (Boissy and

Hornyak, 2006). The ocular melanocytes are of significance for pigment cell research

because the different layers of pigmented tissues originate from two different embryonic

origins: the uveal melanocytes – found in the iris and choroid – are derived from the

neural crest, similar in origin to the cutaneous melanocytes, whereas the retinal pigment

epithelium (RPE) and iridal pigment epithelium (IPE) cells are derived from the outer

neuroectodermal sheath of the eyecup (Schraermeyer and Heimann, 1999; Bumsted and

Barnstable, 2000; Boissy and Hornyak, 2006).

1.2   The Structure of Melanin

The complexities of melanins have so far hindered a detailed understanding of

their molecular structures (Prota, 1992; Ito and Wakamatsu, 2006). First, the high

molecular weight pigments are virtually insoluble in most all solvents, resulting in

difficulties of isolation of the melanins in pure form and utilization of common

characterization techniques. Second, the pigments are amorphous and particulate in

nature. And thirdly, these pigments are heterogeneous and are made up of mixtures of

more or less similar polymers comprised of different monomer units linked through

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nonhydrolyzable bonds. To circumvent these obstacles and probe the molecular

compositions of the pigment, powerful and informative approaches were developed that

were based on the oxidative and reductive degradation of the pigments, followed by

identification and quantification of the molecular markers produced (Panizzi and

Nicolaus, 1952; Fattorusso et al., 1968; Benathan and Wyler, 1980; Prota, 1998; Ito and

Wakamatsu, 2006). These markers were further quantitatively related to the presence of

specific chemical moieties present in the original pigment.

Shosuke Ito refined many of these analytical approaches (Ito et al., 2000;

Wakamatsu and Ito, 2002), and his methods are currently employed to derive molecular

information about melanins from different tissues. The pyrrole units, pyrrole-2-3-5-

tricarboxylic acid (PTCA) and pyrrole-2,3-dicarboxylic acid (PDCA), derived from the

oxidation products of eumelanin, are routinely used to infer the relative contributions of

the specific molecular monomers DHICA and DHI, respectively, to the eumelanin

pigment (Piattelli et al., 1962; Benathan and Wyler, 1980; Ito and Fujita, 1985;

Wakamatsu and Ito, 2002). Additionally, the 4-amino-3-hydroxyphenylalanine (4-AHP)

degradation product derived from the reductive hydrolysis of pheomelanin with

hydriodic acid is routinely used to infer the relative contributions of pheomelanin (Ito

and Jimbow, 1983; Wakamatsu and Ito, 2002). In short, these approaches quantify

structural information regarding a) the relative amounts of eumelanin and pheomelanin

present in a sample of pigment and b) the relative amounts of DHI and DHICA present

in eumelanin. Although these methods fail to provide information on the chemical connectivity between the molecular monomers, the ability to determine the relative contributions of the molecular monomers to the overall architecture of the pigment plays an important role in developing structure/function models for melanin.

1.3 Physicochemical Properties and Biological Functions of Melanin

The unique physicochemical properties of melanins include: broad-band absorption through the UV and visible, photoreactivity, anti-oxidant and free-radical scavenging, redox reactivity, and binding of metal ions and organic cations (HIll, 1992; Sarna and Swartz, 2006). These properties, a result of the pigment structures, define the biological functions of melanins. For purposes of this thesis, descriptions of the main properties of eumelanin and pheomelanin related to the specific biological roles of photoprotection and photosensitization are presented.

1.3.1 Melanin as a Photoprotector

The optical absorption spectra observed for natural and synthetic melanins is featureless and increases nearly monotonically with decreasing wavelength through the UV (Crippa et al., 1978; Sarna and Sealy, 1984b; Nofsinger et al., 2002b; Sarna and Swartz, 2006). These spectral properties establish the critical photoprotective biological function of melanin as its broad-band absorption in the skin and eye mitigates the exposure of underlying tissues to harmful radiation (Kollias et al., 1991). However, this characteristic feature is complex and despite significant efforts to understand the

unusual optical properties, melanin absorption has defied explanation (Meredith and Sarna, 2006; Sarna and Swartz, 2006; Pezzella et al., 2009a).

Due to the insolubility and particulate nature of the melanins, the absorption spectrum is likely a result of a complex combination of absorption and scattering at different wavelengths and further depends on the aggregation status of melanin in solution (Nofsinger et al., 2002b; Sarna and Swartz, 2006). As a result, and because of the difficulties of isolating natural melanin in pure form, studies of the optical absorption properties of melanins are often carried out on soluble synthetic model systems (Ito, 1989; Sarna and Swartz, 2006; Riesz et al., 2007; Pezzella et al., 2009a). Optical absorption reported for soluble synthetic eumelanin and pheomelanin were found to be similar and well described by the characteristic monotonic broadband absorption (Sarna and Swartz, 2006). It is important to note, however, that while synthetic samples are widely studied, the absorption spectra of melanins depend on several factors, including the synthetic method used and the oxidation/reduction state of various sites within the resulting polymer (Sarna, 1992).

The ability of melanins to scavenge reactive oxygen species (ROS) and mitigate their damaging effects on cellular processes is yet another photoprotective property attributed to the pigments (Bustamante et al., 1993; Meredith and Sarna, 2006; Sarna and Swartz, 2006). These antioxidant properties have been examined through studies measuring the rate constants of the interaction of different melanins with singlet oxygen

and other free radicals with various EPR techniques (Sarna et al., 1985; Korytowski et al., 1986) and pulse radiolysis methods (Sarna et al., 1986; Rózanowska et al., 1999). The apparent rate constants obtained from the pulse radiolysis methods revealed that although the oxidation of synthetic eumelanin by oxidizing radicals was easier than that of synthetic pheomelanin, both synthetic melanins were efficient scavengers of strongly oxidizing free radicals. Furthermore, recent studies on irradiated natural human RPE cells have suggested that the melanin contained within provided protection against light-induced apoptosis through the quenching of photogenerated ROS (Seagle et al., 2005).

1.3.2 Melanin as a Photosensitizer

Despite the photoprotective properties of melanins established above, the pigments can also act as photosensitizers and generate harmful ROS upon aerobic photoexcitation (Chedekel et al., 1978; Felix et al., 1978; Chedekel et al., 1980; Sarna and Sealy, 1984b; Korytowski et al., 1987; Hubbard-Smith et al., 1992; Sarna and Swartz, 2006). Action spectra for photoinduced oxygen consumption were reported for a variety of synthetic and natural melanins and while found to resemble each other, they generally differed significantly from their absorption spectra (Sarna et al., 1984; Sarna and Sealy, 1984a). An explanation was put forth for this disparity in the case of eumelanin through comparisons of the optical properties of different particle-sized fractions with their free-radical generation (Nofsinger et al., 1999; Nofsinger et al.,

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2002b). The action spectra were found to match the absorption spectrum of the

oligomeric constituents of the natural eumelanins. Furthermore, subsequent studies

established that aggregation of these constituents affected their aerobic reactivity

(Nofsinger et al., 2002a); the unaggregated oligomeric constituents were found to have a

higher efficiency towards ROS photogeneration than the aggregated oligomers. In the

case of pheomelanin, it was demonstrated that different molecular constituents are

responsible for the emission, transient absorption, and oxygen photoconsumption

exhibited by the pigment (Ye et al., 2008). These later studies also observed increased

aerobic reactivity of pheomelanin in the UV-A.

The UV photosensitization of pheomelanin has been hypothesized to be linked to

an increased susceptibility of skin and ocular cancers for individuals of lighter-colored

skin, hair and eyes (Chedekel et al., 1978; Wenczl et al., 1998b; Davies et al., 2002;

Wakamatsu et al., 2007; Hu et al., 2008). In exploring this hypothesis, Prota and co-

workers have reported that UV sensitivity is associated with high pheomelanin and low

eumelanin levels (Vincensi et al., 1998). In contrast, it was reported that factors other

than the amount of pheomelanin may be important in determining cancer susceptibility

based on an examination of the eumelanin and pheomelanin concentrations in human

skin before and after exposure to UV radiation (Hennessy et al., 2005). Douglas Brash

and co-workers examined the induction of DNA lesions and apoptosis upon UV

exposure of congenic mice with black, yellow, and albino coats (Takeuchi et al., 2004). In

their experiments, UV-B-induced cyclobutane dimerization and apoptosis measured by

sunburn cells or keratinocytes containing active caspase-3 were found to be strain

independent. However, the UV irradiation of melanin was found to photosensitize

adjacent cells to caspase-3 independent apoptosis at a frequency greater than the

apoptosis induced through UV absorption by DNA. In particular, pheomelanin

exhibited a 3-fold greater activity than eumelanin, supporting the hypothesis that

melanin-induced apoptosis may contribute to the observed epidemiology of skin cancer

for black-, blond-, and red-haired populations. A suggestion in support of a causative

role of pheomelanin is also provided by experiments on UV-A-induced DNA single-

strand breaks in human melanocytes differing only in the type of pigment produced;

such reactivity occurs in cells expressing pheomelanin, arguing that the origin is an

intrinsic chromophore, most likely pheomelanin itself and/or a molecule produced along

the pigment's synthetic route (Wenczl et al., 1998b).

Initially reported studies on the photoreactivity of synthetic pheomelanin found

that the activation of molecular oxygen resulted from pigment photoionization by UV-A

radiation (Chedekel et al., 1978; Chedekel et al., 1980). Building on this mechanistic

work, photoemission electron microscopy (PEEM) was used to quantify the

photoionization threshold for natural eumelanin and pheomelanin using melanosomes

isolated from human black- and red-hair respectively (Samokhvalov et al., 2005; Ye et

al., 2006). The PEEM data revealed a common ionization threshold at 282 nm

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characteristic for both melanosomes, reflecting the presence of eumelanin in both

samples. Pheomelanosomes (melanosomes exhibiting a large concentration of

pheomelanin), however, exhibited a unique second ionization threshold at 326 nm,

which was attributed to the benzothiazine structural motif present in pheomelanin. The

fact that pheomelanin has a lower ionization potential than eumelanin is supportive of

the view that pheomelanin could be an active contributor to the greater incidence rate of

UV-induced cancers observed for red-haired vs. black-haired individuals. Compelling

evidence in support of this assignment was determined by probing the threshold

ionization potentials for synthetic pheomelanin by time-resolved spectroscopic detection

of solvated electrons and EPR measurements of the photoconsumption of oxygen

following excitation of dissolved synthetic pheomelanin in water (Ye et al., 2006). All

studies were consistent with the photoionization threshold determined for the

pheomelanin contained in human red-hair, and further established the relevance of the

threshold potential data collected under the high vacuum conditions of PEEM to those

exhibited by pigments in physiologically relevant buffer solutions.

1.3.3 The Current State of Melanin

As described, melanin's photoprotective properties are interrelated to their

potentially deleterious side effects of photoinduced oxidative stress and a delicate

balance may exist between the two. Based on the increased photoreactivity of synthetic

pheomelanin to eumelanin and the observed epidemiological correlation between skin

and ocular cancers and pheomelanin concentrations, the photoreactivity of the two pigments are generally thought to reflect an antioxidant, protective eumelanin contrasted to a reactive and pro-oxidant pheomelanin (Chedekel et al., 1978; Harsanyi et al., 1980; Persad et al., 1983; Takeuchi et al., 2004; Ye et al., 2006). Further studies of the interaction of UV and visible light of melanins contained within intact natural melanosomes are needed to fully understand the dichotomy of the attributed biological functions in cutaneous and extracutaneous tissues.

1.4 *Overview of This Dissertation*

In this dissertation, the structural and photophysical properties of natural, intact melanosomes were probed with PEEM, a unique, surface sensitive, imaging microscopy, to examine the potential role they may play in the epidemiology of skin and uveal cancers.

As described, intact melanosomes are not amenable to quantitative assessment through conventional techniques and thus, established and novel applications of PEEM have been utilized to assess their complex properties. **Chapter 2** presents the historical perspective of biologically focused PEEM and provides specific details of the instrument (Peles and Simon, 2009). Experimental methodologies are described for the determination of threshold potentials and absorption coefficients. The PEEM assessment of these two properties is of biological importance and provides key insight into the photoproperties of melanins within the melanosome.

Chapter 3 presents a structural analysis of mixed-melanic uveal melanosomes isolated from the iridal stroma of different colored human irides (Peles et al., 2009). The different colored iridal stroma melanosomes vary in their eumelanin:pheomelanin ratios and provide an excellent system to study the structural morphology of mixed melanogenesis. Atomic force microscopy (AFM) and scanning electron microscopy (SEM) were used to examine the size and overall morphology of these melanosomes. PEEM was used to measure their photoionization potentials to examine the structural morphology of the two pigments contained within. The structural observations provided by the aforementioned microscopies contribute to the understanding of the potential contribution iridal stroma melanosomes may have in inducing oxidative stress and accounting for the observed iris-color-dependent epidemiology of uveal melanoma.

The optical UV absorption coefficients were quantified for intact, natural melanosomes to further measure the photophysical properties of melanin. Preliminary measurements were carried out on melanosomes isolated from different ocular tissues of bovine eyes (Peles et al., 2010; Peles and Simon, 2010a). These melanosomes are eumelanic, but vary in their relative DHICA/DHI content and their embryonic origin, thus providing a model system to probe the eumelanic absorption as a function of monomer composition and embryonic origin (**Chapter 4**). After the optical properties of eumelanin had been established, the absorption coefficients were measured for the dark brown and blue-green human iridal stroma melanosomes, described in **Chapter 3**, to

determine the absorption coefficient of pheomelanin (**Chapter 5**) (Peles and Simon, 2010b). These data provide the first directly quantified absorption coefficients from single, intact melanosomes.

2.   Applications of Photoemission Electron Microscopy to Pigment Research[1]

2.1   Introduction to PEEM

PEEM is a unique, surface-sensitive technique capable of providing real time

images of the surface of a sample under high spatial resolution. The physical principle

underlying this technique is the photoelectric effect. Simply, electrons are emitted from

the surface of the sample when the associated photon energy of the incident light is

above the photoionization threshold value characteristic of the sample of interest. The

generated photoelectrons are accelerated through a series of electron optics and the

surface of the sample is imaged at a high magnification. The first PEEM images were

published in 1933 (Brüche, 1933). Following these preliminary images tremendous

development in the understanding and instrumentation of PEEM has been achieved

(Griffith and Rempfer, 1988; Bauer, 1994).

Although, PEEM is currently used extensively in the field of materials science, O.

Hayes Griffith, a pioneer of PEEM technology, initially applied the technique to

biological samples and reported first PEEM images of rat epididymis (Griffith et al.,

1972). Griffith and his group realized the capabilities of PEEM as the electron-optics

analog of fluorescence microscopy and used colloidal gold and silver enhanced colloidal

gold particles to selectively label portions of a biologic with a higher spatial resolution (Birrell et al., 1985; Griffith and Birrell, 1985; Griffith and Rempfer, 1988). PEEM images for cells (Dam et al., 1977; Nadakavukaren et al., 1981; Habliston et al., 1995), viruses (Houle and Griffith, 1983b; Birrell et al., 1994), and DNA (Houle and Griffith, 1983a; Griffith et al., 1990; Habliston et al., 1993; Birrell et al., 1994), among others (Grund et al., 1979; Birrell et al., 1983; Nadakavukaren and Griffith, 1985), were reported using both labeling and non-labeling techniques. In addition to the experimental evidence, reviews were written establishing PEEM as a powerful tool for biological imaging (Griffith and Rempfer, 1985; Griffith, 1986; Birrell et al., 1991).

Despite the capabilities demonstrated by Griffith and others, the technique did not flourish in the biological sciences and applications of PEEM to biological systems have lagged behind complementary electron imaging techniques, such as SEM and TEM. Instead, as an ultra high vacuum (UHV) instrument suited for relatively flat samples, PEEM found prominence in the fields of surface and materials sciences (Mundschau, 1991; Bauer, 1994). PEEM's applications in surface and materials sciences go beyond imaging. For instance, surface dynamics can be imaged during in situ experiments (e.g. heating, cooling, exposure to reactive gases, etc.) by monitoring the changes in the electronic properties of the surface (Bethge and Klaua, 1983; Mundschau et al., 1990; Mundschau, 1991; Rotermund, 1993; Ehsasi, 1994; Kordesch, 1995; Zeman et al., 2006). If the resulting work function of the modified surface is below (above) the

photon energy of the incident light an image will (will not) be observed. Subsequently,

and more importantly for the purposes of this thesis, tunable monochromatic light

sources have made it possible to scan a series of photon energies to obtain the work

function or selectively turn on or off a particular region of the sample (Ade et al., 1998;

Li et al., 2005; Nakano et al., 2008). Thus, PEEM has become a critical tool in surface and

materials sciences that is capable of achieving valuable and unique information.

Motivated by the challenges of melanin research, a result of the complex nature

of the pigments, the Simon group, in collaboration with Robert Nemanich's group,

extended the application of biological PEEM to mirror its use in surface and materials

sciences. As a result, photoionization thresholds (work functions) were determined for

different types of intact natural melanosomes (Samokhvalov et al., 2004; Samokhvalov et

al., 2005; Bush et al., 2006; Hong et al., 2006; Ye et al., 2006; Garguilo et al., 2007). The

photoionization thresholds determined for eumelanin and pheomelanin are of biological

importance for understanding the potentially phototoxic role of pheomelanin as

compared to eumelanin. This technique (described herein) and these results have

provided a foundation for the research described in **Chapter 3** on the examination of the

morphology of mixed melanins in intact melanosomes. Furthermore, in continuing to

expand applications of PEEM to probe critical photophysical properties of melanosomes,

the changes in the electronic properties of the substrate were analyzed at different UV

wavelengths to measure optical absorption spectra in a similar fashion to the

measurement of the surface dynamics for surface and materials sciences. This novel

technique, which has enabled the direct quantification of the absorption coefficients of

single, intact melanosomes of variable pigment composition, as described in **Chapters 4**

and **5**, is also developed herein.

2.2 The PEEM Instrument in Detail

A schematic of the PEEM is shown in **Figure 2**. The instrument consists of a

main chamber, a lens column and a detection center, all of which are held under UHV.

Emission of photoelectrons occurs when light of an appropriate wavelength is

illuminated on the surface of a sample. A variety of photon sources are available to

excite the electrons (X-ray, synchrotron radiation, or UV arc lamps and lasers). UV

excitation is generally required to photoionize the valence electrons of biological

materials, and is the spectral range utilized for the pigment research described

throughout this thesis. As a consequence of the instrumental design the sample must be

illuminated with a photon beam at a high incident angle, ~ 77º from the surface normal.

This is necessary because the maximal resolution of the instrument is achieved only

when there is a short working distance between the anode and the sample (~ 4mm),

shown in **Figure 2**.

Typically, the sample is held at a negative potential (-20kV) and an electric field

is created between it and a grounded anode. This potential accelerates the generated

Figure 2: A schematic of the PEEM instrument. The instrument consists of the main chamber, lens column and detection center. The anode and sample cartridge are depicted in the main chamber. Reprinted from: A Free Electron Laser – Photoemission Electron Microscope System (FEL-PEEM) by H. Ade, W. Yang, S. L. English, J. Hartman, R. F. Davis, R. J. Nemanich, V. N. Litvinenko, I. V. Pinayev, Y. Wu and J. M. J. Madey in Surface Review and Letters, Copyright @ 1998 World Scientific.

photoelectrons through a series of electron optics. After passing through the lens column, the emitted electrons are amplified by a microchannel plate and subsequently imaged onto a phosphor screen. A CCD camera captures the data from the screen. The electron optics project the image plane of the sample onto the microchannel plate, thus enabling the direct collection of a spatially-resolved image.

For a geometrically flat sample, the current lateral instrumental resolution is about 10 nm (Xiong et al., 2006). However, biological samples are characterized with significant morphology and subsequently reduce the instrumental resolution through sample dependent accelerating field aberrations. Thus, the spatial resolution of the PEEM used herein for melanosomes is on the order of tens of nanometers, a resolution far exceeded by other imaging microscopies.

In addition to the lateral resolution, it is necessary to also briefly address the depth of information of PEEM. The depth of information is the distance between the surface and a point at which information from the sample contributes to the image at a specified resolution (Houle et al., 1982). To generate photoelectrons, light that penetrates the sample is absorbed, induces photoionization, and the photoionized electrons are transported to the surface where they then escape from the sample to the vacuum (Habliston et al., 1995). The depth-dependent yield of photogenerated electrons is not known. Estimates for organic materials suggest that electrons are generated within the first 10 nm (Houle et al., 1982). However, it is reasonable to assert that the

yield will decrease with depth from the surface and so while light may be absorbed deep

in the sample, the volume relatively near the surface will comprise the dominant

contribution to the signal (Griffith and Rempfer, 1988). Furthermore, as melanosomes

are effective electron scavengers it is likely that the electrons generated below the

surface are efficiently trapped.

2.2.1 Spatial Dependence of the Signal

As previously described, the light source must illuminate the sample at a high

incident angle with respect to the surface normal. Consequently, only a portion of the

sample surface is directly illuminated because the morphology of the spheroidal

melanosomes; the curved side of the specimen facing the incident beam will have a

significantly higher photoelectron flux than the opposite side. Given the geometrical

considerations, spherical samples will result in an asymmetric photoemission image.

The long axis of the sampled image is defined as the axis perpendicular to the incident

light. This is the axis that is not influenced by the asymmetry that results from the

geometrical constraints of the PEEM and the incident light source (Nepijko et al., 2001).

The three-dimensional topography of biological specimens will also result in

photoelectron emission at various angles. Thus, "shapes" revealed in the PEEM image

may reflect the interplay between the illumination angle and the surface geometry, not

simply the shape of the biomolecule. Emitted electrons possess kinetic energy and the

large accelerating potential is used to collect these electrons with a minimum loss of

lateral resolution (Griffith and Engle, 1991). However, spreading or bunching of the

emitted electrons results from topographical electric field variations by complex three-

dimensional surfaces (Rempfer et al., 1980). As a result, the image may have darker or

brighter regions according to the topographic features of the sample. An aperture

located in the lens column is used to prevent deflected electrons from reaching the

detection area. Deflection of the electrons typically occurs from the sides of a protrusion

whereas the electrons arising from surrounding flat regions and the top regions of

protrusions pass through the electron-optics system undeflected. Thus, the top regions

of protrusions and surrounding flat areas appear brighter while the sides of protrusions

are darker.

A two-dimensional image detection (the CCD serves as a two-dimensional image

plane, with each pixel having the same area) creates a non-linear distance scale for the

image of the melanosome. Consider the projection image of a sphere, **Figure 3a**. The

region of a curved three-dimensional surface imaged onto an individual pixel increases

with the angle from the surface normal. Therefore, a curved melanosome will further

affect the resolution achieved by the instrument. With increasing angle from the surface

normal, the photo-ejected electrons initially have increased velocity projections in the

plane of the substrate, and fan out with different degrees of curvature prior to being

accelerated towards the lens column. As a result, if the accelerating potential is reduced,

the size of the image will increase. **Figure 3b** displays the length of the long axis of the

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Figure 3: Spatially dependent signal considerations for spherical objects. (a) A three-dimensional sphere projected onto a two-dimensional surface. (b) A plot showing the dependence of the image diameter of a ~150 nm *Sepia* eumelanin granule on the accelerating potential of the instrument.

image of a ~150 nm spherical *Sepia officinalis* eumelanin melanosome (recall that

spherical structures appear as ellipsoids) with respect to the accelerating voltage. A

linear dependence is observed for the acceleration potential examined. Nepijko has

developed a theoretical analysis of such an experiment and provides functional

relationships for determining the actual size of the three-dimensional spherical particle

from such data (Nepijko et al., 2001).

2.3 *Determination of Threshold Potentials*[2]

To acquire the photoionization threshold of an intact melanosome, UV

wavelengths are scanned and the integrated brightness of the image is analyzed as a

function of the excitation energy (Samokhvalov et al., 2004; Samokhvalov et al., 2005;

Bush et al., 2006; Hong et al., 2006; Ye et al., 2006; Garguilo et al., 2007).

2.3.1 Substrate Preparation

Substrates were prepared from ⟨100⟩-oriented silicon (N-type, P-doped)

purchased from VA Semiconductor, Inc. (Fredericksburg, VA). Sample preparation was

initiated by cleaving wafers to square regions of approximately 1 cm². These wafers

were cleaned using the standard RCA wet chemical procedure without the hydrofluoric

acid step (Senturia, 2001). Isolated melanosomes were suspended in a Nanopure water

solution and deposited onto the cleaned silicon wafers in 0.5 μL aliquots. The samples

were allowed to air-dry in a sterile Petri dish before experimentation with care taken to avoid sample exposure to UV light. After drying (< 30 min), the samples were transferred under UHV into the PEEM chamber for data collection. To ensure a large sampling, the instrumental field of view was set at 50 μm. All images were captured with a DVC-1312 M digital camera from DVC Company, Inc. (Austin, TX). The digital camera resolution was 1300 × 1030 pixels × 12 bits. Acquisition times were 9 sec.

2.3.2 Excitation Source

The spontaneous emission mode of the Duke UV-free electron laser (FEL) was used to acquire thresholds. Details of the Duke FEL have been described elsewhere (Ade et al., 1998). The FEL was tuned through the spectral range from 240 to 310 nm (5.17 – 4.00 eV) in 5 nm increments.

2.3.3 Measurement Procedure

The measured brightness of the melanosome within the photoelectron image was taken to be proportional to the photocurrent as the emission current cannot be accessed in a standard PEEM. An image was obtained for each wavelength and the melanosome's integrated brightness, S, was measured by a summation of all of the pixel gray scale depths in the selected region. The minimum and maximum gray scale values are regarded as black or white saturated, respectively. Images with a large fraction of the pixel counts located at these values result in a loss of the information regarding the brightness of a melanosome and the photoelectron current is no longer proportional to

the integrated brightness. To ensure the intensity histogram of the image spanned a

distribution across the pixel gray scale depths and avoided black and white saturation,

the voltage on the multichannel plate and the software gain of the imaging program was

adjusted.

Prior to analysis of the threshold curves, standard data corrections were needed

for the obtained measurements. The integrated brightness was corrected for the

software gain adjustment through the equation:

$$gain(db) = 20 \log_{10} G. \tag{1}$$

To calibrate for the multichannel plate voltage changes on S, PEEM images were

collected at fixed wavelength and software gains, but with different voltages. The

correction factor was provided by the ratio of S between the two images. Also, because

of absorption and the dependence of the incident FEL power on the current in the FEL

storage ring, the photon flux of the incident wavelength changed as one scanned

through the UV region. This was measured and compensated for with each experiment.

To obtain the photoionization potential of the sample, threshold curves were

functionally fit with early theoretical models proposed to explain photoelectric curves.

In 1931, Fowler described the photoelectric curve of solid metallic surfaces with an

expression that was a function of temperature and the frequency of the incident light

(Fowler, 1931). The integrated brightness of the sample, S, proportional to the

photocurrent, I, is in proportion to the product of the square of the temperature of the sample, T, and a function $f(u)$ given by:

$$S \propto I(\chi_0 - hv)^{1/2} \propto T^2 f\left(\frac{hv - \chi}{k_B T}\right), \text{ where} \qquad (2)$$

$$f(u) = e^u - \frac{e^{2u}}{2^2} + \frac{e^{3u}}{3^2} - \ldots, \qquad (u \leq 0)$$

$$= \frac{\pi^2}{6} + \frac{u^2}{2} - \left[e^{-u} - \frac{e^{-2u}}{2^2} + \frac{e^{-3u}}{3^2} - \ldots\right], \quad (u \geq 0).$$

In the above expressions, χ_0 is the threshold potential and χ is the thermionic work function ($\chi = \chi_0 - \varepsilon^*$, where ε^* is the energy of the highest occupied molecular orbital). Thus, the surface photoionization threshold potentials of melanosomes (discussed in **Chapter 3**) were determined after functionally fitting the threshold curves of S/T^2 vs. $hv/k_B T$. This technique is the same used previously to determine the photoionization threshold potentials of eumelanin and pheomelanin (Samokhvalov et al., 2004; Samokhvalov et al., 2005; Bush et al., 2006; Hong et al., 2006; Ye et al., 2006; Garguilo et al., 2007) and while these photoionization threshold potentials are obtained in isolation and under UHV with PEEM, remarkable consistency has been observed with respect to how the melanosomes behave under physiologically relevant conditions (Ye et al., 2006).

2.4   Determination of Absorption Coefficients[3]

The procedure for using the PEEM to directly and quantitatively determine

absorption coefficients for single intact melanosomes is established herein.

2.4.1 Substrate Preparation

Two different substrates were utilized in the absorption coefficient calculation.

Titanium substrates were used in a single wavelength (λ = 244 nm) analysis. These

substrates were prepared from wafers of $\langle 100 \rangle$-oriented silicon (P-type, B-doped),

purchased from VA Semiconductor, Inc. (Fredericksburg, VA). These wafers were

cleaved to square regions of approximately 1 cm^2 and then cleaned using a simple

solvent procedure. Specifically, wafers were added, in subsequent steps, to a

trichloroethylene bath, an acetone bath, and a methanol bath for 5 min each. The wafers

were then placed on lens paper in a sterile Petri dish and dried with a steady flow of

nitrogen gas. Afterward, the cleaned wafers were coated with a thin Ti film (2.5kA˚)

using the Kurt J. Lesker PVD 75 E-beam and Thermal Metal Evaporator (Pittsburgh,

PA).

Substrates prepared from a wafer of n-type 4H-SiC, purchased from Cree, Inc.

(Durham, NC) were used to determine wavelength dependent absorption coefficients

and are the predominate substrate used in the studies reported in **Chapters 4** and **5**. The

wafer was chemomechanically polished by NOVASIC (Le Bourget-du-Lac, France) and

cleaved to square regions of 1 cm² by American Precision Dicing, Inc. (San Jose, CA).

Individual substrates were then subjected to the same simple solvent cleaning process

described for the Ti wafers. Similar to the photoionization threshold sample

preparation, isolated melanosomes were suspended in a Nanopure water solution and

deposited on the substrates in 0.5µL aliquots immediately prior to experimentation. The

samples were air-dried (< 30 min) with minimal exposure to UV light and subsequently

transferred under UHV into the PEEM chamber for data collection. To ensure a large

sampling, melanosomes were imaged at fields of view of 150 µm.

2.4.2 Excitation Source

An intracavity doubled Ar⁺ ion continuous wave laser was used to illuminate the

sample at λ = 244 nm for preliminary investigations on Ti and later for purposes of

finding and focusing the sample prior to wavelength scans on SiC with the tunable laser

source. Laser excitation utilized for the wavelength dependent absorption experiments

was provided by a solid-state tunable nanosecond laser system from Opotek, Inc.

(Carlsbad, CA). Data was collected at the following wavelengths: 244 nm (5.08 eV), 257

nm (4.82 eV), 270 nm (4.59 eV), 280 nm (4.43 eV), 290 nm (4.28 eV), 300 nm (4.13 eV) and

310 nm (4.00 eV). The polarization of the light was parallel to the plane of incidence. To

avoid noted space-charge effects (Buckanie et al., 2009) and physical disruption of the

melanosomes (Glickman et al., 1996) resulting from pulsed laser sources, a neutral

density filter was inserted in the beam path to reduce the average laser power. The

maximum pulse energy, after the neutral density filter, was at most 12 mJ/cm^2 for λ =

244 nm and decreased at longer wavelengths. The maximum pulse energy used herein is

< 10% of the previously calculated photodisruption threshold of 153.6 mJ/cm^2 for

melanosomes (Glickman et al., 1996). At each wavelength, laser power and optical

alignment were optimized prior to acquisition of the digital image. All images were

captured with a cooled DVC-1500 M digital camera from DVC Company, Inc. (Austin,

TX) with a resolution of 1392 x 1040 pixels x 12 bits. As a result of varied laser power

across wavelengths, the acquisition times were 5, 7, 14, 28, 42, 50 and 50 min for λ = 244,

257, 270, 280, 290, 300 and 310 nm, respectively.

2.4.3 Measurement Procedure

For reasons provided below, the Ti and SiC substrates were purposely utilized as

their work functions are lower in energy than that of the associated incident light at the

single wavelength λ = 244 nm and through the UV wavelengths examined (5.07 eV at

244 nm and 4.00 eV at 310 nm). As a result, at shorter wavelengths, both the substrate

surface and the melanosomes emit electrons and are imaged by the microscope. The

melanosomes have a higher efficiency for ionization (electrons per area) than the

substrate and are subsequently easily distinguished in the image.

The approach for determining the absorption coefficients is shown schematically

in **Figure 4**. As previously described, laser light was focused on the sample at a high

Side View   **Top View**

Surface Normal

Melanosome

hν

θ

Shadow

SiC

Melanosome

hν

Shadow

SiC

Figure 4: Experimental geometry for PEEM of single melanosomes. Laser excitation hits the melanosome at an angle of incidence of approximately 77º with respect to the surface normal. Light is attenuated and a shadow is generated on the far side of the melanosome.

angle of incidence (~ 77º with respect to the surface normal). As light travels through the melanosome, absorption attenuates its intensity, causing a reduction in the photoemission of the substrate immediately behind the melanosome, and a shadow is visualized as a result. However, as simulated in **Figure 5**, illumination of an ellipsoidal object with dimensions comparable to a melanosome produces significant scattering of the electric field. Consequently, the absorptive properties of the melanosomes, which are manifested by changes in the image intensity of the substrate, are determined from the region of the substrate immediately behind the melanosome that is void of significant scattering contributions.

Because the molecular structure of melanin is not known, pigment absorption coefficients are represented by the product εc, where ε is the extinction coefficient and c is the concentration. The Beer-Lambert Law defines εc through the following equation:

$$\varepsilon c = A\ell^{-1} = \ell^{-1}\log\left(I_0/I\right) \tag{3}$$

with units of inverse length. As shown, the calculation of εc requires knowledge of the pathlength, ℓ, and the incident and transmitted intensities of light, I_0 and I, respectively. The values of I_0 and I are derived from the PEEM image; an average of the photoemission current of the substrate, where there is no obstruction by absorbing melanosomes gives I_0, and an average of the photoemission current of the shadow region adjacent to the melanosomes gives I.

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Figure 5: A top view (x-y dimension) simulation of the interaction of electromagnetic radiation with an ellipsoid. The interaction was simulated with λ = 244 nm light and the model ellipsoid was characterized with a refractive index of 3.4. The dimensions of the ellipsoid are comparable to the melanosomes studied in this thesis (260 nm x 560 nm). Simulations of the electrogmagnetic field were performed with the finite-difference time-domain method (Taflove and Hagness, 2000) using a freely available software package (Oskooi et al., 2010). The colors reflect the peaks and valleys of the electromagnetic radiation.

2.4.3.1 Disaggregating Absorption and Scattering

To disaggregate the effects of absorption and scattering within the shadow

region, the portion of the shadow region that is void of significant scattering

contribution was determined. Following the methodology outlined by Chelaru et al.

(Chelaru et al., 2006), diffraction will influence the intensity in the shadow region

beginning at a distance from the melanosome corresponding to the first maximum in the

diffraction pattern of regular Fraunhofer diffraction. Note, according to Babinet's

principle, the Fraunhofer diffraction pattern for an opaque object is the same as it is for

an aperture of the same shape except that the intensity distributions are inverted

(Guenther, 1990). Thus, calculating the dimensions of the first maximum diffraction

disk from an elliptical aperture is equivalent to the dimension of the first minimum

diffraction disk observed for opaque ellipsoidal melanosomes.

The intensity distribution for the illumination of an elliptical aperture with

coherent plane waves is described by

$$I \propto \left[\frac{J_1(x)}{x} \right]^2, \tag{4}$$

where $J_1(x)$ is a first-order Bessel function and $x = 2\pi a \varepsilon p / \lambda z$, $2a$ is the length of the

major ellipsoidal axis of the melanosome, ε is the ellipsoidal axial ratio (b/a), p is the

radius of the diffraction pattern produced on the plane of observation, z is the distance

between the aperture and the plane of observation and λ is the wavelength of the

incident light (Kathuria, 1985). The first maximum of the diffraction pattern

corresponds to the first zero of the function $J_1(x)$, occurring at $x = 3.83166$ (m_0). Thus,

the dimension of the first maximum observed on the plane of observation can be

expressed as

$$p = \frac{\lambda m_0 z}{2\pi a \varepsilon} \quad . \tag{5}$$

This expression then gives the distance in the shadow region from the melanosome that

is void of any significant contribution from scattering, and thereby defines the region

from which the absorption coefficient is calculated. Under the experimental geometry,

z is equivalent to $2b/\tan(\alpha)$ where $2b$ is the length of the minor axis of the ellipse (the

height of the melanosome from the surface plane) and $\alpha = 13°$, the angle between the

surface plane and the incident light. As expected this region is contained within the first

diffraction ring that is visible in the obtained images and it is this region through which

the average photoemission current is subsequently determined. Specifically, the upper

threshold values of the image were set to visually segment the image into shadow and

substrate features. Each region was reduced to an average value by averaging the

intensities of the included pixels. These values, taken with the calculated average

pathlength, are then used to determine the absorption coefficients.

2.4.3.2 Pathlength Calculation

In principle, the spatial dependence of the photoemission current of the shadow

maps directly onto the different pathlengths through the melanosome. In practice,

however, it is only possible to precisely know the dimensions of the melanosome to

within a few tens of nm, the resolution of the PEEM. This uncertainty covers the

majority of the range of the size distribution of the melanosomes as determined by SEM

in **Chapter 3** and in (Liu et al., 2005b), and so the following approach was developed to

determine ℓ from average SEM values for the melanosome dimensions.

SEM characterization of the ocular melanosomes examined in **Chapters 4** and **5**

reveal ellipsoidal morphologies, **Chapter 3** and (Liu et al., 2005b). As a result, points in

the shadow correspond to different chord lengths through the ellipsoid. To compute the

absorption coefficient, an average ℓ is calculated and used in **Equation 3** along with the

average photoemission intensity obtained from the shadow and the unobstructed

substrate regions. The average ℓ calculation is derived as follows. Consider an

ellipsoid described by:

$$\frac{x^2}{a^2} + \frac{y^2}{b^2} + \frac{z^2}{c^2} = 1 \ , \tag{6}$$

where a and b are the equatorial radii along the x- and y-axes, respectively (in the

plane of the substrate), and c is the polar radius along the z-axis (normal to the

substrate), **Figure 6**. Solving this equation for y produces two functions that

correspond to the two parabolas of a 2-D horizontal ellipse at a height, z, shown in

Figure 6. The distance, s, between points on each parabola as a function of x and z

can thus be calculated from

$$s(x,z) = 2[b^2 - (b^2 x^2 / a^2) - (b^2 z^2 / c^2)]^{1/2} . \tag{7}$$

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Figure 6: Geometrical model of an ellipsoid with dimensions a, b, and c. Parabolas are diagramed within the ellipsoid as the two halves of the dashed 2-D ellipses. An example of a single chord distance, s, is highlighted in black between the points (x, y_1, z) and (x, y_2, z). The equation for the highlighted curvature of a melanosome along the x-axis is shown.

To find the average distance (ℓ) of all of the different chord lengths, s, within the

collection of 2-D ellipses that make up the ellipsoid, integration, with appropriate

bounds, is required. Consider, the 2-D ellipse at height $z = 0$. Here, the range can be

limited to [0, a] in the x-axis (by symmetry, the range can be limited to a instead of

$2a$). However, as the height, z, changes for each 2-D ellipse, the range must be limited

to the radius of that particular slice along the x-axis. Thus, with the range $z \in [0, c]$, it

must be that $x \in [0, [a^2 - (a^2 z^2)/c^2]^{1/2}]$ to account for the curvature of the ellipsoid.

Using these bounds, the average pathlength of an ellipsoid is given by

$$\ell = \frac{\int_0^c \int_0^\tau s(x,z)\,dx\,dz}{\int_0^c \int_0^\tau dx\,dz},$$ (8)

where $\tau = [a^2 - (a^2 z^2)/c^2]^{1/2}$. It must be noted, however, that portions of the analyzed

shadow regions generally map onto pathlengths that extend beyond half of the volume

of the melanosome, but not the entire melanosome, due to the geometry of the

experiment. The polar distance, ξ, that extends below $z = 0$ and accounts for the

additional pathlengths associated with the shadow region, can be obtained directly from

the PEEM image. Once ξ is determined, the averaged experimental pathlength is

calculated by integrating Equation 4 with the integration bounds $z \in [\xi, c]$ and $x \in$

$[-\tau, \tau]$.

The above analysis is accurate for ellipsoids whose long axis is oriented

perpendicular to the direction of incident light. However, in a typical sample,

melanosomes are oriented at random angles with respect to the direction of incident

light. Unfortunately, many of the melanosomes examined are characterized with small

dimensions and aspect ratios and determination of the angle between the long axis of

the ellipsoid and the incident light is difficult to achieve with reasonable accuracy in the

PEEM images. In this limit, the average pathlength is calculated by assuming the

melanosomes are oriented perpendicular to the direction of incident light. This is a

reasonable approximation; as the aspect ratio decreases, the melanosomes approach a

spherical shape. In the case of the RPE eumelanosomes, however, the aspect ratio is

such that the orientation of the melanosomes can be determined from the PEEM image.

In this instance, it is necessary to take into account the angle between the incident light

and the long axis of the melanosome. The modeled melanosome, originally oriented

perpendicular to the incident radiation, is rotated in a counter-clockwise rotation around

the z-axis by θ radians through variable substitution

$$x = X\cos(\theta) + Y\sin(\theta) \quad \text{and} \tag{9}$$

$$y = Y\cos(\theta) - X\sin(\theta).$$

The chord lengths through the rotated melanosome are then described by

$$s(X,z,\theta) = 2\{[a^2b^2[-c^2X^2cos(\theta)^4 + cos(\theta)^2[a^2(c^2-z^2) - 2c^2X^2sin(\theta)^2] - \\ sin(\theta)^2[b^2(-c^2+z^2) + c^2X^2Sin(\theta)^2]]]/[c^2[a^2\cos(\theta)^2 + b^2\sin(\theta)^2]^2]\}^{1/2}. \tag{10}$$

Note that in this orientation, the major radius no longer lies along the x-axis, and as a

consequence, the upper limit of the integration bounds for the X-variable becomes

$\tau_2 = \{[(c^2 - z^2)[a^2 Cos(\theta)^2 + b^2 Sin(\theta)^2]]/c^2\}^{1/2}/\{[Cos(2\theta)]^2\}^{1/2}$. Thus, the average ℓ for

half of the volume of a rotated ellipsoidal melanosome is

$$\ell_{rotated} = \frac{\int_0^c \int_0^{\tau_2} s(X,z,\theta)\,dx\,dz}{\int_0^c \int_0^{\tau_2} dx\,dz} \ . \tag{11}$$

Once again, ξ can be obtained directly from the PEEM image, and then, the limits of the

integrals in **Equation 11** are adjusted accordingly.

3. Structural and Morphological Analysis of Human Iridal Stroma Melanosomes[1]

3.1 *Introduction*

The photoreactivities of melanins are generally described as photoprotective and antioxidant for eumelanin and photoreactive and pro-oxidant for pheomelanin (Chedekel et al., 1978; Harsanyi et al., 1980; Persad et al., 1983; Takeuchi et al., 2004; Ye et al., 2006). Furthermore, as the epidemiology of skin cancer and uveal melanoma indicates an observed increased incidence for increased relative concentrations of pheomelanin, the eumelanin:pheomelanin ratio within naturally occurring melanosomes is often discussed as a biologically significant marker (Vincensi et al., 1998; Wenczl et al., 1998b; Wakamatsu et al., 2007; Hu et al., 2008). The fact that pheomelanin has a lower ionization potential than eumelanin is also supportive of the view that pheomelanin could be an active contributor to the greater incidence rate of UV-induced cancers (Ye et al., 2006). Upon comparison of the threshold ionization energies assigned to eumelanin (282 nm) and pheomelanin (326 nm) with the solar irradiance at the Earth's surface for several different solar zenith angles, it is observed that one is exposed to wavelengths of

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light that are sufficient to ionize pheomelanin, but that the atmosphere nearly

completely blocks the light required to photoionize eumelanosomes (Ye et al., 2006).

For pheomelanin to be active in inducing such oxidative stress in natural

systems, however, it must be present on or near the surface of the melanosomes. If the

pheomelanin is present in the core of melanosome, then it is highly unlikely that

photoionization will lead to electrons that can escape the organelle. This becomes even

more improbable upon consideration of the excellent scavenging properties of

eumelanin. Thus, determination of the pigment composition at the surface of mixed

melanic melanosomes is of biological importance. Understanding whether or not the

morphology of mixed melanins is such that pheomelanin lies at the core may provide

insight into the balance of the dichotomy of the pigments and the possible roles

melanosomes may play in the molecular mechanisms of cutaneous and uveal

melanoma.

The melanogenesis of the two pigments is described by the Raper-Mason

scheme, **Figure 1**. Applying data from pulse radiolysis studies reported by Tad Land

and Patrick Riley (Land and Riley, 2000; Land et al., 2003), Ito and co-workers were able

to quantify kinetics for several steps of the Raper-Mason scheme (Ito, 2003; Ito and

Wakamatsu, 2008). The data indicate that one can think of the synthesis of melanins in

terms of a 3-step process. Initially cysteinyldopa formation will occur to the exclusion of

eumelanin synthesis, as long as the concentration of cysteine is greater than 0.13 μM.

Second, oxidation of cysteinyldopa to pheomelanin occurs for concentrations of

cysteinyldopa greater than 9 µM. Finally, eumelanin is generated – occurring after most

of the cysteinyldopa and cysteine levels are depleted. While there are no studies that

provide a real time view of melanin formation within the melanosome, electron

microscopy of melanosomes at differing degrees of melanization indicate that pigment

granules gradually grow to diameters of ~30 nm within the melanosome, and once they

reach this size, no free space can be detected (Liu and Simon, 2003; Simon et al., 2008).

Given this morphology, the 3-step process characteristic of samples containing both

pheomelanin and eumelanin would predict a structural motif where the ~30 nm granule

would have pheomelanin at their core and eumelanin at the surface. This type of

molecular structure has been previously proposed based on biochemical studies of

mixed melanogenesis and solubility properties of synthetic model systems (Agrup et al.,

1982; Ozeki et al., 1997a). However, despite the compelling evidence presented from the

in vitro studies, direct evidence supporting a "casing model" architecture for mixed

melanic melanosomes isolated from the skin or uvea has not been reported.

Because different photoionization thresholds characterize the two pigments,

PEEM imaging of the melanosome surface can uniquely determine whether or not both

pigments comprise its surface. Melanosomes isolated from the stroma of human irides

are an optimal system for such a study as different colored irides contain melanosomes

of varying eumelanin:pheomelanin ratios (Wakamatsu et al., 2007). This system

presents the opportunity to investigate the surface photoreactivity as the relative

pheomelanin concentration changes through a single type of human melanosome with

wavelength-dependent PEEM imaging.

3.2   Materials and Methods

3.2.1 Sample Isolation

Extraction of the iris was performed on donor eyes from two patients of different

colors and ages, dark brown, age 14, and blue-green, age 66. The posterior IPE was

isolated from the iris with a previously described method (Prota et al., 1998), and the

remaining IPE-scraped iris tissues were stored at -70 °C until further use. An enzymatic

extraction was used to isolate the iridal stroma melanosomes from the iris tissue using

the same procedure as that reported on melanosome isolation from bovine iris (Liu et al.,

2005b). Briefly, wet iris tissue was cut into small pieces and subjected to three steps of

enzyme digestions at 37 °C in PBS under argon, collagenase (68 U/mL, with 2 mM

$CaCl_2$) for ~16 h, pancreatin (2.5 mg/ mL) for 2 h, and trypsin (646 U/mL, with 2 mM

$CaCl_2$) for 2 h. After each digestion, the remaining material was washed with water

several times and then subsequently subjected to further enzyme digestions. Following

these treatments, the materials were suspended in 1% (wt/vol) Triton X-100 solution and

stirred for 1.5 h at room temperature under argon. The particles were then washed with

methanol/water 1:1 (vol/vol) once and then with water four times. Afterward, the

pellets were treated with collagenase (68 U/mL, with 2 mM $CaCl_2$) for 16 h at 37 °C

under argon. The resulting black to brown melanosomes were washed with water five times and kept at -70 °C until use.

3.2.2 Scanning Electron Microscopy

Melanosomes were suspended in a water suspension and dropped onto freshly cleaved mica slides in 2 µL aliquots. The slides were then mounted on stainless steel pegs with double stick tape and air-dried in the dark. The mounted samples were coated with Au/Pd under argon plasma for 2 min at 15 mA using a Hummer V sputter coater (Anatech, Springfield, VA). A Philips XL30 ESEM (FEI company, Hillsboro, OR) equipped with a backscattering secondary electron detector was used to examine the samples in high vacuum mode. Multiple SEM images were captured at magnifications of 10,000× for each of the different samples. Care was taken to ensure that each image collected covered a completely different surface area. The dimensions of the melanosomes captured in the SEM images were measured using Scandium Imaging Software (Olympus Soft Image Solutions, Lakewood, CO). For each sample, over 1500 granules were measured from 6 to 14 images taken at 10,000× magnification. The histograms of the lengths of the short and long axes of the measured melanosomes were plotted and fit to a single Gaussian function.

3.2.3 Free Electron Laser-Photoemission Electron Microscopy

The photoionization thresholds of the samples were analyzed with the same procedures as described in **Chapter 2**.

3.2.4 Atomic Force Microscopy

A water suspension of iridal stroma melanosomes was pipetted in 0.5 μL aliquots onto freshly cleaved mica and allowed to air-dry in the dark. The instrument and method of imaging has been previously described in detail (Clancy and Simon, 2001). Briefly, AFM height and phase images were collected with a NanoScope IIIa BioScope AFM (Digital Instruments, Santa Barbara, CA) operated in tapping mode. The AFM head was mounted on a Zeiss (Thornwood, NY) Axiovert S100 TV inverted optical microscope, allowing visual observation of the sample.

3.3   Results and Discussion

3.3.1 Shape and Size Analysis of Iridal Stroma Melanosomes

SEM imaging was used to analyze the morphology of blue-green and dark brown iridal stroma melanosomes from donors of age 66 and 14, respectively (**Figure 7**). The shape and size observed in the analysis revealed a consistent morphological distribution across samples regardless of their respective age or iris color origins. The long and short axes of the melanosomes were measured from these images, and the resulting histograms were fit to a single Gaussian function, **Figure 8**. The outcomes of the fitting are given in **Table 1**. The morphology revealed for these iridal stroma melanosomes by SEM is in agreement with previously reported morphological results on iridal stroma melanosomes by TEM (Hu et al., 1992; Hu et al., 1993).

Figure 7: **SEM images of (A) dark brown iris melanosomes and (B) blue-green iris melanosomes.**

Figure 8: The distributions of the widths and lengths of dark brown iris melanosomes (A, B) and blue-green iris melanosomes (C, D). As shown, the distributions of lengths of the long and short axes are represented by single Gaussian distributions.

Table 1: Summary of the size analysis of the melanosomes isolated from human irides. The error in measuring the dimensions is < 5%. The histograms of the lengths of both the long and short axes were fit to a single Gaussian function. The peak of the distribution is tabulated. The half-width of the distribution is given in parentheses.

Iris Color	Length/nm	Width/nm
Dark Brown	560 (140)	260 (80)
Blue-Green	540 (160)	250 (70)

During the sample preparation, the IPE is carefully removed from the posterior surface of the iris. However, complete dissection of the IPE is nearly impossible to achieve, and the anterior IPE remains tightly bound to the iris tissue (Prota et al., 1998). As a consequence, melanosomes isolated from this "IPE-scraped" iris tissue comprise those from anterior IPE and iridal stroma. Morphologically, the IPE melanosomes are spherical, with an average diameter of 1.02 µm, whereas the iridal stroma melanosomes are smaller, ovoid-shaped organelles with width and length dimensions of 0.25 and 0.64 µm, respectively (Hu et al., 1992; Hu et al., 1993). The SEM size distributions results revealed a morphology that is consistent with previously reported dimensions for iridal stroma melanosomes (**Figure 8**). Additionally, the representative SEM images shown in **Figure 7** illustrate the dominance of ovoid shapes rather than larger spheres. From the size and shape analysis of the melanosomes isolated from the blue-green and dark brown irides, it is concluded that the samples are dominated by iridal stroma melanosomes rather than anterior IPE.

3.3.2 Surface Photoionization Potentials of Iridal Stroma Melanosomes

The surface composition of melanosomes isolated from dark brown and blue-green iridal stroma tissue was examined using FEL-PEEM. The integrated intensity (S) of the PEEM images was determined as a function of the excitation wavelength generated by the FEL and the acquired data from individual melanosomes was functionally fit. Despite the fact that melanosomes isolated from the iridal stroma tissue

contain both eumelanin and pheomelanin in varying ratios depending on iris color

(Prota et al., 1998; Wakamatsu et al., 2007), an analysis of the plots generated with the

FEL-PEEM technique for dark brown and blue-green iridal stroma melanosomes

revealed data that was best fit by a single photoionization threshold of 4.9 ± 0.2 eV (253

nm) (**Figure 9**). This threshold was attributed to eumelanin since it is comparable to the

eumelanic threshold established in previous reports on purely eumelanic systems (e.g.,

Sepia ink and black hair melanosomes) (Ye et al., 2006; Garguilo et al., 2007). For

reference, the pheomelanin threshold is characterized as 3.8 ± 0.2 eV (326 nm) from

comparison studies on red hair melanosomes (Ye et al., 2006). Consistent with this

conclusion is our inability to obtain PEEM images on iridal stroma melanosomes using a

wavelength that clearly photoionizes pheomelanin in red hair melanosomes. These

results suggest that despite the color origin of the melanosome, only eumelanic

components are present on or near the surface.

3.3.3 Morphological Surface Organization of Iridal Stroma Melanosomes

A more detailed view of the surface of the melanosomes is obtained with the

AFM images (**Figure 10**). The height and phase images for dark brown iridal stroma

melanosomes reveal that the surface is comprised of smaller substructures. This result

was expected based on previous studies of naturally occurring pigments isolated from

various sources (Clancy and Simon, 2001; Liu et al., 2005a; Liu et al., 2005b; Hong et al.,

2006). All of the pigments reveal smaller substructures with lateral dimensions of a few

Figure 9: Integrated wavelength-dependent FEL-PEEM data for A) dark brown iridal stroma melanosomes and (B) blue-green iridal stroma melanosomes.

Figure 10: AFM images of dark brown iridal stroma melanosomes. The small substructures of the iridal stroma melanosomes are observed in both A) the height image and B) the phase image.

tens of nanometers. These results are additionally consistent with the view of melanogenesis proposed by Brumbaugh in the late 1960s (Brumbaugh, 1968). In that report, Brumbaugh showed that eumelanin premelanosomes comprise zigzagging longitudinal strands with cross-links that occur every 200 Å. Further analysis of the electron micrographs during the final stages of melanogenesis revealed melanin deposition occurring around and upon these matrixes. As a result of the studies, the FEL- PEEM technique would be probing the surface of these smaller substructures along the surface. Thus, it is concluded that these substructures found along the surface of the iridal stroma melanosomes all have eumelanin coats.

Kinetic studies of the initial steps of the Raper-Mason scheme for melanogenesis established the branching between eumelanin and pheomelanin production. Although initiation of the formation of each pigment is achieved by the enzymatic oxidation of tyrosine by tyrosinase, the kinetic data suggests a three-step process for melanogenesis following this oxidation (Land and Riley, 2000; Ito, 2003; Ito and Wakamatsu, 2008). First, the initial production of cysteinyldopa occurs when the cysteine concentration is greater than 1.3 μM. Then, when the cysteinyldopa concentration is greater than 9 μM, subsequent oxidation of cysteinyldopa to pheomelanin occurs. It is only after the depletion of most of the cysteinyldopa and cysteine levels that eumelanin formation occurs. These kinetic results suggest a structural design with a pheomelanin core encased by a eumelanin exterior. Studies on the biochemistry of mixed melanogenesis

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(Agrup et al., 1982) and the solubility properties of synthetic model systems (Ozeki et al., 1997a) also proposed this bimolecular casing model. As both of the colored iridal stroma melanosomes analyzed displayed characteristics of pure eumelanin on the surface, the FEL-PEEM results provide strong evidence for the casing model in human iridal stroma melanosomes.

A chemical analysis investigating the relationship between the amount and type of melanin and iris pigmentation was recently described in the literature (Prota et al., 1998; Wakamatsu et al., 2007). For different colored irides, the amount of pheomelanin remained fairly constant, while the eumelanin component increased in darker-colored irides. As result of the casing model and the FEL-PEEM results, the presence of a relatively constant pheomelanic "core" with a varying eumelanic exterior is proposed for colored iridal stroma melanosomes. The variation of the thickness of the eumelanic shell is attributed to the difference of the eumelanin:pheomelanin ratio. In lighter-colored irides (blue, hazel, etc.), melanosomes have a low eumelanin:pheomelanin ratio, and subsequently, a thin eumelanic shell is expected. On the other hand, darker-colored irides, which possess significantly higher eumelanin:pheomelanin ratios, are expected to have a thick eumelanic exterior.

From the report published recently by Wakamatsu et al., the eumelanin:pheomelanin ratios for uveal melanocytes from eyes with blue-green (hazel) and dark brown irides are identified as 1.3 and 14.8, respectively (Wakamatsu et al.,

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2007). Using these ratios, conclusions can be drawn about the variation of the thickness

of the eumelanic coats. Specifically, calculations show that the eumelanin exterior is 2.48

times thicker for dark brown irides than that for hazel irides, assuming the density of the

subunits remains constant for both colors. If we model the substructure of the

melanosomes as spheres of diameter 30 nm, the eumelanic exterior would be ~9 and 3.6

nm thick for the dark brown and blue-green iridal melanosome, respectively, and the

corresponding diameter of the pheomelanin cores would be ~12 and 22.8 nm,

respectively. As described in **Chapter 2**, the electron escape depth for melanosomes is

expected to be a few nanometers; thus, the absence of the pheomelanic signature is not

surprising despite the thin eumelanic shell.

The photoreactivity of the melanin pigment within the melanosome becomes

important as a consequence of these results. A dark-colored iris with lower relative

pheomelanin concentration (high eumelanin:pheomelanin ratio) is presumed to have a

thick eumelanic exterior. Therefore, after the oxidation of the surface pigment, there is

still a large amount of eumelanin that can be protective against oxidative stress.

However, in melanosomes from light-colored irides, after the oxidation and degradation

of the eumelanic surface, a certain amount of pheomelanin molecules are exposed and

show their pro-oxidant properties, causing tissue damage. These results are consistent

with the incidence of uveal melanoma in different races and in eyes from different

colored irides. Epidemiological studies on the relationship between iridal color and

incidence of uveal melanoma suggest that the light-colored eye (blue, hazel, etc.) and

Caucasian is at a higher risk than the dark-colored eye and African-Asians (Michelson

and Shields, 1977; Tucker et al., 1985; Holly, 1990; Seddon et al., 1990; Vajdic et al., 2001;

Hu et al., 2005). Subsequent exposure of the photoreactive and pro-oxidant

pheomelanin coupled to the lower total amount of melanin in lighter-colored irides

could be a contributor to the observed epidemiology of uveal melanoma.

It is interesting to consider the potential implications of these findings. While

ocular melanosomes have been the focus herein, it has previously been established that a

similar encapsulation of pheomelanin by eumelanin pigments is characteristic of the

neuromelanin granules isolated from various regions of the human brain (Bush et al.,

2006; Ito, 2006; Bush et al., 2009). This suggests that the structure of melanosomes

containing both classes of pigments may share a common structural morphology,

independent of tissue type. A recent study on the eumelanin and pheomelanin content

in uveal melanoma cells found that melanoma cells have a very low eumelanin content

and eumelanin:pheomelanin ratio, significantly lower than that from normal

melanocytes. These differences likely render melanoma cells more susceptible to

mutagenic effects of UV radiation and oxidative stress and may enhance their

proliferation, thereby accelerating the progression of melanoma (Hu et al., 2009).

Epidemiological studies indicate that the incidence of cutaneous melanoma in

individuals with light-colored skin is greater than that from individuals with dark-

colored skin. The relative risk of white/black varies from 12.6 to 17.1 in different reports (Neugut et al., 1994; Cress and Holly, 1997; Tsai et al., 2005). Studies of eumelanin and pheomelanin content of epidermal melanocytes from different donors indicate that the eumelanin:pheomelanin ratio correlates with the color of the skin and the ethnic background of the donors. Melanocytes from dark-colored skin and African- American donors have a greater amount of eumelanin and a high ratio of eumelanin:pheomelanin as compared to that with lighter-colored skin and Caucasian (Wakamatsu et al., 2006). If the melanosomes in uveal melanoma cells and epidermal melanocytes have a similar structure as that in iridal melanocytes melanosomes, the different incidence of cutaneous melanoma in individuals with various colored skins and the progress of uveal melanoma could be explained, at least partly, by the structure of melanosomes and the different thicknesses of eumelanic coats.

3.4 Conclusions

The surface potentials of iridal stroma melanosomes from human dark brown and blue-green irides have been studied with FEL-PEEM, and further analysis of the surface morphology was probed with AFM. All melanosomes show pure eumelanin on their surfaces, independent of their respective eumelanin:pheomelanin ratios. These results provide very strong evidence for the casing model in the human irides and imply that the smaller substructures all have eumelanin coats of varying thickness. This result provides new insights into the molecular mechanisms underlying the epidemiology of

uveal melanoma. As the reactive pheomelanin is encased by the photoprotective eumelanin, degradation of the eumelanic exterior is necessary to expose the pro-oxidant, photoreactive properties of the pheomelanic core. Degradation of the eumelanic surface can be achieved through several processes (photobleaching, oxidation), but exposure of the pheomelanic core will result more quickly in lighter-colored irides with thinner eumelanic shells. These results are consistent with the epidemiological studies, which show that the incidence of uveal melanomas is greater in Caucasian and in eyes with light-colored irides. Further studies are needed, however, to confirm that the outer protective layer thins with the lightening of the color of the iris.

4.   Ultraviolet Absorption Coefficients of Bovine Ocular Melanosomes Containing Eumelanin[1]

4.1   Introduction

The eumelanin contained within cutaneous and ocular melanosomes has been identified as having a variety of functions, but it is the role of photoprotection that is often the most cited (Kollias et al., 1991; Sarna, 1992; Meredith and Sarna, 2006). Central to understanding the photochemical properties of eumelanosomes, melanosomes predominately comprised of eumelanin, is a direct measurement and thorough description of their absorption coefficients. Within darkly colored mammalian eyes, eumelanosomes are contained in several layers of pigmented tissues, including the choroid, iris and RPE (Dryja et al., 1979; Liu et al., 2005b; Hu et al., 2009). As discussed in **Chapter 1**, these tissues are of varying embryonic origin: RPE cells are derived from the outer neuroectodermal sheath of the eyecup and the uveal melanocytes are of neural crest origin, the same embryonic origin as cutaneous melanocytes (Nordlund, 1989; Schraermeyer and Heimann, 1999; Bumsted and Barnstable, 2000; Boissy and Hornyak, 2006). Furthermore, studies of the eumelanin isolated from these different tissues in bovine eyes display a range of DHICA/DHI monomer ratios (Liu et al., 2005b). Thus, bovine eyes provide a unique system for examining how the absorption coefficients of

eumelanosomes vary for differing molecular composition and embryonic origin to ultimately provide insight into the macroscopic biological function of photoprotection.

Approaches to date for determining the optical properties of intact melanosomes involve indirect measurements and require assumptions about the thermodynamic properties of the intact organelle (Jacques and McAuliffe, 1991; Jacques et al., 1996; Strauss et al., 1997; Brinkmann et al., 2000; Glickman et al., 2001; Neumann and Brinkmann, 2005). Recall (**Chapter 2**), the absorption coefficients of melanosomes are reported as εc, in units of inverse length, as the lack of knowledge of the melanin structure prevents calculation of the concentration c. Jacques et al. utilized the indirect technique of explosive vaporization to calculate internal absorption coefficients of several types of melanosomes. In this method, the absorption coefficient is deduced from assumed thermodynamic properties of the melanosome and the threshold temperature measured after heating with a pulsed laser *in situ*. While most of this work has been focused in the mid-visible wavelength range, a general wavelength-dependent absorption coefficient is predicted based on the scaling of an in vivo spectrophotometer measurement of cutaneous melanin to the acquired data (Jacques and McAuliffe, 1991; Jacques et al., 1996). As a result, the determined wavelength-dependent absorption coefficient for cutaneous melanosomes is predicted to be $\varepsilon c(\lambda_{nm}) = 1.70 \times 10^{12} \lambda^{-3.48} cm^{-1}$, while that for RPE melanosomes is predicted to be ~3.8-fold greater.

Despite the considerable effort directed to determining the absorption coefficient of melanosomes, the results do not directly reflect the absorption coefficient of a single intact melanosome. However, a novel application of the PEEM enables the direct measurement of the absorption coefficients of intact natural melanosomes (**Chapter 2**). Thus, in an effort to fully examine the photochemical properties of eumelanosomes, the PEEM technique is employed to measure the absorption spectra of single, intact natural eumelanosomes of varying DHICA:DHI ratios and embryonic origin over the spectral range from 244 nm to 310 nm to quantitatively probe the relationship between the absorption coefficients and pigment variations.

4.2 Materials and Methods

4.2.1 Sample Isolation

Details of the isolation of the studied melanosomes from bovine RPE cells and bovine iris and choroid tissues were previously published along with their chemical analyses (Liu et al., 2005b). Briefly, mature (> 2 years old) and newborn (< 1 week) bovine eyes were obtained from Animal Technology, Inc. (Tyler, TX). Dissection of the eyes occurred immediately upon receipt with a procedure described by Boulton et al. (Boulton et al., 1990). Through this procedure, each eye was hemisected at the equator and separated into the anterior and posterior eyecups. After removing the lens and vitreous body from the anterior segment, the iris and cilliary body were taken out and temporarily stored in Dulbecco's phosphate buffered saline without calcium and

magnesium (PBSA). Similarly, the vitreous body and neural retina were removed from

the posterior segment and the RPE cells were then scraped off the Bruch's membrane

and temporarily stored in PBSA. Following this removal, the choroid membrane was

lifted and separated from the non-pigmented sclera and also temporarily stored in

PBSA. Tissues from the two different age groups were pooled from 20-50 individual

eyes. The samples were stored at -70º until further use.

As described (Liu et al., 2005b), the RPE melanosomes were extracted with the

procedure described by Boulton et al., but with some modifications (Boulton et al., 1990).

The RPE cell suspension was homogenized using a Teflon/glass homogenizer and was

subsequently centrifuged at 60 g at 6°C for 6 min. The resulting supernatant was

collected and the pellet was re-homogenized and centrifuged.   The supernatants from

both processes were subsequently combined and centrifuged at 3000 g at 6°C for 6 min.

The melanosomes were re-suspended and subjected to the following purification

procedures. First, melanosomes were washed in triplicate with PBSA using

centrifugation at 2000 g at 6°C for 10 min. Second, the resulting melanosomes were

subjected to a simplified discontinuous sucrose gradient centrifugation with 2.0 M and

1.4 M sucrose for 1 h at 4°C at 3200 g. Third, the resulting pellet was washed five times

with pure water and lyophilized after being re-suspended in water.

The summarized enzymatic extraction used to isolate the iridal stroma melanosomes from the iris tissue described in **Chapter 3** is the same procedure that was utilized for melanosome isolation from bovine iris and choroid tissues (Liu et al., 2005b).

4.2.2 Photoemission Electron Microscopic Analysis

The UV absorption coefficients of the samples were quantified with the same procedures as described in **Chapter 2**.

4.3 Results

Figure 11 shows representative PEEM images of bovine ocular melanosomes on Ti substrates at $\lambda = 244$ nm at 50 µm fields of view. At this excitation wavelength, both the substrate and the melanosomes photoemit, but the organelles are resolved in the image as the efficiency of ionization (electrons per area) differs between the substrate and the melanosome. The shape and dimensions of the observed melanosomes are consistent with previously reported scanning electron microscopy analyses (Liu et al., 2005b). In each image, the substrate reveals shadows on the far side of the melanosomes that are oriented in the direction of the incident light, represented as the white arrow at the left bottom of panel (A). The shadows reflect a decreased photoionization intensity of the substrate as a result of the absorption of the incident light by the melanosomes.

An intensity contour plot of the shadow generated by the absorption of $\lambda = 244$

Figure 11: PEEM images of melanosomes isolated from A) adult and B) newborn bovine RPE, C) adult and D) newborn choroid melanosomes, and E) adult bovine iris. The images clearly reveal the shadows generated as a result of absorption of the incident light ($\lambda = 244$ nm). The shadows are oriented in the direction of incident light (indicated by the white arrow).

nm light by a newborn RPE melanosome is shown in **Figure 12-A**. Absorption of the

melanosome is observed by the reduction of the photoemission intensity. Line plots

reveal the spatial dependence of the photoemission intensity throughout the shadow.

Plots of the intensity along the direction of incident light and perpendicular to the

incident light are shown in **Figure 12-B** and **12-C**, respectively. The ellipsoidal shape of

the melanosome dictates the shape of these plots. The intensity is symmetric about the

center of the melanosome. As one moves away from the center toward the ends of the

long axis, the pathlength through the melanosome decreases causing more UV light to

reach the surface and subsequently produce higher photoemission from the substrate

behind the melanosome. Accordingly, as one moves away from the equator toward the

top of the melanosome, the pathlenghts through the melanosome decrease, and higher

photoemission is observed in the corresponding shadow regions.

PEEM images of a single adult bovine RPE eumelanosome are shown in **Figure

13** for each wavelength examined on SiC at 150 μm fields of view. In each image, a

shadow is visualized behind the melanosome and was oriented in the direction of the

incident light, represented as the white arrow at the left bottom of panel (A). Note,

although the physical angle of the incident of light remains constant, the apparent

direction on the obtained image shifts with different fields of view as a result of changes

in the currents throught the electromagnetic optics. Eumelanosomes were not observed

Figure 12: Spatial dependence of the shadows depicted through A) an intensity contour plot and B, C) intensity line scans. The intensity contour plot is of the shadow resulting from a newborn bovine RPE melanosome (not pictured). Line scans of the image are drawn through the shadow (solid and dashed lines in A) and are plotted as B) a function of perpendicular distance from the center of the melanosome, solid line drawn in A, and C) a function of distance parallel to the melanosome at a distance of 2.2 μm from its center, dashed line drawn in A.

Figure 13: A sequence of PEEM images showing the wavelength-dependence of a single adult bovine RPE melanosome and its shadow on a SiC substrate. Images A - G were obtained upon illumination with laser wavelengths 244, 257, 270, 280, 290, 300, and 310 nm, respectively. The white arrow drawn in the bottom left of panel A) represents the direction of incident light on the sample.

in the PEEM images generated with wavelengths greater than ~ 270 – 280 nm as the

characteristic photoionization threshold potential of eumelanin is ~ 4.5 ± 0.2 eV (276 nm)

(Garguilo et al., 2007). The substrate, however, is photoionized at all wavelengths

examined and thus the generated shadows were visualized throughout the experiment.

The images shown in **Figure 13** are representative of the wavelength-dependent PEEM

images obtained for each of the studied eumelanosomes.

As described in the experimental methods section of **Chapter 2**, the absorption

coefficients measured required using a tunable pulsed laser source and SiC substrates so

that the shadow could be visualized over the wavelength region examined.

Furthermore, as a result of the low-pulsed energy of the laser and a desire to ensure a

large spatial sampling, fields of view of 150 μm were required. These experimental

details were different than those where images were collected using a continuous wave

Ar$^+$ ion laser on Ti at 50 μm fields of view. As shown in **Figure 14**, small changes are

observed between calculated absorption coefficients when measured with different

substrates, magnifications and laser sources; however, there is no systematic variation

between the calculated values based on the methodology. Therefore, the different

experimental conditions are not determined to have a significant influence on the

calculated absorption coefficients and reflect the precision of the PEEM technique. The

measured wavelength-dependent absorption coefficients are all obtained under the

same experimental conditions and thus, variations between the observed εc

Figure 14: Average absorption coefficients measured at λ = 244 nm with different experimental conditions. Experimental conditions were A) 50 μm field of view, Ar⁺ continuous wave laser and Ti substrate, B) 150 μm field of view, Ar⁺ continuous wave laser and SiC substrate, and C) 150 μm field of view, pulsed nanosecond laser and SiC substrate.

measurements on eumelanosomes throughout the UV do not arise from substrate or laser sources used.

The measured absorption coefficients at λ = 244 and 300 nm are listed in **Table 2** along with the previously reported DHICA:DHI ratios for the bovine eye eumelanosomes (Liu et al., 2005b). The ratio of the yield of PTCA by $KMnO_4$ oxidation of the melanosomes to the total melanin (TM) determined by the absorbance at 500 nm for the samples dissolved in Soluene-350 serves as an excellent measure of the percentage content of DHICA-derived units (Ozeki et al., 1997b). The reported εc values are a result of an average of the full data set collected for each type of eumelanosome examined. Considerable variability was observed within the individual data sets throughout all wavelengths. As has been previously discussed in **Chapter 2**, the observed variability is a result of the fact that the pathlength calculations use the previously reported (Liu et al., 2005b) SEM average size dimensions of each eumelanosome as the limited resolution of the PEEM precluded the possibility of dimension measurements directly. Thus, the error of the averaged absorption coefficients arises through propagation of error of the eumelanosome size analyses. Errors calculated in this way are reported in **Table 2**. The error for each εc calculated at all wavelengths for the newborn and adult RPE, newborn and adult choroid and adult iris was determined to be 25.4, 32.8, 19.7, 17.0 and 21.3%, respectively.

Table 2: Summary of the chemical degradation analyses and absorption coefficients of eumelanic melanosomes. The chemical degradation analyses have been previously published (Liu et al., 2005b); products are expressed in units of per mg melanosomes. PTCA was determined from oxidation by $KMnO_4$.

Melanosomes	Age	PTCA μg/mg	TM A_{500}/mg	PTCA/TM μg/A_{500}	DHICA:DHI	εc (244 nm) cm^{-1}	εc (300 nm) cm^{-1}
RPE	Newborn	7.7	5.91	1.30	1.30:1	4970 (1260)	2685 (730)
	Adult	4.2	5.26	0.80	0.80:1	5100 (1675)	3495 (1145)
Choroid	Newborn	14.7	6.34	2.32	2.32:1	3175 (625)	1720 (340)
	Adult	5.6	4.60	1.21	1.21:1	4495 (765)	1935 (330)
Iris	Adult	5.2	5.19	1.00	1.00:1	3270 (695)	1780 (380)

The averaged εc values for all adult eumelanosomes are plotted as a function of

wavelength in **Figure 15**. Newborn melanosomes show similar trends. Generally, εc

values decreased with increasing wavelength, as expected from previous measurements

reported on synthetic and natural melanosomes (Sarna and Sealy, 1984b; Jacques and

McAuliffe, 1991; Jacques et al., 1996; Sardar et al., 2001; Meredith and Sarna, 2006).

Furthermore, as evidenced in **Figure 15**, the absorption coefficients of bovine RPE

eumelanosomes were on average about 1.5-fold greater than those for eumelanosomes

isolated from bovine choroid or iris tissues, regardless of age.

4.4   Discussion

When evaluating the attenuation of light, absorption and scattering must be

considered due to the complex nature of the eumelanosomes. While it is difficult to

evaluate the contribution of the scattering phenomena to our absorption measurement,

we have attempted to disaggregate the two effects within the shadow region. As

previously discussed (**Chapter 2**), the portion of the shadow region that is void of

significant scattering contributions was determined following the methodology outlined

by Chelaru et al. (Chelaru et al., 2006). Briefly, diffraction will influence the intensity in

the shadow region beginning at a distance from the eumelanosome corresponding to the

first maximum in the diffraction pattern observed on the substrate. This distance can be

calculated from the Fraunhofer diffraction pattern of an elliptical aperture of the same

dimensions as the eumelanosomes as the two diffraction patterns are the same except for

Figure 15: Summary of the average measured absorption coefficients of the bovine eumelanosomes versus wavelength. Adult bovine RPE (squares), choroid (circles) and iris (triangles) are plotted.

an inversion of the intensity distributions. This calculation, outlined in the experimental methods section (**Chapter 2, Equation 5**), was used for each eumelanosome at all wavelengths. As long as the region of the shadow analyzed is determined in this manner, it is the case that the calculated εc values for the eumelanosomes predominately reflect absorption of melanin.

It is important to acknowledge that although eumelanosomes are mainly comprised of eumelanin, they also contain proteins, lipids and various ions. While most lipids and amino acids exhibit negligible absorption between $\lambda = 244 - 310$ nm, the aromatic amino acids do absorb in this spectral range. The absorption maxima (extinction coefficient) of the aromatic amino acids Phe, Tyr and Trp are 257.4 nm (165 $M^{-1}cm^{-1}$), 274.6 nm (1,430 $M^{-1}cm^{-1}$), and 279.8 nm (6,000 $M^{-1}cm^{-1}$), respectively (Wetlaufer, 1962), with the extinction coefficients of the latter two on the order of the observed εc values. The contribution of the protein to the absorption measurements can be deduced from reported amino acid profiles of eumelanosomes. The protein content of the studied eumelanosomes increases from RPE to iris to choroid and ranges from $9 - 15\%$ (Liu et al., 2005b). The distributions of the amino acids, however, are similar and the aromatic amino acid content makes up less than 10% of the protein composition for each type of eumelanosome (Liu et al., 2005b). Therefore, the contribution of the aromatic amino acids is expected to be at most 1.5% of the total absorption throughout the spectral range studied. This total contribution to the measured absorption is negligible.

Therefore the measured absorption predominately reflects that of the eumelanin pigment and as a result **Figure 15** represents quantitative absorption spectra of the pigment contained within bovine RPE, choroid and iris eumelanosomes.

Eumelanin is built from varying amounts of the monomeric dihydroxyindole building blocks, DHI and DHICA, (**Table 2**) (Liu et al., 2005b). Initial single-wavelength PEEM experiments performed on intact eumelanosomes (measured with the experimental conditions reported as "A" in **Figure 14**) at $\lambda = 244$ nm suggest that as the DHICA content increases, the εc of the melanosome increases. This observation was compared to the UV absorption spectra and extinction coefficients for the DHI and DHICA monomers (Benigni and Minnis, 1965; Ito and Nicol, 1974; Murphy and Schlutz, 1985; Tran et al., 2006; d'Ischia et al., 2008; Gauden et al., 2009). The extinction coefficients of DHI and DHICA are somewhat sensitive to pH; an average of the ε values from the published literature gives 2610 and 5530 M^{-1} cm^{-1} at $\lambda = 244$ nm for DHI and DHICA, respectively. On the basis of the extinction coefficients of the contributing monomers, an increased absorption is expected with increased DHICA/DHI ratios.

If the absorption of the melanosome was modeled as arising from the sum of the absorption of contributing DHI and DHICA monomers, it is written as

$$A = \ell(\varepsilon c)_{eumelanin} = \ell(\varepsilon_{DHI}\left[DHI\right] + \varepsilon_{DHICA}\left[DHICA\right]). \tag{12}$$

In terms of mole fraction, X, **Equation 12** becomes

$$\varepsilon_{eumelanin} = \varepsilon_{DHI}X_{DHI} + \varepsilon_{DHICA}X_{DHICA}. \tag{13}$$

Because the total mole fraction is unity, $1 - X_{DHICA} = X_{DHI}$, and **Equation 12** can be written as

$$\varepsilon_{eumelanin} = X_{DHICA}(\varepsilon_{DHICA} - \varepsilon_{DHI}) + \varepsilon_{DHI}. \qquad (14)$$

Under this assumption, a plot of the melanosome absorption coefficients as a function of the percentage of DHICA content would have limiting values at the end points $X_{DHICA} =$ 0 and 1, given by ε_{DHI} and ε_{DHICA}, respectively. **Figure 16** shows a plot of εc as a function of the mole fraction of DHICA contained in the melanosomes. The line shown in the plot is drawn between the average ε values of the two monomers (2610 and 5530 M^{-1} cm^{-1}). This line represents the predicted absorption coefficients, assuming that this value is given by the sum of the constituent concentration of contributing DHI and DHICA monomers, **Equation 12**. The observed εc values are consistent with those with **Equation 14**, suggesting that the absorption of natural melanins can be quantified in terms of relative content of the two constituent monomers, weighted by their relative mole fractions. However, the region probed (% DHICA 0.4 - 0.7) and the spread of the data certainly indicates that this conclusion requires further testing to be substantiated. Such a trend was not observed with the $\lambda = 244$ nm εc values measured with SiC substrates and a tunable pulsed nanosecond laser (experimental conditions "C" of Figure 14), **Table 2**; the lack of correlation may be a reflection of the precision of the PEEM technique, **Figure 14**. To further examine whether εc for the melanosome

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Figure 16: Plot of the observed absorption coefficient versus DHICA content at λ = 244 nm. The line is representative of the predicted absorption as arising from the sum of the absorption of contributing DHI and DHICA monomers.

maintains an additive relationship with the constituent monomers across the absorption

spectrum, the longer UV wavelength region is of importance.

The shapes of the absorption spectra of DHI and DHICA are similar in the

spectral region from 240-270 nm, but differ significantly in shape in the UV-A with the

absorption maxima (extinction coefficient) being $\lambda = 296$ nm (3,310 M^{-1} cm^{-1}) and $\lambda = 320$

nm (~14,000-16,000 M^{-1} cm^{-1}), respectively (Murphy and Schlutz, 1985; Tran et al., 2006).

Note, as mentioned, the exact literature values for the extinction coefficients of DHI and

DHICA vary depending on the solvent or pH and the aforementioned spectra, taken in

deaerated water and NaOH solution, respectively, were used throughout the presented

analyses presented. If we were to model the absorption of the adult RPE eumelanosome

as arising from the sum of the absorptions of its contributing DHI and DHICA

monomers, weighted by their relative mole fractions, an absorption spectra similar to

Figure 17A would be obtained, where the DHICA extinction coefficient increases the

absorption at wavelengths nearing the UV-A. The measured absorption spectra

presented in **Figure 15**, however, are in stark contrast to the predicted spectra. A

decrease in the absorption is observed in the longer wavelength regions. Furthermore,

as shown in **Figure 17B**, a plot of the eumelanosome absorption coefficients as a function

of the percentage of DHICA content, at $\lambda = 300$ nm, revealed a decrease in εc with an

increase of DHICA content. The observed trend did not extrapolate to the limiting

values of the DHI and DHICA extinction coefficients as would have been expected if the

Figure 17: Predicted absorption spectra as arising from the sum of the absorption of contributing DHI and DHICA. The absorption spectra plotted in A) is the prediction based on the determined monomer composition for adult bovine RPE melanosomes (0.44 % DHICA). A plot of the observed absorption coefficients versus the % DHICA content at $\lambda = 300$ nm (open squares) is shown in B). The data points 0 and 1 % DHICA (black circles) are representative of the DHI and DHICA extinction coefficients at this wavelength and the solid line drawn between the points is the predicted absorption as arising from the sum of the absorption of the contributing monomers. The observed trend (dashed line) is in stark contrast to this prediction.

absorption of the eumelanosomes arose from the sum of the constituent concentration of contributing DHI ad DHICA monomers. This trend was observed with all calculated absorption coefficients in the 280-310 nm region of interest. It was therefore unequivocally concluded that the predicted additive relationship with the constituent monomers is not maintained across the absorption spectrum.

Despite extensive experimental studies conducted on synthetic and natural eumelanin, including its soluble fraction, the structures of the natural oligomers formed from DHI and DHICA have evaded characterization. As a result, a thorough understanding of the unique optical properties of eumelanins has remained an immensely complex topic. The absorption data on intact, natural eumelanosomes offers a direct view into the oligomeric structure of the eumelanin pigment. As has been discussed, the natural oligomeric structure is not reflective of the electronic structure of the monomers throughout the ultraviolet. This is most notable in the UV-A region where the low energy band of DHICA appears to have a negligible effect on the absorption of the pigment. These results indicate that polymerization of DHICA into the melanin pigment disrupts the electronic structure of these monomeric building blocks; that polymerization modifies the monomers in the resulting oligomer. Recently, papers have been published that provide insight into UV-visible absorption properties of the evolution of the DHI and DHICA monomers into larger oligomeric structures (dimer to tetramer) (Tran et al., 2006; Riesz et al., 2007; d'Ischia et al., 2008; Pezzella et al., 2009a;

Pezzella et al., 2009b). The collective work observes absorption spectral shapes of the

DHI and DHICA monomers that gradually shift towards the broadband eumelanin

spectrum upon oxidative polymerization. In the studies of the DHICA polymerization

(Tran et al., 2006; Riesz et al., 2007), the absorption maximum at λ = 320 nm was found to

decrease as a function of time and a 20% hyperchromism was observed for the DHICA

polymer compared to that of the monomer. These model studies have shown that the

chemical linkages between the monomers affect the spectroscopic properties of the

resulting oligomers. While the relevance of these model studies to the actual eumelanin

oligomer remains to be assessed, similarities between the optical properties of the model

oligomers are in accord with those of the natural eumelanosomes. The electronic

structure of the eumelanic oligomer does not resemble the electronic structure of the

monomeric building blocks, DHI and DHICA, suggesting that the monomers are in fact

modified upon polymerization to the eumelanin pigment.

In an effort to further examine the natural oligomeric structure of the eumelanin

pigment, a comparison of the absorption coefficients of the eumelanosomes as related to

their embryonic origins is of interest. As plotted in **Figure 15**, the absorption coefficients

calculated for the bovine RPE eumelanosomes were on average 1.5-fold greater than

those measured for the uveal eumelanosomes. These results suggest that either the

eumelanin pigment is present in greater density or that there is an underlying difference

in the eumelanic molecular structure. The greater absorption coefficient between the

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eumelanosomes derived from the primitive forebrain compared to those derived from the neural crest is consistent with previous estimates, but is less than the predicted 3.8-fold difference (Jacques et al., 1996). **Figure 18** compares the measured absorption coefficients of the RPE and uveal eumelanosomes with the previously reported and predicted cutaneous (Jacques and McAuliffe, 1991; Jacques et al., 1996) and RPE (Williams et al., 1985; Jacques et al., 1996; Strauss et al., 1997; Brinkmann et al., 2000; Glickman et al., 2001; Sardar et al., 2001; Neumann and Brinkmann, 2005) melanosome absorption coefficients. At the mid-visible wavelengths, the absorption coefficients of the RPE melanosomes were predominately larger than those for the cutaneous melanosomes in accord with the predictions. As seen, however, there is a large variance in the reported absorption coefficients; the values for RPE and cutaneous melanin ranged from 5 cm^{-1} to 11,000 cm^{-1} and 78 cm^{-1} to 997 cm^{-1} at the mid-visible wavelengths, respectively. The large variance may reflect differences in sample preparation, methodology and perhaps, to a lesser degree, individual variation (Sarna, 1992; Sardar et al., 2001; Riesz et al., 2006). In contrast to the previous studies, however, the absorption coefficients obtained herein are measured directly from single, intact natural eumelanosomes. The results suggest that while the RPE eumelanosome absorption coefficients are larger than those of the uveal eumelanosomes, the differences between the two are not comparable to the previously predicted differences and that the

Figure 18: Comparison of the PEEM measured absorption coefficients with those previously reported in literature. Comparisons are made between the absorption coefficients calculated herein on uveal (iris and choroid) and RPE melanosomes (shaded triangle and rectangle data points, respectively) with previously reported cutaneous (Jacques et al., 1996) and RPE (Jacques et al., 1996; Strauss et al., 1997; Brinkmann et al., 2000; Glickman et al., 2001; Sardar et al., 2001; Neumann and Brinkmann, 2005) melanosome absorption coefficients (open circle and diamond data points, respectively) and previously predicted (Jacques et al., 1996) cutaneous and RPE spectra (solid and dashed lines, respectively).

calculated RPE eumelanosome absorption coefficients are comparable to the previously predicted cutaneous melanosome absorption coefficients.

4.5 Conclusions

The absorption coefficients of natural intact eumelanosomes from bovine eye tissues have been directly quantified. The lower-energy UV-A region provides support that the electronic structure of the naturally occurring eumelanic oligomers do not reflect the electronic structure of the monomers. Furthermore, an analysis of the absorption coefficients of the RPE eumelanosomes compared to the uveal eumelanosomes found a 1.5-fold increase, less than the difference predicted by previous reports. The observed difference suggests that either the eumelanin pigment present in RPE is of in greater density or that there is an underlying difference in the eumelanic molecular structure between the eumelanin derived from different embryonic origins.

5. The Ultraviolet Absorption Coefficients of Human Iridal Stroma Melanosomes[1]

5.1 Introduction

Epidemiological data for uveal melanoma, the most common intraocular

malignant tumor in human adults, suggests a link between incidence and the relative

concentrations of pheomelanin (Wakamatsu et al., 2007; Hu et al., 2008). This

observation is consistent with studies that indicate that pheomelanin is more pro-

oxidant than eumelanin (Chedekel et al., 1978; Harsanyi et al., 1980; Persad et al., 1983;

Takeuchi et al., 2004; Ye et al., 2006; Ye et al., 2008). However, because pheomelanin is

naturally encased by the photoprotective eumelanin (**Chapter 3**), exposure of the

pigment is necessary to induce oxidative stress. One mechanism by which this could

occur is through oxidative damage to the melanosome over time, exposing or releasing

the phototoxic pigment. Because the outer eumelanin layer becomes thinner with

increasing pheomelanin content, such a model of "damage-induced" exposure of

pheomelanin is consistent with the epidemiological observations. But other properties

of the melanosomes may also change significantly with composition to correlate with the

epidemiological data. Thus, it is of interest to also consider whether other photophysical

properties, such as photoprotection, vary significantly with pigment composition in a way that may further correlate with the epidemiological data.

The relative extinction coefficient, ε, of eumelanin and pheomelanin is necessary to understand and compare the quantum efficiency of photon absorption and subsequent photochemical reactions, such as oxygen activation, that are of biological importance. Unfortunately, the complex character of these natural melanins (highly insoluble, heterogeneous and difficult to isolate in pure form) has precluded detailed characterization of their macromolecular structure (Prota, 1992). As a result of these difficulties, studies on absorption properties of the pigments are usually conducted on synthetic model systems dissolved or suspended in buffer solutions that mimic physiological conditions (Sarna and Swartz, 2006). The absorptions exhibited by the synthetic melanins are normalized for concentration and extinction coefficients are expressed in terms of $cm^{-1}(mg/mL)^{-1}$, units commonly used because of the lack of molecular structure information even in soluble synthetics. Thus, the absorption properties of natural eumelanin and pheomelanin contained in intact melanosomes have remained elusive.

The PEEM approach, developed in **Chapter 2**, has enabled a direct measurement of the absorption properties of melanins within intact melanosomes, **Chapter 4**. In this chapter, the first measurements of the absorption coefficients, εc, of intact iridal stroma melanosomes isolated from dark brown and blue-green human irides are described for

the spectral range $\lambda = 244 - 310$ nm. The iridal stroma melanosomes are an excellent

system for quantifying the natural absorption coefficients of the two classes of pigments

because different colored irides produce organelles of varying eumelanin:pheomelanin

ratios (Wakamatsu et al., 2007) with similar size and morphology, **Chapter 3**. Chemical

degradation analyses reveal eumelanin:pheomelanin ratios of 14.8 and 1.3 for

melanosomes present in dark brown and blue-green human irides, respectively, and

therefore, this single natural system enables a systematic approach for determining how

the absorption coefficient changes with varying pigment composition. The experimental

spectra measured within are compared with both the extinction coefficient spectra

obtained on soluble synthetic model systems and the monomeric precursors to each

pigment. Modeling the pigment in terms of the currently known monomeric

chromophores enables the measured εc to be surveyed in units of $M^{-1}cm^{-1}$ instead of

cm^{-1}, providing opportunities to compare extinction coefficients per monomer.

5.2 Materials and Methods

5.2.1 Sample Isolation

The isolated iridal stroma melanosomes from different colored iris tissues used

to determine the size distributions and surface photoionization potentials in **Chapter 3**,

are the same samples used in this chapter. Details of their enzymatic extraction were

described in **Chapter 3**.

5.2.2 Photoemission Electron Microscopic Analysis

The UV absorption coefficients of the samples were quantified with the same

procedures as described in **Chapter 2**.

5.3   Results and Discussion

Human iridal stroma melanosomes isolated from different colored irises were

imaged with PEEM to directly quantify absorption coefficients of the eumelanin and

pheomelanin pigments in the spectral range $\lambda = 244 - 310$ nm. **Figure 19** shows acquired

PEEM images for dark brown iridal stroma melanosomes on SiC at $\lambda = 244$ nm (A) and

310 nm (B). The energy associated with the laser light at $\lambda = 244$ nm surpassed the

photoionization threshold of the substrate and the melanosome and both were

visualized in **Figure 19A**. In comparison, only the substrate was visualized in the PEEM

image acquired with $\lambda = 310$ nm, **Figure 19B**. The photoionization threshold of the dark

brown (and blue-green) iridal stroma melanosomes surpasses the laser energy at $\lambda \sim 260$

nm (**Chapter 3**) and, as a result, does not photoemit at the longer wavelengths examined

herein. The shadows generated behind the melanosomes, however, continued to be

visualized as the substrate is characterized with a low work function and subsequently

provided the necessary contrast for visualization. The images presented in **Figure 19**

were representative of the PEEM images acquired for all melanosomes studied,

regardless of iris color.

Absorptive properties were determined from changes in the intensity of the
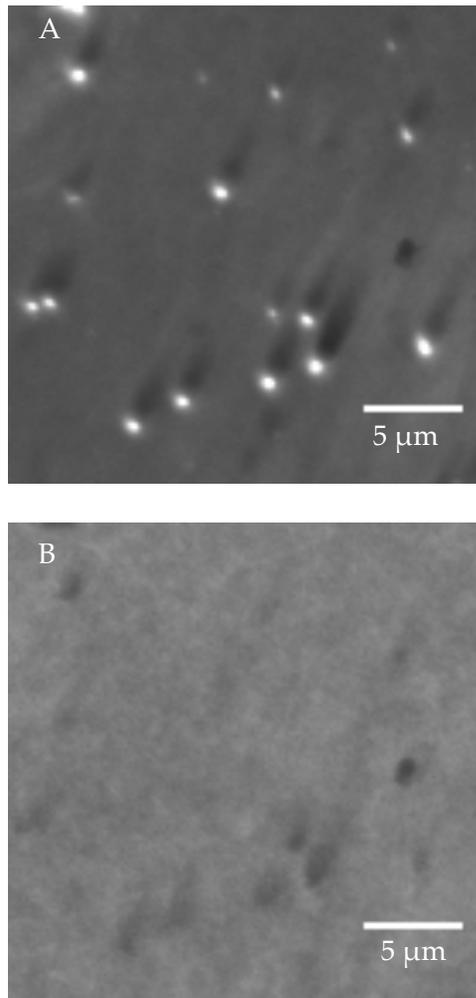
Figure 19: PEEM Images of dark brown iridal stroma melanosomes at A) λ **= 244 nm and B)** λ **= 310 nm.** The white arrow at the bottom left of panel A) identifies the direction of incoming light. The black spot observed in the right center of each image is the result of damage of the PEEM multichannel plate and is not part of the sample surface.

portion of the shadow identified as the first maximum of the diffraction pattern

produced immediately behind the melanosome as this region is void of significant

scattering contributions, **Chapter 2** (Chelaru et al., 2006). The total absorption of light,

however, is a result of the absorption from all constituents within the melanosome:

proteins, lipids and melanin. However, in the case of human iridal stroma

melanosomes, the quantities of each of these constituents are not known. Although

several studies have examined the protein content of melanosomes isolated from

different human and animal tissue sources, the majority of these were based on

melanosomes that had been isolated with acid/base extraction techniques (Borovansky

and Hach, 1973; Borovansky and Duchon, 1974; Sarna et al., 1980). This particular

extraction method was widely used to extract melanosomes from tissue, but this

approach is now known to both alter the physical and biological properties of the

melanosome and result in isolation of a larger protein content than that associated with

intact melanosomes (Liu et al., 2003).

As a specific example, melanin comprises ~86% of the material present in

enzymatically extracted melanosomes from black human hair, whereas the acid-base

extraction of melanosomes from the same hair sample alters the melanosome structure,

and the isolated materials contain 40-52% protein, depending on the method used (Liu

et al., 2003). Thus, although previous isolation techniques produced samples with large

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protein content, enzymatic isolation of intact melanosomes reveals that the dominant

constituent in the organelle is the melanin pigment.

In addition, most lipids and amino acids exhibit negligible absorption in the UV

spectral range studied. Noted exceptions are the aromatic amino acids Phe, Tyr and

Trp, which are characterized with extinction coefficients of 165, 1430 and 6000 $M^{-1}cm^{-1}$ at

λ = 257.4, 274.6 and 279.8 nm, respectively (Wetlaufer, 1962). As discussed in **Chapter 4**,

the aromatic amino acid composition makes up less than 10% of the proteins in bovine

iride melanosomes, with the relative melanin to protein content approximately 7:1

(wt/wt) (Liu et al., 2005b). These aromatic amino acids are thus expected to contribute of

at most 1.5% to the total measured absorption. This is a negligible contribution and as a

result it is concluded that the measured absorption does not reflect lipid and protein

content, but instead is reflective of the eumelanin and pheomelanin pigments contained

within the melanosomes.

Wavelength-dependent absorption coefficients for dark brown and blue-green

human iridal stroma melanosomes are shown in **Figure 20**. The plotted absorption

coefficients were determined from an average of the full data set collected on each type

of melanosome. Variability was observed between the individual melanosomes within

the measured data sets as a result of using average SEM dimensions (**Chapter 3**) in the

pathlength calculation. The error of the reported absorption coefficients was calculated

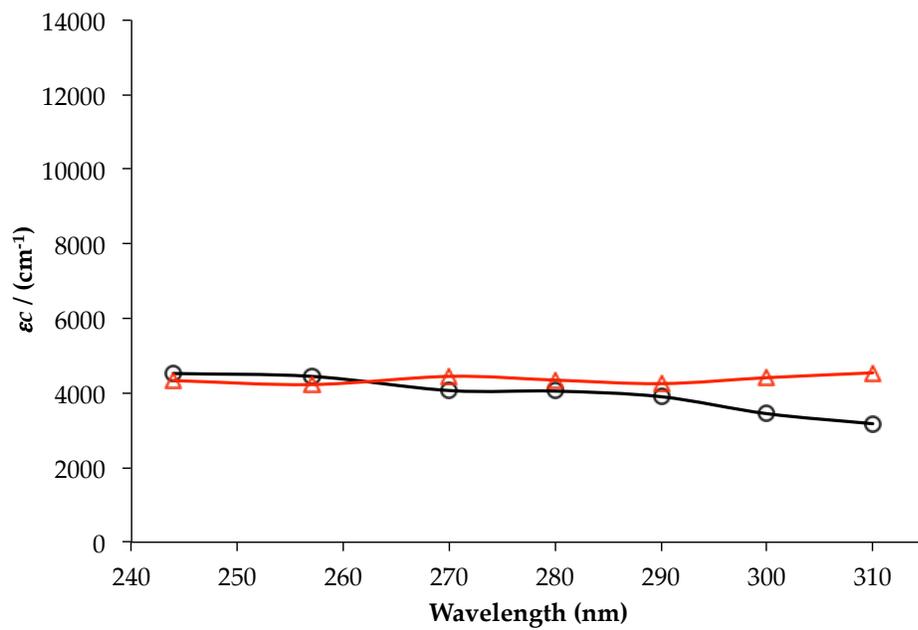through the propagation of error of the melanosome size analyses and was determined

Figure 20: Average absorption coefficients versus wavelength for melanosomes isolated from dark brown and blue-green iridal stroma tissues. Absorption coefficients calculated from dark brown and blue-green iridal melanosomes are represented as open circles and triangles, respectively.

to be 33.6 and 34.7% for dark brown and blue-green human iridal stroma melanosomes, respectively.

In comparing the spectra from the dark brown and blue-green iridal stroma melanosomes, the same spectrum was observed within reasonable error. As the dark brown and blue-green iridal stroma melanosomes have relative pheomelanin compositions of 6 and 43%, respectively, the results suggest that the absorption of the melanosomes containing a mixture of eumelanin and pheomelanin does not significantly differ from those containing pure eumelanin. Because the absorption coefficients of the melanosomes are found to be independent of the melanic composition it is of interest to subsequently examine these values in terms of the pigment monomers to obtain extinction coefficients for further comparisons with synthetic samples.

The molecular chromophores of eumelanin are DHI and DHICA whereas the chromophore of pheomelanin is built from molecules such as dihydro-1,4-benzothiazine-3-carboxylic acid (DHBTCA) and 6-(2- amino-2-carboxyethyl)-4-hydroxy-benzothiazole (BZ) (Wakamatsu et al., 2009). These precursors have different molecular volumes. AM1 calculations of the molecular volumes of DHI, DHICA, BZ, and DHBTCA give 0.129, 0.172, 0.195, and 0.268 nm^3, respectively (http://www.molinspiration.com/services/ volume.html). The chemical degradation analyses used to determine the eumelanin and pheomelanin content is a measure of the number of these monomer building blocks present in the intact pigment, and for a

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constant total volume, an increase in the number of DHBTCA units must result in a loss

of a larger number of DHI/ DHICA units because DHBTCA occupies a larger volume

than a DHI/DHICA molecule. Therefore, if these species comprise the pigment and act

as the "chromophore", the total number of monomeric chromophores (eumelanin and

pheomelanin combined) decreases, with increasing pheomelanin content. Taking the

volume of the melanosome (calculated from the average dimensions determined from

the SEM size analysis presented in **Chapter 3**), the eumelanin:pheomelanin ratio

determined from chemical analyses, and the above volumes for the molecular building

blocks (using an average of the DHI/DHICA volumes to represent the eumelanin

precursors), a 32% decrease in the number of chromophores is found in blue-green iridal

stroma melanosomes as compared with dark brown iridal melanosomes. Taking this

decrease into account with the absorption data for intact melanosomes presented in

Figure 20, we predict that the extinction coefficient (per monomer) for pheomelanin is

on average 1.9 fold greater than that of eumelanin throughout the spectral range

studied.

Sarna and co-workers reported extinction coefficients for synthetic eumelanin

and pheomelanin plotted as a function of $cm^{-1}(mg/mL)^{-1}$ versus λ (Sarna and Swartz,

2006) and the spectra of the two synthetic pigments were quite similar (**Figure 21A**).

Steven Jacques subsequently converted melanin concentration expressed as (mg/mL)

into (moles/liter) by multiplying the respective spectra by the molecular weight of the

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Figure 21: Extinction coefficients as a function of wavelength for eumelanin and pheomelanin. A) Absorption spectra for synthetic eumelanin (solid black line) and pheomelanin (dashed red line) obtained from (Sarna and Swartz, 2006). Extinction coefficients per monomer (B) were calculated from data reported on synthetic melanins in A) (Jacques, 2001). The eumelanin (solid black line) is reported per DHI monomer and pheomelanin is reported per benziothiazine monomer (dashed red line).

monomer plus added molecular weights for linkages (Jacques, 2001). In doing this,

several assumptions were made: 1) the melanin polymer is comprised only of the

monomers DHI/DHICA or benzothiazines with known molecular weights, 2) the

linkages between the subunits are minimal, and 3) the mass measured is reflective of the

pigment without attached protein or optically inert moieties. The wavelength

dependence of the extinction coefficients of eumelanin and pheomelanin per monomer

in units of (cm^{-1}(moles/liter)$^{-1}$) are shown in **Figure 21B**. The pheomelanin:eumelanin

ratio is ~1.8 throughout the spectra range examined herein, which is in excellent

agreement with the determination of ~1.9 arrived at by modeling the calculated εc for

the pigments with their currently known monomeric chromophores. This agreement

suggests that the optical properties of the synthetic and natural pigments are similar.

It is interesting to compare the directly measured absorption spectra for

pigments in intact melanosomes with the extinction coefficients of the eumelanin and

pheomelanin monomers presented in **Figure 22** (Murphy and Schlutz, 1985; Tran et al.,

2006; Wakamatsu et al., 2009). Generally, at wavelengths greater than λ ~ 257 nm,

pheomelanic monomer extinction coefficients are smaller in magnitude than those for

eumelanin. Furthermore, while the relative concentrations of DHBTCA and BZ

comprising the pheomelanin of blue-green iridal stroma melanosomes have yet to be

quantified, the measured pheomelanin spectrum does not reflect the spectral trend of

the two monomers. Additionally, the low energy band of the DHICA monomer does

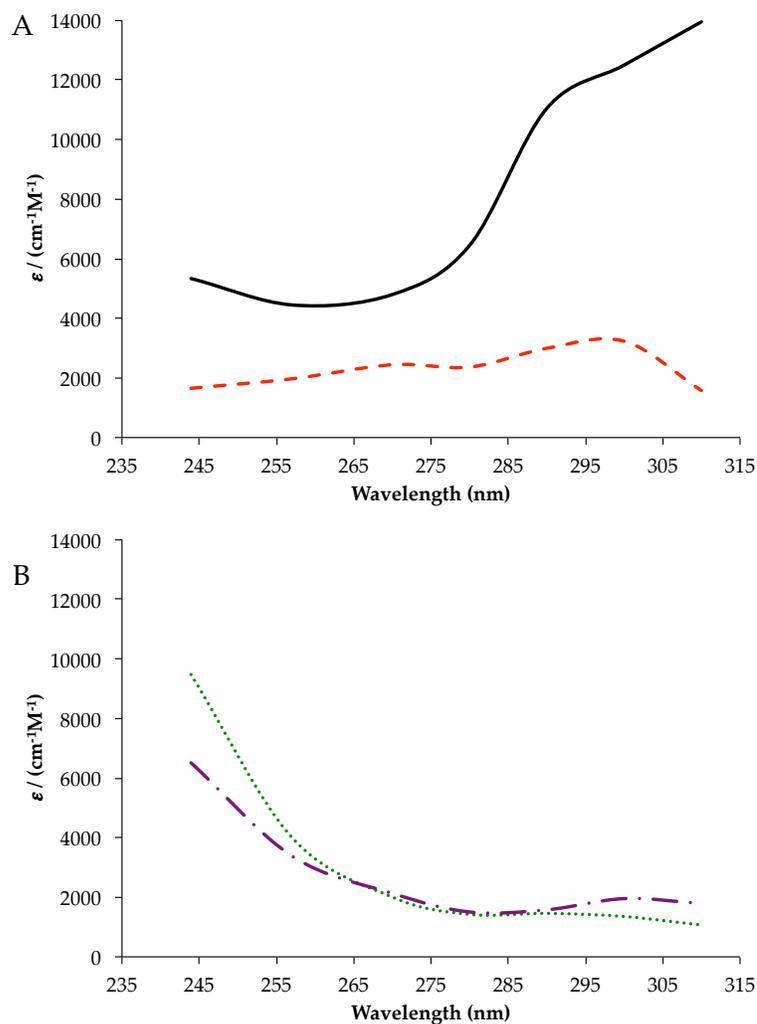**Figure 22: Extinction coefficients of the A) eumelanin and B) pheomelanin monomers.** The eumelanin DHICA (——) and DHI (– –) monomers are reported from spectra taken in NaOH (Tran et al., 2006) and deaerated water (Murphy and Schlutz, 1985; Jacques and McAuliffe, 1991), respectively. The pheomelanin monomers are reported from spectra of BZ (— ·) and DHBTCA taken in HCl (· · ·) (Wakamatsu et al., 2009).

not influence the measured eumelanin spectrum. These results suggest that the monomers are modified upon polymerization to the resulting melanin oligomers. This result is not unexpected based the work comparing the UV absorption spectra of eumelanin comprised of different DHI:DHICA ratios to the monomeric extinction coefficients (**Chapter 4**). It is important to acknowledge, however, that all of the melanin monomers are known to have fluctuations depending on the solvent and/or pH, but generally maintain the spectral trends plotted in **Figure 22** (Ito and Nicol, 1974; Murphy and Schlutz, 1985; Tran et al., 2006; Gauden et al., 2009; Wakamatsu et al., 2009). The values reported herein for the DHICA and DHI monomers are taken from spectra in NaOH (Tran et al., 2006) and deaerated water (Murphy and Schlutz, 1985), respectively, while the BZ and DHBTCA monomers are taken from spectra in HCl (Wakamatsu et al., 2009).

Mixtures of eumelanin and pheomelanin are produced in various ratios throughout natural mammalian melanosomes (Ito, 2003; Wakamatsu et al., 2006; Wakamatsu et al., 2007) and epidemiological data has observed increased incidences of skin and ocular cancers for increased relative concentrations of pheomelanin (Vincensi et al., 1998; Wenczl et al., 1998b; Hu et al., 2008). In the context of these observations, a significant effort has been directed towards understanding the functional significance of increased incidences of pheomelanin. To date, however, there is data that both supports (Vincensi et al., 1998; Wenczl et al., 1998a; Wenczl et al., 1998b; Takeuchi et al., 2004;

Samokhvalov et al., 2005; Ye et al., 2006; Ye et al., 2008) and refutes (Sarna and Sealy, 1984b; Sarna et al., 1985; Hennessy et al., 2005) the possible causative role of pheomelanin. The work described herein on intact melanosomes offers a new viewpoint to this debate. The present work indicates that the photoprotective role of the melanosomes is maintained with increased pheomelanin content, as the absorption coefficient at biologically relevant wavelengths is similar on a per melanosome basis. The correlation between epidemiological data and the eumelanin/pheomelanin ratio is predicted to not be a reflection of the melanosome's decreased ability to attenuate biologically relevant wavelengths, but perhaps is instead a reflection of the different photoreactivities of the melanin pigments contained within. This prediction is further supported by the casing model, **Chapter 3**, as the photoprotective, antioxidant eumelanin pigment is thinner for increased relative pheomelanin concentrations. The thinner shell is more susceptible to degradation, and subsequent exposure of the reactive, pro-oxidant pheomelanin core. Further progress in this area depends on determining the quantum efficiency for oxygen activation from studies on intact natural melanosomes.

5.4 Conclusions

The wavelength-dependent quantification of the absorption coefficients of the natural melanins in intact melanosomes, presented herein, reveal that the absorption of the melanosome is independent of its eumelanin:pheomelanin ratio. Modeling the

pigment in terms of the currently known monomeric chromophores, suggests that the extiction coefficient of pheomelanin monomer is greater than that of eumelanin throughout the spectral range examined, similar to what has been deduced from studies of soluble synthetic pigments. These results suggest that on the volume of the melanosome, the correlation between epidemiological data and the eumelanin:pheomelanin ratio is not predicted to be a reflection of a decreased ability to attenuate biologically relevant wavelengths. Thus, because photoprotection is conserved with increased concentrations of pheomelanin, photoreactivity differences are suggested to be the cause of the observed correlation.

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Education

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Publications

John D. Simon, Lian Hong and Dana N. Peles, (2008) Insights into Melanosomes from some Interesting Spatial and Temporal Properties, *Journal of Physical Chemistry B.*, **112**, 13201-13217.

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Honors

Phi Lambda Upsilon, Member since January 2007
    Honorary Chemical Society

Paul Mangus Gross Fellowship, June 2008
    Awarded annually to two graduate students who have met and surpassed academic and research expectations in the doctoral program.

William Krigbaum Fellowship, June 2010
    Awarded annually to a stellar physical chemist.

Charles Bradsher Fellowship, June 2010
    Awarded annually to an outstanding graduate student in chemistry.