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IDENTIFICATION OF MELANIN

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ABSTRACT

Melanin is a ubiquitous natural pigment that is present in many organs in different species. It is well recognized as a UV absorber and as a protective molecule. It harbors stable intrinsic free radicals and binds metals, and molecules. It shows a broad band optical spectrum and a single- x-ray line in addition to a paramagnetic behavior. It is water and organic-solvent insoluble and is highly resistant to chemical attack; thus it has survived through millennia as an intact chemical entity. Electrically and optically responsive electron spins and protonic conductors exit in melanin and it is normally conductive as well as photo responsive. Currently melanin is described as an oligmeric polymer made of various combinations of indolic monomers. Active and protective physiological roles have been identified, and assigned to biologically constituted melanin as well as to internally or externally administered melanin. It has often been suggested as possible pharmacological agent in certain physiological and disease situations. Many diseases are accompanied by an increase or decrease of melanin production by the living organism. It has increasingly been suggested as a component in bio-inspired electronics and as a biological interface. This review brings together data on the biosynthesis, physicochemical characteristics, structure and morphological properties of melanin. Contrary to most of the well-known biological molecules melanin is not clearly definable as a single chemical structure with a recognizable molecular weight. General characteristics of the molecule that makes melanin identifiable as a specific chemical entity are discussed here.

KEYWORDS: Biosynthesis, morphological, physicochemical properties, spectroscopy, melanin.

1. INTRODUCTION

Melanin is a ubiquitous pigment in nature and is a well-known biomolecule that can also be synthetically prepared. It is also recognized as a robust biomolecule that has shown full conservation and chemical stability over millennia; as has been reflected by its discovery in intact form in ancient samples from the Jurassic period.[1,2] It has been hypothesized that melanin has formerly, during some ancient course of the evolutionary process played an important role as an _organizing molecule‘ assuming functions similar to those of enzymes in contemporary evolutionary-developed systems.[3] In recent times melanin has gained more scientific prominence as a natural pigment of considerable significance in both of the biological systems and physicochemical materials applications. Despite of this, the basic functions and molecular structure of melanin remains poorly understood. Under biological conditions melanin is formed via an oxidative polymerization process of phenolic compounds, which gives rise to diverse oligomeric groups of molecules with high molecular weights.[4] When melanins are isolated from living tissue, they produce an insoluble and amorphous substance that defies analysis by simple chemical and physical classical techniques. The early criteria for provisional identification of a brown or black pigment as melanin were based on a set of chemical tests. These tests included resistance to organic solvents and concentrated acids, solublization and degradation by an alkali, oxidation by ammonia-silver salts, and bleaching by oxidizing agents.[5,6] Various physicochemical techniques can be added to this list, including electron spin resonance (ESR), UV-visible spectrophotometry, Fourier transform infrared (FTIR), fluorescence spectra, X-ray diffraction (XRD), nuclear magnetic resonance (NMR), mass spectrometry, and other more recent microscopy techniques and photonic methods. A general approach to the photo-physics and photochemistry of melanin was presented by Zeise and Chedekel.[7] Additionally, a _standardized test‘, based on ESR criteria, for the identification and characterization of melanin, was proposed by Enochs e*t al.*[8] Many health effects have been attributed to melanin including radioprotective, therapeutic, immunological, neurological and other effects. Some of these, like the UV-radioprotective role is well known and widely studied. In some of these biological activities, melanin acts as a powerful energy transformer, redox agent, cation chelator, and/or a free-radical sink.[9,10] The reactive quinone intermediates that arise in the biosynthetic pathway of melanin are also thought to exhibit antibiotic

properties.[11] Published information that advocates melanin as a beneficial component of herbals is US patent No. 6,256,200.[12]

The role of melanin as a scavenging or quenching molecule for superoxide anions and singlet oxygen species has been discussed by Tada *et al.* (2010)[13] who used ESR and spectrophotometric methods to show that melanin interacts potently with reactive oxygen species generated under certain physiological reactions.

Although originally believed to be an amorphous organic semiconductor[14,15] it has been shown by Meredith *et al*. that melanin is an oligomeric molecule built up of a combination of dihydroxyindoles (DHI) and dihydroxyindole carboxlic acid (DHICA).[16] They could then attribute the conductive nature of melanin to a dual electronic and protonic contributions from the extensive oligomeric molecular structure of melanin[17] It has therefore been predicted that unusual nature of this structure can open exciting possibilities for bioelectronics applications, such as acting as an ion-to electron transduction agent[18] in addition to its biological roles.

Melanin is usually classified into three groups: *eu*melanin, *phaeo*melanin, and *allo*melanin.[19] Phaeomelanin are present in red hair and feathers. Allomelanin (*allo*-meaning ―other‖) are generally associated with plants and are formed from nitrogen-free precursors such as catechols and 1,8-dihydroxynaphthalene (DHN). Phenolic quinones have been identified as important intermediates in the biosynthesis of allomelanin.[20]

2. Biosynthesis of melanin

In mammals two well-known melanin pigments exit, eumelanin and pheomelanins. The predominant pigment is eumelanin and it shares many common characteristics with pheomelanin. Eumelanins are dark black or brownish black while pheomelanins are yellowish or reddish in color. A distinctive chemical distinction is that pheomelanins constitute Sulphur as an additional element in their structures, (Fig. 1).

The amino-acid Tyrosine is the transformed into melanin through a number of consecutive processes that occur within the melanosome organelle residing in the melanocyte cells. This transformation is catalyzed via an enzymatic process by tyrosinase. In many living systems the tyrosinase enzyme plays a similar role. The biosynthesis pathway for eumelanin and pheomelanin in mammals are assumed to be as shown in Fig 2.

Melanin is known to exit in many plants, fungi and bacteria. Tyrosinase has been successfully extracted from grapes and mushrooms[21] and its synthesis has been identified as a distinct stage leading towards the darkening of many organisms. This copper containing enzyme catalyzes two different reactions by using molecular oxygen: the hydroxylation of monophenols to orthodiphenols (monophenolase activity) and the oxidation of the ortho-diphenols to ortho-quinones (diphenolase activity)[4] This latter reaction is autocatalytic such that the enzyme is activated by the ortho-diphenol products.[22] For example, tyrosine, DOPA, dopaquinone, and leukodopachrome can condense with betalamic acid to form yellow pigments called betalains.[23,24] A wide range of benzoquinones is produced by plants under the activity of monophenol oxidases, including tyrosinase with creolase activity (EC 1.14.18.1), ortho-diphenolases with catecholase activity (EC 1.10.3.1), and laccase with both ortho- and para-diphenol oxidase activities (EC 1.10.3.2).[25] Although the structures of natural melanin pigments in various animal tissues have not been elucidated owing to the difficulty of separating and purifying them, some melanins have been prepared by incubation of substrates with relevant enzymes.[26,27] Other phenolic compounds in plants might serve as precursors for melanin through oxidative polymerization. For example, yellow to red-brown synthetic melanins were obtained from 4-hydroxycinnamic acid derivatives (e.g., ferulic and caffeic acids), by using hydrogen peroxide and peroxidase enzymes.[28]

3. Chemical and physical properties of melanin

Melanin is generally described as a brown-black, high molecular weight, three-dimensional polymer with a number of possible structures.[29] For example, a melanin complex from the medicinal mushroom *Inonotus obliquus* is characterized by its extinction coefficient , and by the various chemical groups that it contains [e.g. COOH, CO, OCH3, aliphatic groups and OH groups.[30] In general, characterization of melanin is performed by both destructive and non-destructive techniques.[8,31-38] Use of a destructive technique, namely pyrolysis gas chromatograph mass spectroscopy, revealed that 1,4-dihydroxybenzene and catechuic acid are the units that form black sesame melanin,[39] whereas animal melanins are commonly covalently linked to matrix proteins, and plant melanin might be cross-linked to carbohydrates.[6,38]

4. Chemical stability of melanin

The remarkable stability of free radicals in melanin was first outlined in a report by Mason[40] who identified that free-radical stability was dependent upon semi-quinones that were

stabilized by resonance in the highly conjugated polymer and by steric restrictions on internal radical annihilation. The general consensus amongst chemists is that natural melanin is a mixture of quinones and hydroquinones and those can readily be oxidized or reduced. Thus, melanin is considered a redox active heteropolymer that mediates electron transfer under various physiochemical conditions.[10]

5. Morphological properties of melanin

Numerous reports have attempted to describe the size and shape of melanin particles, and many studies have been undertaken to determine the natural format of melanin aggregation in living systems. Procedures employing a wet milling method to avoid extremes in pH and temperature to extract melanin yielded naturally shaped granules.[8,41,42] Fig 3 shows a nanoscale image of an aggregated granule structure of melanin. It is now well agreed that melanin exists in a granular form with variable levels of extracellular distribution amongst the keratinocytes in the skin.

An early scanning tunneling microscopy investigation of melanin synthesized from tyrosine led to the identification of a protomolecule of approximately 20 Å laterally and 10 Å in height.[43] This size was found to be consistent with contemporary models constructed to fit data from wide-angle X-ray diffraction experiments on melanin.[44,45] More recently, Watt *et al*. provided direct evidence of supramolecular organization in both natural and synthetic eumelanin. Melanin sheets of the protomolecules stack to form onion-like nanostructure. The inter-sheet spacing within these structures is between 3.7 and 4.0 Å, consistent with non-covalent π–π stacking in hetero-aromatic systems.[46] Optical microscopy studies of plant melanin have also revealed that melanin, in the form of granules, is preferentially distributed around the cell walls of plant seed coats in which they play a role in structurally hardening and strengthening the cell wall.[47] Studies by synchrotron small-angle X-ray scattering reveled that melanin particles exhibit scattering characteristic of sheet-like structures with a thickness of approximately 11 Angstroms. This led to the hypothesis that melanin forms planar aggregates of 6- to 10-nm-sized melanin protomolecules. A sketch of this model as given by Littrell *et al.*[48] is as given in Fig. 4.

6. Optical absorption of melanin

Melanin is characterized by their dark brown to black colors and strong broad-band absorption of both UV and visible light. This unique property, first reported in 1981,[49] has been recorded for all other brown-to-black melanins. The progressively increasing absorption

from the low frequency red light to the UV frequencies, without showing any specific peaks at any part of this broad band has been an intriguing phenomenon for researchers for many decades. Various models have been suggested to account for this behavior.[50,51] This spectrum as given by Tran *et al.* (2006)[52] for eumelanin and pheomelanin is as shown in Fig. 5.

This apparently simple semi-exponential absorption curve relative to the wavelength range (200–800 nm) cannot be linked to specific chromophores, which suggests that melanins are highly disordered oligomeric organic structures. It has also been found that melanin has an interesting dissipative mechanism of the energy it absorbs within the UV visible part of the spectrum. The dissipative mechanism involves a quick energy transfer via an ESIPT mechanism from the carboxylic acid group towards the indole nitrogen moieties of the melanin molecule as reported by Huijser *et al.*[53] Because of this unique mode of broad-band absorption and a very low emission melanin is regarded as a photo-protective pigment capable of non-radiative conversion of UV and visible light to thermal energy at all UV-visible wavelengths.

7. **Other spectroscopic techniques used to study melanin**

7.1 Infra-red spectra of melanin

A number of authors have reported the infra-red spectra of melanin and have shown that a very specific spectrum of the molecule is easily obtained via FT-IR. The information on this spectrum shows that broad peaks related to OH stretching in the molecule dominate the spectrum at low IR frequencies for hydrated melanin.[54] The effect of extent and type of metallic binding has also been discovered to modify the IR spectra of melanin.[55]

7.2 X-ray

The spectrum of melanins is dominated by a broad non-Bragg diffraction pattern such that the x-ray spectrum of melanin shows only single broad peak that corresponds to an average interlayer separation distances falling in the range of 3.5 A to 4.0 A. Although melanin is generally classified as an amorphous polymer it seems to have a unique graphite-like sheet structure ordering that is manifested within a short range within each of the oligmeric groupings of the molecule.[56]

7.3 Fluorescence of melanin

Despite of the fact that melanin is very efficient in capturing various types of electromagnetic radiations and is also known to convert almost all of the captured photons into thermal phonons it does show a weak fluorescence of a low quantum yield near the green visible region, when excited by UV radiations. Melanin at different concentrations in solution shows wideband fluorescence spectra upon excitation by radiations in the region 219–420 nm. The fluorescence bands are usually in the visible range of approximately 430–500 nm and can be observed across the full pH range at which melanin remains soluble. Gallas (1981)[57] used aqueous suspensions of synthetic DOPA melanin to detect and investigate the fluorescence of the molecule. Hoffmann *et al.* (2001)[58] detected selective femtosecond pulse-excitation of melanin fluorescence in tissue by using near IR pulses. Meredith *et al.* (2004)[59] studied the radiative relaxation quantum yield for synthetic melanin and found that it can dissipate greater than 47.9% of absorbed UV radiation as heat. More recently, Perna *et al.* (2009)[60] published a detailed study on the fluorescence of synthetic melanin in solution. Their measurements support the hypothesis that fluorescence in eumelanin is related to chemically distinct, selectively excitable oligomeric units and that fluorescence resulting from large oligomer systems is spectrally differentiated from fluorescence caused by monomers and small oligomer systems.

8. Electric conductivity of melanin

Melanin has been reported as the first organic conductor to behave both as an electrical conductor that shows a switching behavior as well as being a photoconductive material.[61,62]

Thin films as well as pellets of melanin do show a weak conductivity and a slowly increasing photo-current as a response to irradiation by UV or visible frequencies. On cessation of irradiation the photo-current increment of the current relaxes slowly to the value before irradiation.[63,64] It has been shown that the conductivity increase with increase of temperature. This behavior prompted early researchers to mistakenly classify melanin as an organic semiconductor. More recently researchers at Queens college in Australia have shown that there is an ionic contribution to the conductivity resulting from protons contributed by water vapor absorbed by the molecule [The structural impact of water sorption on device-quality melanin thin films]. Some of the intriguing and increasingly studied is the role of melanin as a biological interface where melanin is used as a scaffolding for connecting devices to the physiological system.[17] More recently fast short-pulse studies in the femto-

second region using an excitation-probe technique has shown that protons within the melanin structure play an important role in the processes of absorption of radiation and its efficient dissipation by melanin as heat.[53]

9. Electron spin resonance of melanin

ESR studies have confirmed the presence of intrinsic stable free radicals in melanin.[65] The same study showed that the numbers of the free radicals show a sensitivity to the chemical and physical environment of the molecules. Rapid generation of additional free radicals in melanin upon exposure to UV light, visible light or increased temperature, and their decay in the dark, was reported by Arnaud *et al*. using ESR.[66]

Subsequent paramagnetic measurements showed that the paramagnetic metals that are sequestered by the structure of natural and synthetic melanin are also readily detectable.[8]

These metals are assumed to be bound to the structure of the melanin molecule.[67] They have been shown to drastically affect the number of observable free radicals in melanin; either by increasing or decreasing them depending on the type of the metal atom.[68]

A study by Cano *et al.* in 2008[69] of the magnetic properties of synthetic eu-melanin shows that the free radicals of melanin possess enough density to show a superparamagnetic behavior at low temperatures. The presence of a stable and persistent free radical system within a living body is unusual since most of the known stable free radicals in chemistry are implicated in various diseases or are known to be carcinogenic[70] presence of free radicals in melanin is attributed by Mason *et al.*[71] to the existence of a stabilized semiquinonoid form of the polymeric pigment. These free radicals are assumed to be deeply sequestered within the melanin molecule and can neither react appreciably with the surrounding short-lived free radicals that arise during most of the physiological reactions nor can they chemically attack the surrounding molecules.

FIGURES

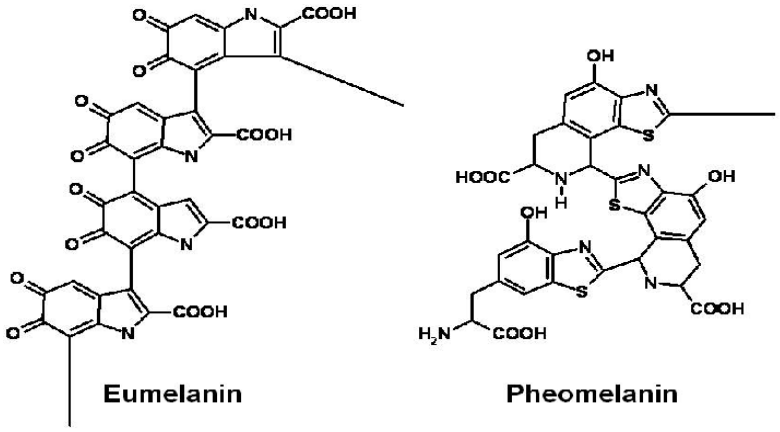

Fig. 1: Example structures of eumelanin and pheomelanin.

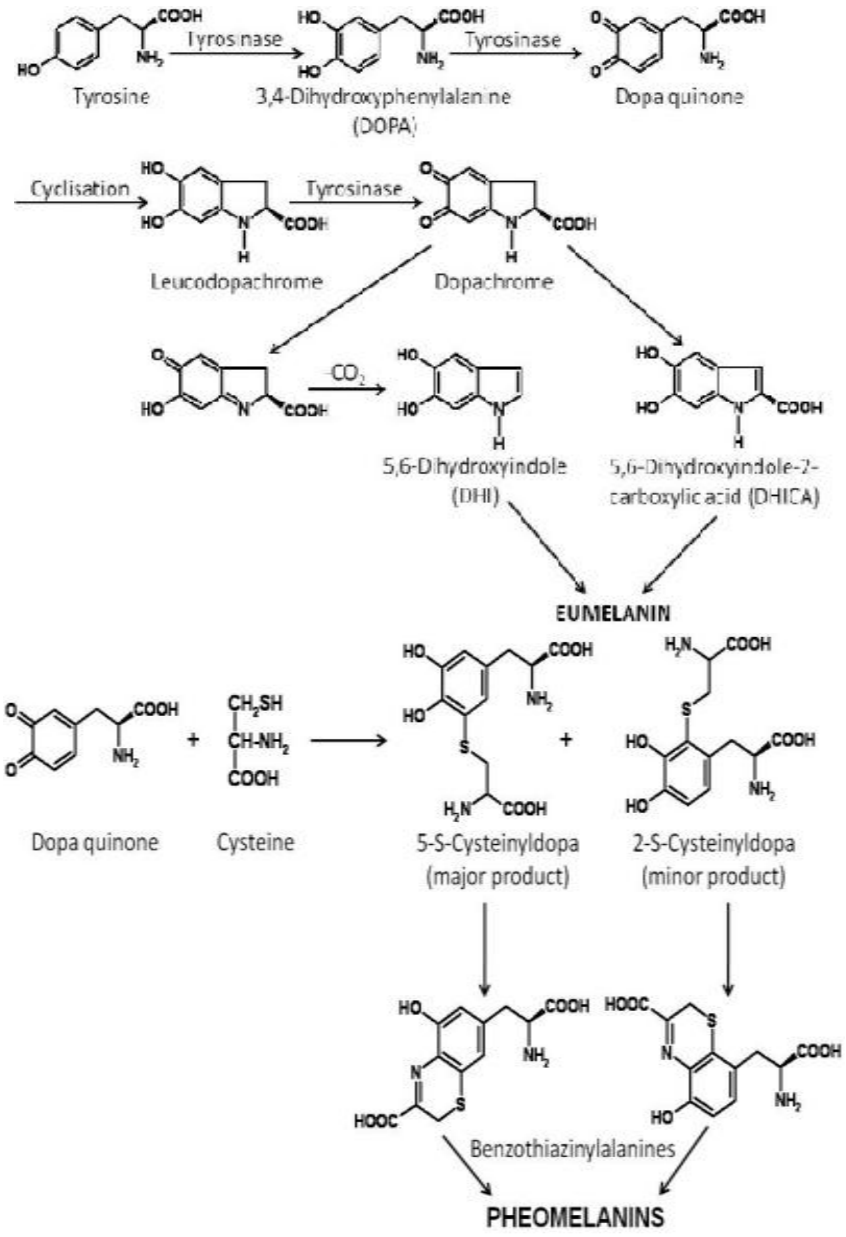

Fig 2: The biosynthetic pathways of eu-melanin and pheomelanin.

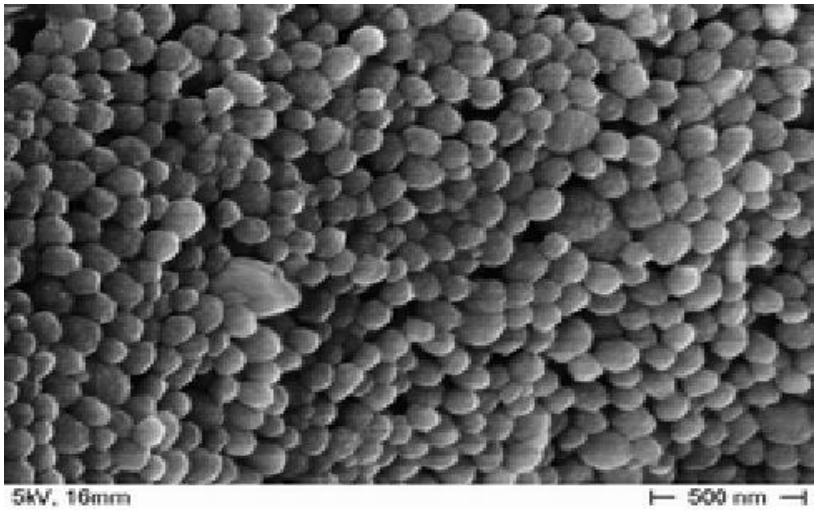

Fig. 3: A nano-scale image of an aggregated granular structure.[16]

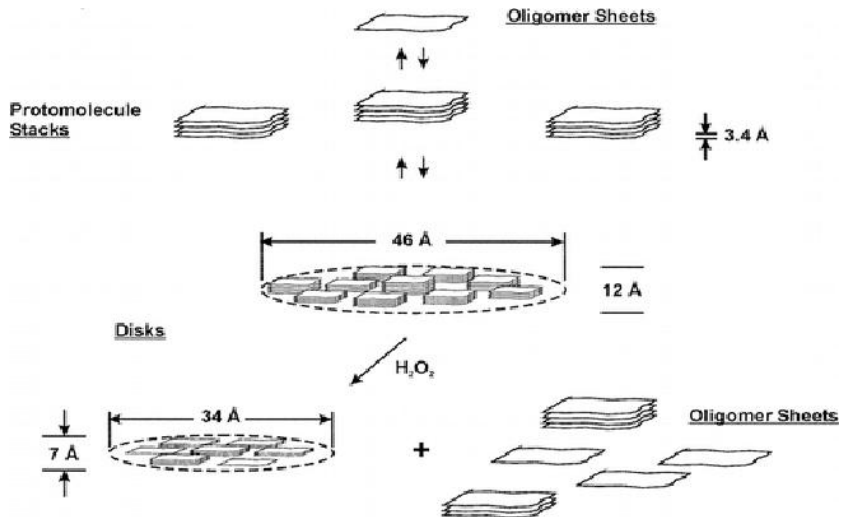

Fig. 4: Planar aggregates of 6–10 nm melanin protomolecules form sheet-like structures approximately 11 Å thick.[48]

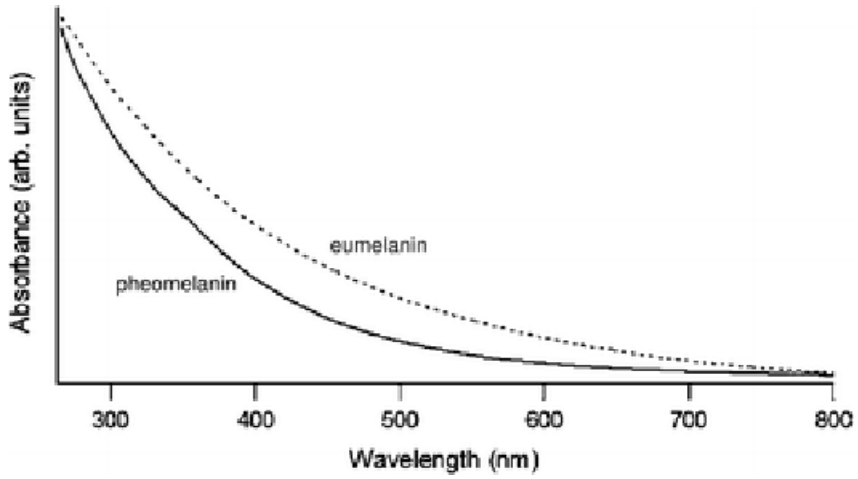

Fig. 5: The broad-band optical absorption spectrum of eumelanin and pheomelanin in the UV-visible region.[52]

DISCUSSION

Although significant efforts are being made to improve the understanding of the structure-function relationship for melanins, the precise determination of their molecular structure and the chemistry and its pathways followed during its myriad set of biological, chemical and physical interactions remain elusive. Indeed, the only accepted approach to describe melanin is through its oligomeric nature and its known variety of functions. For the time being one is only able to identify melanin through various, simultaneously carried out, characterization techniques. The most prominent of these are: ESR, FT-IR, UV-visible, X-rays, electrical conductivity, photoconductivity and FL spectroscopy. Extensive studies on the physicochemical properties of melanin have been undertaken by various authors in order to establish a unique method for identification of the melanin molecule. However, to this date no single, simple and exclusive unique method for identification of melanin is known. An extensive review[72] and an article[73] by Meredith *et al.* in 2006 did point out that a proper understanding of the physical and chemical properties of melanin requires further structure-function-relationship models to be put forward and studied, in order to enable further progress in this field. A large number of reports on the biological, physicochemical and technical applications of melanin have since been published. Many insights have been gained during the past decade on the structure-function relationship for melanin. However, the chemistry of melanin has not yet been fully elucidated to yield a simple definition that may be used for a precise simple identification of it.

ABBREVIATIONS

ESR: Electron Spin Resonance

FTIR: Fourier-transform Infrared

XRD: X-ray Diffraction

NMR: Nuclear Magnetic Resonance

IR: Infra-Red

UV: Ultra Violet

HEV: High Energy Visible

DOPA: Catecholamine 3,4-dihy Hroxyphenylalanine

DHN1: 8-dihydroxynaphthalene

DHI: 5,6-dihydroxy Indole

DHICA: 5,6-Dihydroxyindole-2-carboxylic Acid

T3HN: 1,3,8-trihydroxynaphthalene

DNA: Deoxyribonucleic Acid.

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AUTHORS' CONTRIBUTIONS

ASE, AK, MAKA, AH have collected the literature review, conceived the study, and contributed in writing the final draft of this manuscript. Finally, all authors of this manuscript have read and approved the final manuscript.

COMPETING INTERESTS

The authors of this manuscript declare that they have no competing interests.

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