Biophysical Journal Volume 90 June 2006 4137–4144

Quantitative Scattering of Melanin Solutions

Jennifer Riesz,* Joel Gilmore,† and Paul Meredith*
*Soft Condensed Matter Physics Group, †Condensed Matter Theory Group, Physics Department, University of Queensland, St. Lucia, Brisbane, Australia, QLD 4072

ABSTRACT The optical scattering coefficient of a dilute, well-solubilized eumelanin solution has been accurately measured as a function of incident wavelength, and found to contribute <6% of the total optical attenuation between 210 and 325 nm. At longer wavelengths (325–800 nm), the scattering was less than the minimum sensitivity of our instrument. This indicates that ultraviolet and visible optical density spectra can be interpreted as true absorption with a high degree of confidence. The scattering coefficient versus wavelength was found to be consistent with Rayleigh theory for a particle radius of 38 ± 1 nm. Our results shed important light on the role of melanins as photoprotectants.

INTRODUCTION

Melanin is a biological pigment found in the skin, hair, and eyes of many species, including humans. It is a photoprotectant, but paradoxically has also been implicated in the chain of events that lead to melanoma skin cancer (1–3). Of the two types found in human skin (eumelanin and pheomelanin), eumelanin is the most common, and the most extensively studied. Eumelanin is known to be a macromolecule of dihydroxyindole and dihydroxyindole-carboxylic acid, but the nature of the secondary structure (i.e., the supramolecular organization) is not known (4).

Likely related to its photoprotective role, eumelanin has a broadband absorption spectrum that increases exponentially toward the ultraviolet. This is a highly unusual feature; most biological pigments exhibit distinct absorption bands. The origin of the broadband absorption spectrum of eumelanin has long been the topic of scientific debate, which continues to this day. Galvao and Caldas (5–7) have used Hückel theory to attempt to reproduce the broadband shape, with some success. More recently, density functional theory has been used to predict the optical properties of small eumelanin oligomers (8–14). This has led to the theory that eumelanin may in fact be a collection of different small oligomers of varying electronic structure. The broadband absorption of eumelanin may then be due to the summation of these individual spectra (8,11,12). This idea was recently extended by the suggestion that the broadband absorption may be due to extreme chemical disorder (4,15).

Wolbarsht (16) first suggested that the broadband absorption spectrum may be due to scattering, rather than electronic or physical properties of the eumelanin itself. He noted that Rayleigh scattering would reproduce the broadband spectrum, and account for the increase in optical density at short wavelengths. This has very serious implications; if the measured shape of the absorption spectrum is dominated by scattering, then great care must be taken when calculating optical properties. Despite several studies on the topic, optical scattering remains a significant concern. The published literature on the scattering of eumelanin solutions is sparse and not cohesive, hence it is useful at this point to briefly review past work.

The importance of optical scattering was noted by Nofsinger and Simon (2,17) when they discovered that the shape of the eumelanin absorption spectrum is strongly dependent upon the particle size. Since scattering intensity is very strongly dependent upon particle size, this could indicate that the optical density of eumelanin is dominated by scattering. To test this, they conducted photoacoustic measurements, which suggested that the measured optical density was not dominated by scattering for wavelengths longer than 400 nm for any particle size fraction (2). An earlier photoacoustic calorimetry measurement by Forest and Simon (18) similarly suggested that scattering contributes no more than 15% of the total light extinction at 350 nm. Hence, Nofsinger and Simon concluded that the observed dependence upon particle size was due to electronic and physical properties of the eumelanin.

Recently, a number of optical emission and excitation studies have been published, which report accurate quantitative measurement of key properties such as the radiative quantum yield as a function of wavelength (19–22). Such studies provide valuable insight into energy absorption and dissipation mechanisms, as well as shedding light on the structural question. These measurements require the assumption that scattering is negligible. If this is not the case, the scattering coefficient should be measured and subtracted from the optical density to obtain the true absorption. This was attempted by Krysciak (23), who directly measured the optical scattering from a dilute eumelanin solution as a function of wavelength. He found scattering to be negligible between 500 and 700 nm, but also discovered the puzzling result of negative scattering at shorter wavelengths. He suggested that this was due to multiple

Submitted October 11, 2005, and accepted for publication February 15, 2006.

Address reprint requests to J. Riesz, Tel.: 61-7-3365-3406; Fax: 61-7-3365-1242; E-mail address: riesz@physics.uq.edu.au.

0006-3495/06/06/4137/08 $2.00
doi: 10.1529/biophysj.105.075713

scattering events and absorption (which becomes very large at shorter wavelengths) decreasing the measured scattering below the previously measured baseline. Krysciak's results were nonconclusive, neither confirming nor excluding the presence of scattering at optical wavelengths.

The following year, Kurtz (24) reported on a theoretical prediction of the relative contributions of scattering and absorption to the optical density of eumelanin. He found that in the Rayleigh regime (particle radii much less than the wavelength) absorption dominated over scattering, whereas for larger particles the two contributed equally. He emphasized the very strong dependence of scattering on particle size. The importance of this is experimentally apparent in a 2001 study by Sardar et al. (25), where scattering and absorption coefficients were measured at four optical wavelengths between 633 and 476 nm. They found that scattering far outweighed absorption at all wavelengths, contributing >99% of the optical density at 633 nm. This result contradicts all previous studies, and is almost certainly due to the sample preparation, which resulted in what was described as ''a brown turbid suspension.'' The authors state that the eumelanin particles were not solubilized and remained a particulate suspension. Under these conditions, the particle sizes would most likely be much larger than those in the well-solubilized, dilute solutions typically used for spectroscopic studies (2,17,19–22).

Other studies have attempted to use alternative methods to measure the absorption of eumelanin in the absence of scattering effects. Caiti et al. (26) used photoacoustic phase angle spectroscopy of powdered melanins in the dry state. This technique is insensitive to scattering, and confirmed unambiguously the decrease in the absorption of melanins with increasing wavelength. Unfortunately, the phase spectra do not correspond by visual inspection to absorption spectra, and interpretation remains difficult. Therefore, while this study sheds doubt on the Wolbarsht model, it does not allow correction of absorption spectra for scattering effects in a quantitative way. Similarly, a recent study by Albuquerque et al. (27) used photopyroelectric spectroscopy to measure the optical absorption coefficient of eumelanin in the solid state. Again, the decrease in absorption with increasing wavelength was confirmed, although a direct comparison with solution measurements could not be made due to the different properties of the system. Interestingly, a band gap was observed at 1.70 eV (730 nm), which is possibly hidden in solution spectra by scattering.

A careful study by Vitkin et al. (28) in 1994 gives the most quantitative estimate available of the scattering coefficient of a eumelanin solution. Vitkin et al. (28) conducted photometric measurements with a double integrating sphere system at 580 and 633 nm. They found that scattering contributed 12% and 13.5% of the total attenuation coefficient at each wavelength, respectively. These values, while small, are enough to introduce significant error in the measurement of the radiative quantum yield and other optical parameters, and should ideally be corrected for. A measurement of the scattering coefficient as a function of wavelength would allow the subtraction of scattering effects from the optical density spectrum to achieve this.

If the scattering coefficient as a function of wavelength were known, the shape of the scattering spectrum could be compared with Rayleigh theory. As stated earlier, there remains debate as to the secondary structure of eumelanin: heteropolymer or nanoaggregate (13,14). This is a most fundamental question, since it influences the interpretation of many other experiments. Since Rayleigh scattering is strongly dependent upon particle size, these scattering measurements can also be used to determine a fundamental particle size of eumelanin in solution. Hence, we have conducted an integrated scattering measurement as a function of wavelength over the ultraviolet range, where scattering effects should be most significant.

In addition, the solutions used by Vitkin et al. (28) (0.07–0.12% eumelanin by weight) were more concentrated than those best suited to photoluminescence measurements. The broadband absorption spectrum of eumelanin gives rise to significant reabsorption and inner filter effects at concentrations above 0.0025% by weight (19,20). Although scattering should scale linearly with concentration, it is feasible that there is less aggregation at lower concentrations, giving rise to less scattering. Hence, we have made a direct measurement of the scattering coefficient at the ideal spectroscopic concentration.

In this study, we endeavor to:

1. Measure the integrated scattering from an optical spectroscopy grade eumelanin solution as a function of wavelength from 210 nm to 325 nm.
2. Develop general equations to measure the scattering in broadband absorbing samples, and apply these to the specific case of a eumelanin solution.
3. Show that the measured scattering is consistent with Rayleigh theory, and use this to estimate an approximate particle size.

EXPERIMENTAL METHODS

Sample preparation

Synthetic eumelanin (dopamelanin) derived from the nonenzymatic oxidation of tyrosine was purchased from Sigma-Aldrich (St. Louis, MO), and used without further purification. The powder was solubilized to form a 0.1% solution (by weight) in high purity 18.2 MΩ MilliQ deionized water (Millipore, Billerica, MA). This stock solution was then diluted to a concentration (by weight) of 0.0025%. To aid solubility, the pH of the solution was adjusted to ~pH 11.5 using NaOH, and the solution gently heated with sonication. Under such conditions, a pale brown, apparently continuous eumelanin dispersion was produced. This is identical to the sample preparation typically used for spectroscopic analysis (19,20). This concentration is usually selected since it maximizes the weak photoluminescence signal while minimizing distorting reabsorption and probe beam attenuation effects.

Absorption spectrometry

An absorption spectrum between 200 and 800 nm was recorded for the synthetic eumelanin solution using a Perkin Elmer (Boston, MA) Lambda 40 spectrophotometer. An integration of 2 nm, scan speed of 240 nm/min, and slit width of 3-nm bandpass were used. The spectrum was collected using a quartz 1-cm square cuvette. Solvent scans (obtained under identical conditions) were used for background correction.

Integrated scattering

Scattering measurements were made using a Perkin Elmer Lambda 40 spectrophotometer with an integrating sphere attachment (model No. RSA-PE-20 reflectance spectroscopy accessory, Labsphere, North Sutton, NH). The solution was contained within a 1-mm pathlength quartz cuvette that was placed at the front and back of the sphere as shown in Fig. 1, *b* and *c*, to measure the forward and backward integrated scattering, respectively. Scattering anisotropy was not of concern for this study, since we are interested only in the total scattering signal. Measurements were taken with a scan speed of 120 nm/min, a slit width of 4-nm bandpass, and 2-nm smoothing. Since the scattering intensity was very low, each scan was taken five times and averaged. The 100% reflectance intensity was determined using a Labsphere certified reflectance standard (as shown in Fig. 1 *a*). The solvent alone was measured in both the front and back positions (Fig. 1, *b* and *c*) and subtracted after absorption correction (described in the following section). Some light was inevitably lost due to the nonzero size of the beam entry and exit holes in the sphere, and due to the width of the cuvette. This loss, along with the nonperfect reflectivity of the inside of the sphere, was accounted for by the use of the 100% transmission measurement as a standard. A short pathlength cuvette (1 mm) was used to minimize this loss.

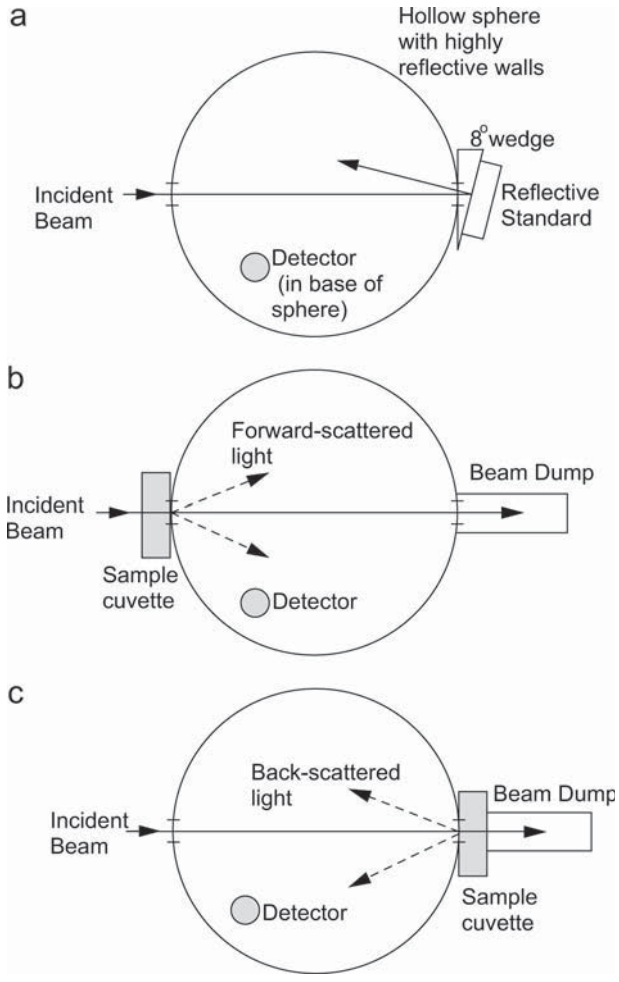

FIGURE 1 (*a*) Geometry for 100% transmission standard. (*b*) Geometry to collect forward scattered light. (*c*) Geometry to collect backward scattered light.

THEORY

Eumelanin solutions have strong, broadband absorbance, and all optical spectroscopic results are therefore affected by reabsorption (attenuation of photoluminescence) and inner filter (attenuation of the incident beam) effects. Although a narrow cuvette and dilute concentration were used to minimize these effects, it was necessary to perform a careful analysis to account for attenuation of the measured scattering by absorption. We derive here a general method for correcting for absorption effects in scattering measurements that can be applied to any strongly absorbing solution.

We define α_{sf} to be the forward scattering coefficient, α_{sb} to be the backward scattering coefficient, and α_s to be the total scattering coefficient, such that $\alpha_{sf} + \alpha_{sb} = \alpha_s$. The absorption coefficient is given by α_a and the total attenuation coefficient is given by α_t. We assume that $\alpha_a = \alpha_t - \alpha_s$ (any attenuation not due to scattering is included in the absorption coefficient). Consider a cuvette of width d, with a beam of light incident from the left, as shown in Fig. 2. By definition, in a small region dx, the attenuation of the beam due to each effect (scattering or absorption) is proportional to each αdx, and to the intensity of the beam in that region ($I(x)$). Therefore,

$$dI(x) = -\alpha_{sf}I(x)dx - \alpha_{sb}I(x)dx - \alpha_a I(x)dxI(x) = I_0 e^{-\alpha_t x},$$

which is the familiar Beer-Lambert law, where I_0 is the intensity of light incident upon the cuvette. Therefore, the intensity of light scattered forward (I_{sf}) is given by

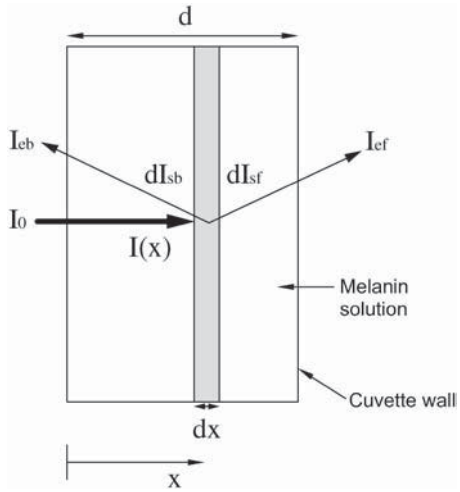

FIGURE 2 Cuvette geometry.

$$I_{sf} = \int_0^d dI_{sf} = \int_0^d \alpha_{sf}[I_0 e^{-\alpha_t x}]dx = \frac{\alpha_{sf}}{\alpha_t} I_0(1 - e^{-\alpha_t d}). \quad (1)$$

Correction for absorption

We make the geometric approximation that the light scattered in the forward direction will travel a pathlength of $d–x$ to leave the cuvette (see Fig. 2). As the scattered light travels this distance through the eumelanin solution, it will be attenuated by absorption. We assume that attenuation is only due to absorption here, and not scattering, since multiple scattering is known to be negligible for melanin solutions at this concentration. (Multiple scattering is negligible if the total attenuation coefficient is linear in concentration (29,30). This has been previously shown to be the case for melanin solutions at the concentrations used in this study (19).) Let the final intensity emitted forward from the cuvette (attenuated by absorption) be given by I_{ef}. Using the Beer-Lambert law,

$$\begin{aligned} I_{ef} &= \int_0^d dI_{ef} \\ &= \int_0^d (e^{-\alpha_a(d-x)} dI_{sf}) \\ &= \int_0^d (e^{-\alpha_a(d-x)} \alpha_{sf} e^{-\alpha_t x} I_0 dx) \\ &= \frac{\alpha_{sf}}{\alpha_t - \alpha_a} e^{-\alpha_a d}[1 - e^{-(\alpha_t - \alpha_a)d}] I_0. \end{aligned} \quad (2)$$

To determine the amount of light that was originally scattered (I_{sf}) from the attenuated intensity that we measure (I_{ef}), we combine Eqs. 1 and 2 to eliminate I_0,

$$I_{sf} = \frac{\alpha_t - \alpha_a}{\alpha_t}\left(\frac{1 - e^{-\alpha_t d}}{e^{-\alpha_a d} - e^{-\alpha_t d}}\right) I_{ef} - B_f, \quad (3)$$

where we must subtract off the background signal (B_f), which is measured from a blank cuvette (containing solvent only) to remove scattering from the solvent and cuvette walls. This process can be repeated in a very similar manner for the backward scattering to find

$$I_{sb} = \frac{\alpha_t + \alpha_a}{\alpha_t}\left(\frac{1 - e^{-\alpha_t d}}{1 - e^{-(\alpha_t + \alpha_a)d}}\right) I_{eb} - B_b, \quad (4)$$

where I_{sb} is the intensity of light scattered backward, I_{eb} is this intensity attenuated by absorption, and B_b is the background scattering in the backward direction. Note that the different form of the equation is due to the fact that the absorption for back scattering is calculated over a distance x rather than $d–x$, as shown in Fig. 2.

We have made several assumptions in the above derivations, which we will now summarize. First, it is assumed that the incident beam is scattered at a small enough angle (either forward or backward) that we can make geometric approximations of the pathlength, as described earlier. This is justified by the $cos^2(\theta)$ angular dependence of Rayleigh scattering, which therefore requires that the scattering particles are small enough to be within the Rayleigh regime (particle diameter much less than the incident wavelength). We also assume that we can define a scattering coefficient as an intensive property of a melanin solution (scattering per unit length). This is true only if no multiple scattering events occur, which is true for sufficiently dilute solutions (as is the case here). Absorption of the scattered light, detection efficiency, and background noise are all accounted for in the method outlined below. Thus, this method can be applied to any sample of small scattering particles (diameter much smaller than the wavelength of the incident light) sufficiently dilute such that the total attenuation is linear with concentration, where absorption is believed to be affecting the measured scattering intensity.

Comparison with experiment

We must now take into account the actual manner in which the intensity of the scattered light was measured. We define S to be the light received by the detector as a percentage of the maximum light received with a standard reflector in place of the beam-dump (see Fig. 1):

$$S = \frac{I_{recorded}}{I_{max}} \times 100\%.$$

Assuming the detector receives a constant fraction of the true scattered light, and 100% of the light is scattered by the standard reflector in the calibration test,

$$S = \frac{I_{scatt}}{I_0} \times 100\%, \quad (5)$$

where I_{scatt} is scattering in either the forward or backward direction. Thus, S is the percentage of incident light scattered by the sample. However, the detected values are affected by absorption. Let S_{mf} be the scattering signal actually measured (affected by absorption):

$$S_{mf} = \frac{I_{ef}}{I_0} \times 100\%.$$

Since S is linear in I we can apply the recorrection given in Eq. 3 to obtain S_f, the true percentage of I_0 that is scattered forward,

$$S_f = \frac{\alpha_t - \alpha_a}{\alpha_t}\left(\frac{1 - e^{-\alpha_t d}}{e^{-\alpha_a d} - e^{-\alpha_t d}}\right) S_{mf} - S_{BGf}, \quad (6)$$

where S_{BGf} is the background scattering signal measured in the forward directions. Similarly for scattering backward,

$$S_b = \frac{\alpha_t + \alpha_a}{\alpha_t}\left(\frac{1 - e^{-\alpha_t d}}{1 - e^{-(\alpha_t + \alpha_a)d}}\right) S_{mb} - S_{BGb}, \quad (7)$$

where S_b is the percentage of incident light scattered backward, S_{mb} is this percentage attenuated by absorption, and S_{BGb} is the percentage scattered backward in the background measurement.

Determining the scattering coefficient

Finally, we must relate these to the total scattering coefficient, α_s. Combining Eqs. 1 and 5 and similar equations for backscattering we find that the total scattering, $S = S_f + S_b$, is given by

$$\frac{S}{100} = \frac{\alpha_s}{\alpha_t}(1 - e^{-\alpha_t d}).$$

Combining this with Eqs. 6 and 7, we find

$$\frac{\alpha_t - \alpha_a}{\alpha_t}\left(\frac{1 - e^{-\alpha_t d}}{e^{-\alpha_a d} - e^{-\alpha_t d}}\right)\frac{S_{mf}}{100} - \frac{S_{BGf}}{100} + \frac{\alpha_t + \alpha_a}{\alpha_t}$$
$$\left(\frac{1 - e^{-\alpha_t d}}{1 - e^{-(\alpha_t + \alpha_a)d}}\right)\frac{S_{mb}}{100} - \frac{S_{BGb}}{100} = \frac{\alpha_s}{\alpha_t}(1 - e^{-\alpha_t d}). \quad (8)$$

Since $\alpha_a = \alpha_t - \alpha_s$, this equation has only one unknown (α_s) and can be solved (S_{mf}, S_{mb}, S_{BGf}, S_{BGb}, α_t, and d are all measurable). This must be done numerically, since α_s appears nontrivially on both sides.

RESULTS AND DISCUSSION

Fig. 3 shows the absorption coefficient for a 0.0025% (by weight) solution of synthetic eumelanin over the visible and UV range. It is typically broadband, and in excellent agreement with previously published absorption spectra of eumelanins (2,16,17,19,23,31–33). The measured scattering coefficient for the same solution is also shown, as a function of wavelength between 210 and 325 nm (calculated using Eq. 8). For wavelengths longer than 325 nm the scattering coefficient was less than the minimum sensitivity of the instrument. We expect that scattering will decrease at longer wavelengths; Rayleigh scattering, for particles with radii smaller than ~50 nm has a λ^{-4} dependence, and Mie scattering, for larger particles, is independent of wavelength. It is therefore reasonable to assume that the scattering coefficient is less than the measured values over the whole visible range.

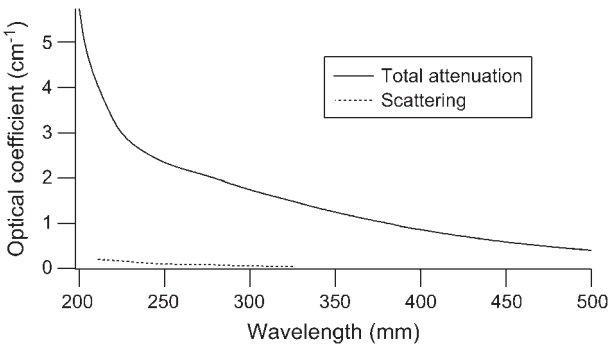

FIGURE 3 Total attenuation and scattering coefficients for a 0.0025% (by weight) solution of synthetic eumelanin.

The percentage of the total attenuation due to scattering ($\alpha_s/\alpha_t \times 100$) was calculated as a function of wavelength, and is plotted in Fig. 4. It can be shown that the ratio of the coefficients is equivalent to the ratio of the intensities,

$$\frac{\alpha_s}{\alpha_t} = \frac{I_s}{I_s + I_a}, \quad (9)$$

where I_a is the intensity of light lost due to absorption and I_s is the intensity of light lost due to scattering. Hence this quantity gives the percentage of the lost intensity that is due to scattering. It can be seen from Fig. 4 that scattering contributes <6% of the total loss at all wavelengths within the measured range. This means that measured absorption spectra (total loss spectra) of eumelanin can be assumed to be primarily due to actual absorption, and used for interpretation of spectroscopic data without further manipulation. This allows accurate determination of important quantities such as the radiative quantum yield of eumelanin (19). This percentage is less than that measured by Vitkin et al. (28) (12% at 580 nm and 13.5% at 633 nm), and possibly indicates less aggregation in our more dilute solutions.

Prediction of scattering coefficient

The scattering coefficient appears to exhibit a dependency upon the wavelength (Fig. 5), which is suggestive of Rayleigh scattering, rather than Mie scattering (which is independent of wavelength). Let us therefore determine whether the measured scattering coefficient is consistent with Rayleigh scattering alone (no Mie scattering). As shown by Jackson (34), in the Rayleigh limit (particles much smaller than the wavelength of the incident light), the scattering coefficient

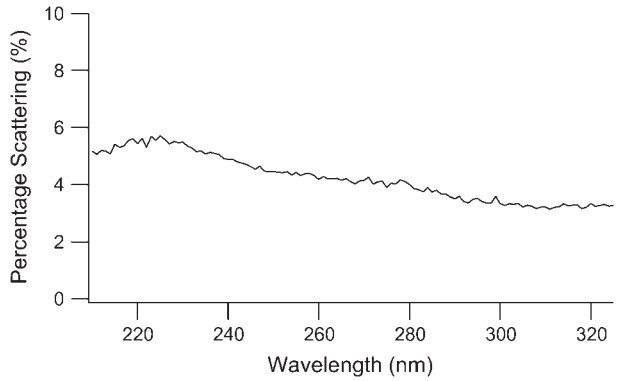

FIGURE 4 The scattering coefficient (as plotted in Fig. 3) as a percentage of the total attenuation coefficient for the same solution. We see that even over this short wavelength range where scattering should be most significant, it contributes <6% of the total attenuation.

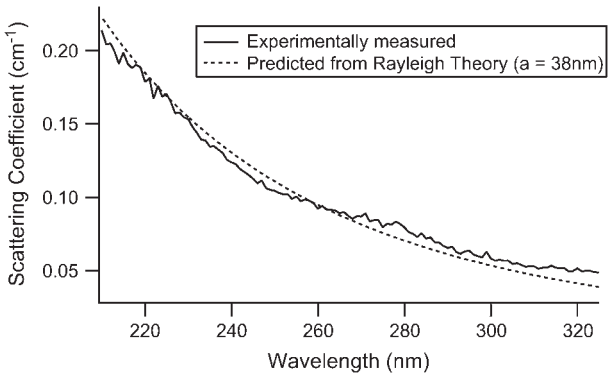

FIGURE 5 The eumelanin scattering coefficient, with the predicted Rayleigh scattering coefficient (from Eq. 11). The best fit (*plotted above*) was obtained with a particle radius of 38 nm.

(α_s) for dielectric spheres of radius a with dielectric constant E in a vacuum is given by

$$\alpha_s = \frac{128\pi^5}{3}\frac{Na^6}{\lambda^4}\left|\frac{\epsilon - 1}{\epsilon + 2}\right|^2,$$

where λ is the wavelength of the illuminating light and N is the number of spheres per unit volume. This calculation can be repeated with the spheres in a solvent of dielectric constant ϵ_s to show that the scattering coefficient is then given by

$$\alpha_s = \frac{128\pi^5}{3}\frac{Na^6}{\lambda^4}\left|\frac{\epsilon - \epsilon_s}{\epsilon + 2\epsilon_s}\right|^2. \quad (10)$$

Hence, knowing the way that the scattering coefficient depends upon the wavelength, we can estimate the size of the particles giving rise to scattering. Unfortunately, it is nontrivial to apply this to melanins, since the structure of the fundamental particles is unknown. This makes determining the number of particles per unit volume challenging. Nevertheless, we can make some assumptions about the structure to determine an estimate of the particle size.

In the absence of a better structural model, it is a fair assumption that eumelanin monomers form globular particles (approximately spherical). The volume of each particle will be equal to the number of monomers per particle (n_p) multiplied by the ''volume of a single monomer'' (V_m), which can be estimated to be 1.2×10^{-28}m^3 (36–38). Hence,

$$n_p = \frac{4}{3}\pi a^3\frac{1}{V_m}.$$

The molecular weight of a dihydroxyindole monomer is 149 g/mol. The molecular weight of an aggregate will therefore be 149 n_pg/mol. Let C be the concentration of our solution in weight percent, such that $C = 2.5 \times 10^{-5}$ for a solution that is 0.0025% eumelanin by weight. Taking the density of the solvent (water) to be 1 g/cm^3, 1 cm^3 of solution will contain C grams of eumelanin, or $C/(149\ n_p)$ moles of eumelanin aggregates. The number of aggregates per cm^3 of solution will then be given by

$$N = \frac{N_A C}{149 n_p} = \frac{3N_A C V_m}{596\pi a^3},$$

where $N_A = 6.02214 \times 10^{23}$ is Avogadro's number. Applying this to Eq. 10, we find

$$\alpha_s = \frac{32}{447}N_A\pi^4 C V_m\frac{a^3}{\lambda^4}\left|\frac{\epsilon - \epsilon_s}{\epsilon + 2\epsilon_s}\right|^2. \quad (11)$$

The dielectric constant for eumelanin (ϵ) has been measured to be $\approx$2.72 at optical frequencies (633 nm) (38,39). The dielectric constant for water (ϵ_s) is known to be $\approx$1.81 at optical frequencies (40,41). The value V_m has been estimated to be 1.2×10^{-28} m^3, as discussed above. Knowing these parameters, we can fit the scattering coefficient versus wavelength curve by varying the particle size, a. Although we have used several very rough assumptions about the structure of eumelanin, the particle radius is to the third power in the equation for the scattering coefficient. The scattering is therefore strongly dependent upon the particle size and it can be determined somewhat accurately from a measurement of scattering.

This was done over the range 210–325 nm, where accurate scattering data was available, as shown in Fig. 5. The best fit was found for a particle radius of 38 $\pm$ 1 nm. The good fit of the data to Rayleigh scattering theory suggests that we are in fact measuring scattering, and not another phenomenon (instrumental or otherwise). This particle size is larger than that predicted by Cheng et al. (35,36), and possibly suggests that the protomolecules further aggregate. Larger particles were measured by Vitkin et al. (28), who report a particle size distribution for a similar sample preparation that has most particles with radii in the range 10–70 nm. Hence an approximate particle size of 38 nm is reasonable.

It is not clear how these in vitro particle sizes relate to those in vivo. Biologically, melanin is synthesized in cellular organelles called melanosomes whose size and shape varies widely depending upon the species and where the cell is located in the organism. The ultrastructure of these melanosomes also varies widely, and is very poorly characterized (42). The structure will also be heavily influenced by the protein to which melanin is strongly bound in vivo. By studying synthetic melanin samples (protein free, and much simpler than the complete biological system), we aim to understand the behavior and structure of the fundamental melanin particles. This will hopefully shed light on the ultrastructure of melanosomes and therefore their biological functionality.

Melanosomes can be quite large (2–3 μm) and therefore could be expected to scatter light strongly (as will tissue generally, being a complex collection of organelles of varying sizes). It has been questioned whether this scattering could potentially contribute to photoprotection, or whether

melanin absorption is solely responsible. If the outer layer of skin were highly scattering this would shield inner cells in an identical manner to an absorbing layer of the same optical density. In light of these results, however, we believe that melanin functions primarily by absorbing light and dissipating it nonradiatively, rather than scattering the incident radiation.

CONCLUSION

The integrated scattering of a eumelanin solution was measured as a function of incident wavelength, and found to contribute $<6\%$ of the optical density between 210 and 325 nm. This means that eumelanin absorption spectra can be interpreted as actual absorption with a high degree of confidence, and allows the calculation of many other optical spectroscopic quantities, such as the radiative quantum yield, without direct subtraction of scattering (19). Hence, as long as eumelanin spectroscopic solutions are appropriately prepared and well solubilized, scattering is not a concern. The scattering coefficient versus wavelength was found to fit Rayleigh Theory with a particle radius of 38 ± 1 nm. This is a larger estimate of the fundamental particle size than those previously reported from x-ray scattering and microscopy studies (35–37), and perhaps indicates that in our samples the fundamental particles have aggregated. This is consistent with other optical studies (28). Knowing the physical structure of eumelanin particles is essential for interpretation of spectroscopic results, and therefore for understanding the de-excitation pathways in eumelanin and its biological functionality.

This work has been supported by the Australian Research Council, the University of Queensland Center for Biophotonics and Laser Science, and the University of Queensland (Research Infrastructure Fund scheme).

REFERENCES

1. Hill, H., L. Zeise, M. Chedekel, and T. Fitzpatrick. 1995. Is melanin photoprotective or photosensitizing? *In* Melanin: Its Role in Human Photoprotection. Vladenmar Press, Overland Park, KS.
2. Nofsinger, J., S. Forest, and J. Simon. 1999. Explanation for the disparity among absorption and action spectra of eumelanin. *J. Phys. Chem. B*. 103:11428–11432.
3. Menon, I., and H. Haberman. 1997. Mechanisms of action of melanins. *Br. J. Dermatol.* 97:109–112.
4. Tran, L., B. Powell, and P. Meredith. 2006. Chemical and structural disorder in eumelanins: a possible explanation for broadband absorbance. *Biophys. J.* 90:743–752.
5. Galvao, D., and M. Caldas. 1988. Polymerization of 5.6-indolequinone: a view into the band structure of melanins. *J. Chem. Phys.* 88: 4088–4091.
6. Galvao, D., and M. Caldas. 1990. Theoretical investigation of model polymers for eumelanins. I. Finite and infinite polymers. *J. Chem. Phys.* 92:2630–2636.
7. Galvao, D., and M. Caldas. 1990. Theoretical investigation of model polymers for eumelanins. II. Isolated defects. *J. Chem. Phys.* 93:2848–2853.
8. Stark, K., J. Gallas, G. Zajac, J. Golab, S. Gidanian, T. McIntire, and P. Farmer. 2005. Effect of stacking and redox state on optical absorption spectra of melanins—comparison of theoretical and experimental results. *J. Phys. Chem. B*. 109:1970–1977.
9. Il'ichev, Y., and J. Simon. 2003. Building Blocks of eumelanin: relative stability and excitation energies of tautomers of 5,6-dihydroxyindole and 5,6-indolequinone. *J. Phys. Chem. B*. 107: 7162–7171.
10. Bolivar-Marinez, L., D. Galvao, and M. Caldas. 1999. Geometric and spectroscopic study of some molecules related to eumelanins. 1. Monomers. *J. Phys. Chem. B*. 103:2993–3000.
11. Stark, K., J. Gallas, G. Zajac, M. Eisner, and J. Golab. 2003. Spectroscopic study and simulation from recent structural models for eumelanin. I. Monomer, Dimers. *J. Phys. Chem. B*. 107:3061–3067.
12. Stark, K., J. Gallas, G. Zajac, M. Eisner, and J. Golab. 2003. Spectroscopic study and simulation from recent structural models for eumelanin. II. Oligomers. *J. Phys. Chem. B*. 107:11558–11562.
13. Powell, B. 2005. 5,6-Dihydroxyindole-2-carboxylic acid (DHICA): a first-principles density-functional study. *Chem. Phys. Lett.* 402:111–115.
14. Powell, B., T. Baruah, N. Bernstein, K. Brake, R. McKenzie, P. Meredith, and M. Pederson. 2004. A first principles density-functional theory calculation of the electronic and vibrational structure of the key melanin monomers. *J. Chem. Phys.* 120:8608–8615.
15. Meredith, P., B. Powell, J. Riesz, S. Nighswander-Rempel, M. Pederson, and E. Moore. 2006. Towards structure-property-function relationships for eumelanin. *Soft Matter*. 2:37–44.
16. Wolbarsht, M., A. Walsh, and G. George. 1981. Melanin, a unique biological absorber. *Appl. Opt.* 20:2184–2186.
17. Nofsinger, J., and J. Simon. 2001. Radiative relaxation of sepia eumelanin is affected by aggregation. *Photochem. Photobiol.* 74:31–37.
18. Forest, S., and J. Simon.1998. Wavelength-dependent photoacoustic calorimetry study of melanin. *Photochem. Photobiol.* 68:296–298.
19. Meredith, P., and J. Riesz. 2004. Radiative relaxation quantum yields for synthetic eumelanin. *Photochem. Photobiol.* 79:211–216.
20. Riesz, J., J. Gilmore, and P. Meredith. 2004. Quantitative photoluminescence of broad-band absorbing melanins: a procedure to correct for inner filter and re-absorption effects. *Spectrochim. Acta A*. 61:2153–2160.
21. Nighswander-Rempel, S., J. Riesz, J. Gilmore, J. Bothma, and P. Meredith. 2005. Quantitative fluorescence excitation spectra of synthetic eumelanin. *J. Phys. Chem. B*. 109:20629–20635.
22. Nighswander-Rempel, S., J. Riesz, J. Gilmore, and P. Meredith. 2005. A quantum yield map for synthetic eumelanin. *J. Chem. Phys.* 123: 194901.
23. Krysciak, J. 1985. Light Scattering and absorption spectrum of alkaline melanin solutions. *Folia Biol.* (*Krakow*). 33:49–54.
24. Kurtz, S. 1986. Light scattering calculations for melanin pigments from the Rayleigh to the Mie regime. *J. Invest. Dermatol.* 87:400.
25. Sardar, D., M. Mayo, and R. Glickman. 2001. Optical characterisation of melanin. *J. Biomed. Opt.* 6:404–411.
26. Caiti, E., P. Crippa, and C. Viappiani. 1993. Application of photoacoustic phase angle spectroscopy (ΦAS) to eumelanins and pheomelanins. *Pigment Cell Res.* 6:140–144.
27. Albuquerque, J., C. Giacomantonio, A. White, and P. Meredith. 2005. Study of optical properties of electropolymerised melanin films by photopyroelectric spectroscopy. *Eur. J. Biophys.* 35:190–195.
28. Vitkin, A., J. Woolsey, B. Wilson, and R. Anderson. 1994. Optical and thermal characterization of natural *Sepia officinalis* melanin. *Photochem. Photobiol.* 59:455–462.
29. Bricaud, A., A. Morel, and L. Prieur. 1983. Optical efficiency factors of some phytoplankters. *Limnol. Oceanogr.* 285:816–832.
30. Nelson, N., and B. Prezelin. 1993. Calibration of an integrating sphere for determining the absorption coefficient of scattering suspensions. *Appl. Opt.* 32-33:6710–6717.
31. Ou-Yang, H., G. Stamatas, and N. Kollias. 2004. Spectral responses of melanin to ultraviolet A irradiation. *J. Invest. Dermatol.* 122:492–496.

32. Nofsinger, J., T. Ye, and J. Simon. 2001. Ultrafast nonradiative relaxation dynamics of eumelanin. *J. Phys. Chem. B.* 105:2864–2866.

33. Nofsinger, J., E. Weinert, and J. Simon. 2002. Establishing structure-function relationships for eumelanin biopolymers. *Biospectroscopy.* 67:302–305.

34. Jackson, J. 1999. Classical Electrodynamics, 3rd Ed. John Wiley and Sons, New York.

35. Cheng, J., S. Moss, and M. Eisner. 1994. X-ray characterization of melanins. I. *Pigment Cell Res.* 7:255–262.

36. Cheng, J., S. Moss, M. Eisner, and P. Zschack. 1994.X-ray characterization of melanins. II.*Pigment Cell Res.*7:263–273.

37. Clancy, C., and J. Simon. 2001. Ultrastructural organization of eumelanin from *Sepia officinalis* measured by atomic force microscopy. *Biochemistry.* 40:13353–13360.

38. Kurtz, S., S. Kozikowski, and L. Wolfram. 1986. Optical constants of solid melanins determined from reflection measurements in the visible spectrum. *J. Invest. Dermatol.* 87:401.

39. Kurtz, S., S. Kozikowski, and L. Wolfram. 1986. Non-linear optical and electro-optical properties of biopolymers. *In* Electro-Optic and Photorefractive Materials. P. Gunter, editor. Springer-Verlag, Berlin, Germany. 110–130.

40. Pope, R., and E. Fry. 1997. Absorption spectrum (380–700 nm) of pure water. II. Integrating cavity measurements. *Appl. Opt.* 36:8710–8723.

41. Segelstein, D. 1981. The complex refractive index of water. M.S. thesis. University of Missouri, Kansas City, MO.

42. Sarna, T., and H. Swartz. The physical properties of melanins. *In* The Pigmentary System. J. Norlund, R. Boissy, V. Hearing, R. King, and J. Ortonne, editors. Oxford University Press, Oxford, UK. 333–357.