Evaluation of the Practicality of Melanin as a Photodynamic-Inactivation Photosensitizer by Its Nanonization

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It has been proposed that melanin may play a role in the antimicrobial defense system of a human body. Furthermore, it has been found that melanin has efficacy against oxidative stress, tumor, venin, virus, and heavy metal ions. In view of the great potential of melanin in medical applications revealed by its photodynamic actions, we develop techniques based on mechanical stir and photo-fragmentation with femtosecond laser pulses respectively for nanonization of melanin to produce nanometer-sized and water-dispersible melanin, in order to more reliably study melanin as a photodynamic-inactivation photosensitizer. In vitro experiments on the antimicrobial efficacy of nanonized melanin were conducted. It was found that both Sepia melanin and synthetic melanin do not have a significant effect on lowering the survival rate of Gram-positive bacteria Streptococcus mutans with or without light irradiation. Therefore, it is concluded that melanin, though capable of generating melanin radicals and reactive oxygen species, is not a practical agent for photodynamic inactivation.

Keywords: melanin, nanoparticle, radical, water-dispersible, photodynamic inactivation

1. Introduction

Melanin is a heterogeneous light-absorbing polymer widespread in the animal and plant kingdoms. Melanocytes in the human epidermis have numerous enzymes with capabilities in antimicrobial defense and functional links to the immune system, in which melanin may play an activating role [1-3]. In addition, some researches revealed that melanocytes play a key role in protecting our skin from damaging effects of solar UV radiation by scavenging free radicals and reactive oxygen species (ROS), besides simply attenuating the radiation [4,5]. On the other hand, it has been found that melanin has great potential in many industrial and medical applications [6-8].

In all potential applications, insolubility and indispersibility of both natural melanin and synthetic melanin in pure water and biological medium (aqueous medium at physiological pH) lead to quick precipitation and formation of large aggregates, drastically reducing the efficacy in in vivo and in vitro experiments. On the other hand, some experiments utilized melanin dissolved in basic solution or nonpolar solvent for ex vivo study of melanin properties and functions, but these solvents are not bio-compatible, prohibiting its direct medical use, and the properties resolved may not be the same as that in a biological medium. Therefore, to circumvent these problems, a way to produce water-dispersible natural melanin and synthetic melanin is crucial. Nanonization also provides further merits in biological function assays and industrial and medical applications. For instance, melanin nanoparticles could be ingested by cells via endocytosis at a rate and a proportion much larger than micrometer-sized aggregates. Furthermore, when nanonized the exposed surface area of melanin is increased by many orders of magnitude compared to that of micrometer-sized aggregates, the efficiency of chemical and biological interactions will be dramatically increased. In previous published work we developed the technique based on mechanical and photo-fragmentation processes for nanonization of synthetic melanin to improve its antioxidant efficacy [9]. In this report we show natural melanin can also be nanonized by these methods.

Recently, photodynamic inactivation (PDI) of microbes has gained much attention as a route to fight infection, especially in view of the increasingly serious
problem of drug resistance of microbes against antibiotics. In PDI, a photosensitizer is used to turn molecular oxygen into singlet oxygen $^{1}O_2$ upon irradiation with light. The singlet oxygen produced and other ROS evolved from it subsequently have microbicidal ability, as a result of their strong oxidative power which leads to damage of vital molecules or organelles of microbes [10-12]. It is well established that free radicals, being also strongly oxidative, express similar microbicidal activity [13]. Previous studies revealed by measurement with electron paramagnetic resonance (EPR) spectroscopy [14-17] have found that melanin possesses permanent and light-induced free radicals which can react with $O_2$ to form ROS [18-20]. In addition, it has been found that melanin under irradiation can generate ROS, similar to what the photosensitizers used in PDI do, although with a lower quantum efficiency [15,21-23]. In our previous work [9], we found that water-dispersible synthetic melanin exhibits two contradicting functions of scavenging ROS (photoprotection) and generating ROS (phototoxicity). Therefore, it seems reasonable to hypothesize that melanin may be able to damage bacteria by intrinsic melanin radicals, extrinsic (light-induced) melanin radicals, or light-induced melanin-generated ROS. To examine this hypothesis, in this work we experimented on the antimicrobial efficacy of both Sepia melanin (natural melanin) and synthetic melanin in order to get better understanding of the role of melanin in human body and its application of a practical PDI photosensitizer.

2. Method

2.1. Nanonization of melanin

The photo-fragmentation and mechanical stir methods were used to disintegrate melanin aggregates (powder particles) to produce nanoparticles of melanin dispersed in purified water (reverse osmosis and deionization) or in brain heart infusion (BHI) broth. A 10-ml suspension of melanin powder at 1 mg/ml was contained in a beaker of 5-cm base diameter situated on a magnetic stirrer operated at 800 rpm and room temperature with a stir bar of 8 mm in diameter and 30 mm in length. The solubility/dispersibility of the melanin was characterized by using a spectrophotometer (Newlab, SP7205) to measure the absorbance of the supernatant and by observing the color of the supernatant and the amount of precipitation at the bottom of the container after the melanin suspension was settled for various periods of time. For processing with photo-fragmentation, a collimated femtosecond-pulsed laser beam was propagated through a quartz cuvette (10-mm width, 10-mm optical path, and 5-cm height) containing a suspension of melanin powder in 1 ml of purified water. The laser beam was of 800-nm central wavelength, 50-fs pulse duration, 10-Hz repetition rate, and 7.2-mJ pulse energy [24]. The laser beam size was 6.7 mm in clear aperture and the peak fluence was 94 mJ/cm$^2$. The quartz cuvette was situated on a magnetic stirrer operated at 800 rpm and room temperature with two stir spheres of 3-mm diameter to vigorously stir the suspension to ensure all melanin particles passed through the irradiated region frequently. When the laser beam was switched off, the disintegration of melanin aggregates was accomplished by just the mechanical stir. When the laser beam was switched on, the disintegration of melanin aggregates was dominated by photo-fragmentation. The relative rates of the two different processing methods could be compared in this way.

2.2. Atomic force microscopy

Atomic force microscopy (AFM) was used to measure the morphology and size of melanin particles with and without nanonization. An aliquot of melanin suspension from each sample was transferred onto a mica substrate, dried with an oven, and viewed with an AFM (NanoWizard II, JPK Instruments) using a cantilever of $<12$ nm tip radius of curvature. Particle size distribution was analyzed with ImageJ.

2.3. Electron paramagnetic resonance

Electron paramagnetic resonance (EPR) spectroscopy was used to measure the amount of melanin radicals present in the melanin colloidal dispersion with and without continuous light exposure. Melanin dispersed in purified water or the same medium as that for the microbe experiment was contained in a flat cell with a sample volume of 150 $\mu$l and characterized with an EPR (EMX-10/12, Bruker) with a microwave frequency of 9.771 GHz. Light exposure was done with a laser (MRL-III-660D, Changchun New Industries) with a wavelength of 660 nm at an intensity of 40 mW/cm$^2$, same as that for the microbe experiment.

2.4. Reagents

Although natural melanin and synthetic melanin have similar optical absorption spectrum and EPR spectrum,
natural melanin contains proteins and thus may differ significantly from synthetic melanin in both the processes of endocytosis by melanocytes and interaction with microbes. Therefore, both natural melanin and synthetic melanin were used in this study. Natural melanin from cuttlefish *Sepia officinalis* (consisting of mainly eumelanin and likely polypeptide remnants) and synthetic melanin (eumelanin) produced by oxidation of tyrosine with hydrogen peroxide were purchased from Sigma-Aldrich. For the experiment of antimicrobial efficacy, melanin was suspended in BHI broth at 1.4 mg/ml and then processed with a magnetic stirrer for 48 h. Chlorin e6 was purchased from Frontier Scientific and dissolved in BHI broth at a concentration of 10 μM. BHI broth was purchased from Bio Star, Taiwan. BHI agar for bacterial viability (survival rate) measurement was purchased from either Sigma-Aldrich or BD. All reagents were sterilized with an autoclave before use.

2.5. Photosensitization setup and procedure

A diode laser with a wavelength of 660 nm was used for excitation of both melanin and chlorin e6, since melanin has a smooth broadband absorption spectrum extending from UV to near infrared and chlorin e6 exhibits an absorption peak near 660 nm, both verified with the spectrophotometer. An aliquot (1 ml) of the bacteria suspension with an optical density of ≈0.9 at 600-nm wavelength was centrifuged (8000 × g, 10 min) and the supernatant was discarded. The bacteria pellet was resuspended in 1 ml of BHI broth with 1.4-mg/ml melanin or 10-μM chlorin e6. After incubated for 1 h, the suspension was transferred to a 35-mm-diameter dish and exposed to the laser beam from above at a laser power of 385 mW for various exposure times. The laser beam fully covered the area of the dish at an intensity of 40 mW/cm². With this configuration, the transmittance of the laser beam through the culture was over 60% for all cases, ensuring that all melanin particles were effectively excited by the laser beam. Additionally, a diode laser with a wavelength of 532 nm was also used for excitation of melanin to test the antimicrobial efficacy.

2.6. Bacterial strain

Previous studies [25-27] have shown that while it works well for Gram-positive bacteria, with only a few exceptions photodynamic inactivation is ineffective in eradicating Gram-negative bacteria as a result of their additional outer layer of cell wall. It is so unless the bacteria are treated with reagents such as ethylenediaminetetraacetic acid (EDTA) or polymyxin nonapeptide (PMNP) to alter the permeability of the bacterial outer membrane before photosensitization [28,29], or a cationic photosensitizer was employed instead [30]. Therefore, Gram-positive bacteria *Streptococcus mutans* was chosen as the representative microbe model for the experiment. Stock culture of *Streptococcus mutans* was purchased from Bioresource Collection and Research Center, Hsinchu, Taiwan. It was cultured aerobically in BHI broth at 37°C with aeration.

2.7. Bacterial survival rate measurement

To measure the numbers of viable bacteria in the bacterial suspensions with and without photosensitization, aliquots of the bacterial suspensions were diluted with purified water at various proportions and then seeded onto BHI agar. The seeded agar plates were cultured at 37°C for 48 h and then the numbers of colony-forming units were counted. The survival rate is defined as the ratio of the number of colony-forming units per milliliter of the bacterial culture treated with photosensitization to that without the treatment.

3. Results and Discussion

Fig. 1a shows the absorbance of *Sepia* melanin at 355-nm wavelength as a function of processing time under mechanical stir. The inset shows the photographs of the *Sepia* melanin suspension before (left) and after (right) 48-h mechanical stir. When *Sepia* melanin or synthetic melanin powders was suspended in water, without the processing the majority (>90%) of the suspended particles precipitated on the bottom of the cuvette within 10 min. The supernatant was transparent with visible suspended black particles which continued to precipitate at a later time. With 48-h processing under mechanical stir the suspension became a homogeneous black dispersion and there was no discernible precipitate at the bottom of the cuvette. It maintained so for more than a week. Fig. 1b shows the absorbance of *Sepia* melanin processed with 48-h mechanical stir at 355-nm wavelength as a function of settling time. As shown, the absorbance of processed *Sepia* melanin suspension has a half-life exceeding two weeks. Furthermore, it was found that by simply shaking the suspension for 10 s the absorbance of processed *Sepia* melanin suspension after settled for one month can be fully restored to the same value as that right after processing and then decays with
the same half-life. This indicates that processed Sepia melanin will not be converted back to its original form by settling. The results of processed synthetic melanin are very similar to that of processed Sepia melanin [9]. The long half-life and the ease of re-dispersibilization are crucial for practical medical applications.

Fig. 2 shows AFM images of unprocessed Sepia melanin (a) and Sepia melanin processed with 48-h mechanical stir (b). It was observed that the majority of original Sepia melanin are aggregates of many nanometer-sized particles (Fig. 2a). The aggregates were disintegrated by the 48-h mechanical stir. Fig. 3 shows the diameter and height distributions of Sepia melanin processed with the 48-h mechanical stir. The average diameter of the Sepia melanin nanoparticles is ≈47 nm and the average height is ≈0.8 nm, showing that the Sepia melanin nanoparticles are of flaky shape. It was observed that the processed synthetic melanin are very similar to that of processed Sepia melanin [9]. This reveals that the processing turned both Sepia melanin and synthetic melanin suspension into a colloidal dispersion of nanoparticles, which explains the dramatic increase in dispersibility. Furthermore, the size distribution is consistent with the proposal that the secondary structure of melanin is π–π stacking of sheets with an inter-sheet spacing of 0.33–0.37 nm [31,32]. Simulation results obtained by Chen et al. [33] on the self-assembly of tetramers of 5,6-dihydroxyindole (DHI, one of the major building monomeric units of eumelanin) shows that the secondary structure of eumelanin is π–π stacked aggregates with a height distribution which has a maximum value of ≈3.3 nm and an average value of =
Fig. 3. Diameter (a) and height (b) distributions of *Sepia* melanin processed with 48-h mechanical stir.

1.3 nm. The height histogram, maximum height, and average height are all close to our experimental results of nanonized *Sepia* melanin and nanonized synthetic melanin (both also eumelanin) [9].

Fig. 4a shows the UV-visible-NIR absorption spectra of nanonized and dispersed *Sepia* melanin in pure water. As can be seen, the processed *Sepia* melanin displays an optical absorption spectrum similar to those observed for melanin dissolved in various solutions, i.e., a monotonically-decreasing broadband absorption curve extending from UV to NIR. The slope of the absorption curve of nanonized melanin suspension is less steep than that of melanin dissolved in NaOH solution, in which melanin is presumably in oligomeric form. This is consistent with the observation of Nofsinger et al. [23], which used a filtration method to separate oligomers and aggregates and observed similar difference. Fig. 4b shows the EPR spectrum of nanonized and dispersed *Sepia* melanin in BHI broth measured under the conditions with (L+) and without (L-) continuous light exposure respectively. The sample concentration is 1.4 mg/ml and the sample volume is 150 μl. g ≈ 2.00433, line width = 0.7 mT.

Fig. 4b reveals that the process of nanonization by mechanical stir does not lead to degradation of melanin in optical and free-radical properties, which could be critical to biological function studies and applications of melanin.

Fig. 5 shows the absorbance of *Sepia* melanin at 355-nm wavelength as a function of processing time under mechanical stir with and without the femtosecond-pulsed laser beam. As shown, the rate of nanonization (and thus dispersibilization) can be
enhanced by a factor of 1.4 when the femtosecond-pulsed laser is employed. The melanin processed with photo-fragmentation was also characterized, and the results were all very similar to that shown above for the melanin processed with the mechanical stir. The rate of nanonization with the laser-irradiation method could be further increased by increasing the energy fluence of laser pulses or the volume of irradiation. It could also be increased by change in the geometry or motion of the stir bar or the geometry of the container to produce a more thorough and steadier circulation of all melanin particles through the volume of irradiation. The rate of nanonization with the mechanical-stir method could be further increased by change in the geometry or motion of the stir bar or the geometry of the container to produce a stronger smashing force or to produce a more thorough and steadier circulation of all melanin particles through the volume of smashing. Although the mechanical stir

![Graph showing absorbance of Sepia melanin at 355-nm wavelength](image)

Fig. 5. Absorbance of Sepia melanin at 355-nm wavelength as a function of processing time under mechanical stir with (solid square) and without (open circle) irradiation by the femtosecond-pulsed laser beam.

method takes a much longer processing time, it is much simpler and cheaper for mass production, and therefore it was used for the production of nanonized melanin used in the in vitro experiments presented here.

The use of nanonized and dispersed melanin is imperative because both Sepia melanin and synthetic melanin as purchased precipitate quickly in water and thus cannot interact with microbes for a sufficient amount of time. In addition, melanin nanoparticles could be ingested by microbes via endocytosis. Furthermore, when nanonized the exposed surface area of melanin is increased dramatically, raising the rate of interaction with vital molecules and organelles of microbes. Fig. 6 shows the survival rate of Streptococcus mutans in suspension with various photosensitizers and treated with light exposure of various periods. It was found that the survival of Streptococcus mutans culture is not reduced significantly by the treatment with either nanonized Sepia melanin or nanonized synthetic melanin under light exposure. In contrast, the use of chlorin e6 as the photosensitizer led to five orders of magnitude drop in the survival rate after 5-min light exposure, even though the mass concentration of chlorin e6 used (6 μg/ml) is much lower than that of the melanin used. Using the efficacy of chlorin e6 as a positive control [34-36], we conclude that both Sepia melanin and synthetic melanin do not have a significant efficacy in directly inactivating bacteria. Note that the use of 660-nm light for activation of melanin in this experiment is for the purpose of making a quantitative comparison with chlorin e6. Past studies have shown that the efficiency of generation of melanin radicals and ROS increases exponentially with decrease of light wavelength [21,37]. Therefore, the experiment

![Graph showing survival rate of Streptococcus mutans](image)

Fig. 6. Survival rate of Streptococcus mutans in suspension with various photosensitizers and treated with light exposure of various periods – Ce6: 10-μM chlorin e6; nano-Sepia: 1.4-mg/ml nanonized Sepia melanin; nano-synthetic: 1.4-mg/ml nanonized synthetic melanin; none: without photosensitizer. Each data point represents the mean of four samples and the associated error bar enhanced by a factor of 1.4 when the femtosecond-pulsed laser is employed.

Fig. 4b verify that the light exposure in the Streptococcus mutans experiment indeed generated a substantial amount of extrinsic (light-induced) melanin radicals. However, this still did not lead to appreciable efficacy in killing the bacteria. Although it has been found that melanin in oligomeric form exhibits a substantially
higher quantum efficiency in generating ROS than nano-aggregates [23], it is not likely the form in which melanin is present in organisms.

4. Conclusion

The water-dispersible melanin was used to evaluate the efficacy of melanin in directly inactivating bacteria. It is concluded that the antimicrobial function of melanin does not rely on using melanin radical or melanin-generated ROS to directly kill microbes. Whether melanin is the byproduct of an antimicrobial immune response or it play a role of mediating a more complex process requires further investigation. It is shown that Sepia melanin can be nanonized and dispersed in pure water or biological medium by processing of melanin powder in suspension with a magnetic stirrer running for 48 h. It is also shown that irradiation of Sepia melanin in suspension with femtosecond laser pulses can result in photo-fragmentation, which leads to nanonization and dispersibilization at a rate higher than that with mechanical stir. In this report and our previous work [9] we have demonstrated that both Sepia melanin (natural melanin) and synthetic melanin can be nanonized and dispersed in pure water or biological medium by processing of melanin powder in suspension with a magnetic stirrer running for 48 h. Although water-dispersible melanin processed with the techniques reported herein would not be practical photosensitizer for PDI, we expect it could be utilized in other applications such as adjuvant in radiation therapy [38], antioxidant [4,5,9,39], metal-ion scavenger [40], antidote for acute radiation syndrome [38], antitumor drug [41,42], antivenin drug [43], antivirus [44] and contrast-enhancing agent in magnetic resonance imaging (MRI) [45,46].

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