The radioprotective properties of fungal melanin are a function of its chemical composition, stable radical presence and spatial arrangement

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Summary

Melanized microorganisms are often found in environments with very high background radiation levels such as in nuclear reactor cooling pools and the destroyed reactor in Chernobyl. These findings and the laboratory observations of the resistance of melanized fungi to ionizing radiation suggest a role for this pigment in radioprotection. We hypothesized that the radioprotective properties of melanin in microorganisms result from a combination of physical shielding and quenching of cytotoxic free radicals. We have investigated the radioprotective properties of melanin by subjecting the human pathogenic fungi *Cryptococcus neoformans* and *Histoplasma capsulatum* in their melanized and non-melanized forms to sublethal and lethal doses of radiation of up to 8 kGy. The contribution of chemical composition, free radical presence, spatial arrangement, and Compton scattering to the radioprotective properties of melanin was investigated by high-performance liquid chromatography, electron spin resonance, transmission electron microscopy, and autoradiographic techniques. Melanin protected fungi against ionizing radiation and its radioprotective properties were a function of its chemical composition, free radical quenching, and spherical spatial arrangement.

Key words: melanin/ionizing radiation/radioprotection/fungi/Compton scattering/electron spin resonance

Introduction

Melanins are high molecular weight pigments, ubiquitous in nature, with a variety of biological functions (Hill, 1992). Many fungi constitutively synthesize melanin (Jacobson, 2000), which is likely to confer a survival advantage in the environment by protecting against environmental predators, heavy metals toxicity, and physical insults such as UV and solar radiation (reviewed in Nosanchuk and Casadevall, 2003). Melanized microorganisms inhabit some remarkably extreme environments on the planet including high altitude, Arctic and Antarctic regions (Robinson, 2001). Most dramatically, melanized fungal species colonize the walls of the damaged reactor at Chernobyl where they are exposed to a high constant radiation field (Mironenko et al., 2000). Further attesting to the potential radioprotective properties of melanin, cooling pool water in nuclear reactors is sometimes contaminated with melanized microorganisms (Sinilova et al., 1969). These findings and the laboratory observations of the resistance of melanized fungi to ionizing radiation (Mirchink et al., 1972; Saleh et al., 1988) suggest a role for this pigment in radioprotection.

Melanin can play many different roles in microorganisms because of its unique physico-chemical properties. Meredith and Sarna (2006) recently reviewed the relationship between melanin (eumelanin) structure and its optical properties, condensed-phase electric properties, electron exchange, paramagnetic properties, ion exchange capacity, and ability to absorb UV and visible radiation. In contrast to the large body of knowledge accumulated over the past 50 yr in establishing structure–property relationship for the above properties of melanin, we are not aware of any attempt to give a physico-chemical explanation of radioprotective properties of melanin. Recently, we have shown that nonlethal doses of ionizing radiation change the electronic properties of melanin and enhance the growth of melanized fungi (Dadachova et al., 2007). Here we have

investigated the radioprotective properties of melanin by subjecting the melanized fungi to sublethal and lethal doses of radiation. We have hypothesized that the radioprotective properties of melanin in microorganisms result from a combination of physical shielding and quenching of biologically damaging free radicals. Here we demonstrate that melanin protects fungi against ionizing radiation because of its chemical composition, free radical quenching, and spherical spatial arrangement.

Results

To investigate the ability of melanin to protect fungi against radiation damage, we analysed two fungi capable of melanogenesis, *Cryptococcus neoformans* (Cn) and *Histoplasma capsulatum* (Hc). These fungi were selected for the study because they can be evaluated in their non-melanized or melanized states depending on whether they are grown with substrate for melanization. When Cn and Hc are grown with L-dopa (3,4-dihydroxyphenylalanin) – melanin is concentrated in the cell wall (Figure 1A,B). Hollow melanin particles with a roughly spherical shape can be isolated from melanized cells digested in concentrated acid and have been dubbed 'ghosts' because they retain the shape and dimensions of the parent cell (Wang et al., 1996). Melanin in these ghosts is assembled into multiple concentric layers of approximately 100 nm thickness consisting of closely packed much smaller particles (Eisenman et al., 2005). Given that one of our aims was to compare the radioprotective properties of fungal melanin to those of commercially available melanins such as *Sepia officinalis* melanin from the ink glands of the cuttlefish (Zeise et al., 1992), we boiled melanized cells in 6 N HCl to remove fungal cell components (Nosanchuk et al., 2002; Wang et al., 1996). We have previously shown that the acidic treatment of *Sepia* melanin results only in slight difference in elemental composition of untreated and acid-treated samples (Rosas et al., 2000). In fact, resistance to acid degradation is one of the characteristics used to define a compound as a melanin.

Late stationary phase melanized and non-melanized Cn and Hc cells were subjected to very high doses of radiation – up to 8 kGy. For comparison, 0.005 kGy is lethal to humans. As the LD_{10} for these organisms in their non-melanized form is around 0.05–0.1 kGy (Dadachova et al., 2004), high doses were needed to analyse the radioprotective effect of melanin. Melanized Cn cells were significantly less susceptible to external gamma radiation ($P = 0.01$) in the dose range of 0–0.22 kGy than non-melanized cells (Figure 2A) with a dose reduction factor (DRF) calculated to be 1.6. At 1.0–8.0 kGy, the protective effects were not statistically significant ($P = 0.4$). For Hc cells, melanin provided

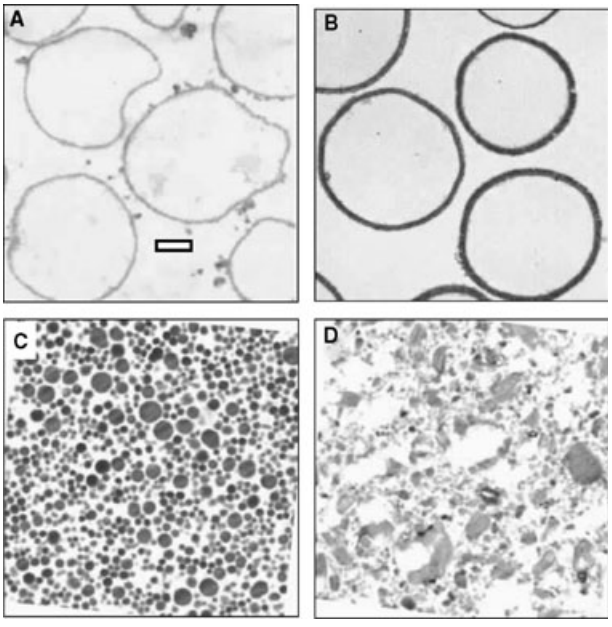

Figure 1. Transmission electron microscopy micrographs of different melanins: (A) intact *Histoplasma capsulatum* ghosts; (B) intact *Cryptococcus neoformans* ghosts; (C) *Sepia officinalis* (cuttlefish) melanin; (D) crushed *C. neoformans* ghosts. Bar in panel A is 1 μm.

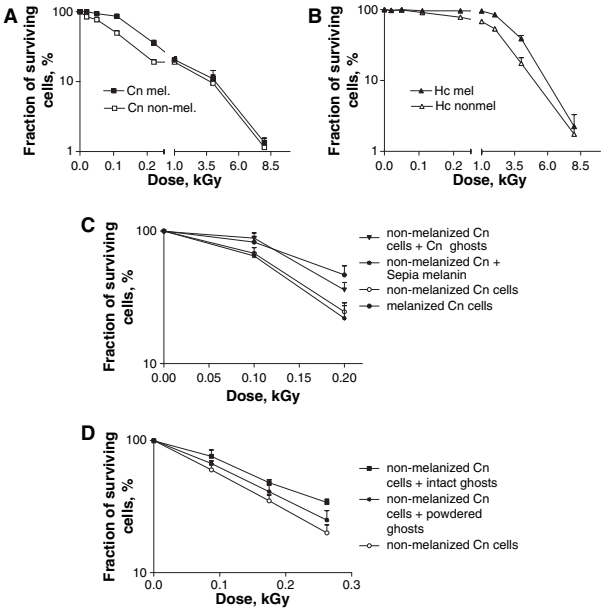

Figure 2. Survival of non-melanized and melanized fungal cells following exposure to external gamma rays: (A) *Cryptococcus neoformans* in phosphate-buffered saline (PBS) up to 0.22 kGy at 0.014 kGy/min and up to 8 kGy at 0.030 kGy/min; (B) *Histoplasma capsulatum* in PBS up to 0.22 kGy at 0.014 kGy/min and up to 8 kGy at 0.030 kGy/min; (C) melanized and non-melanized *C. neoformans* irradiated at 0.014 kGy/min up to 0.2 kGy, 0.4 mg/ml of *Sepia officinalis* melanin or 0.1 mg/ml of *C. neoformans* whole melanin ghosts was added to non-melanized cells; (D) non-melanized *C. neoformans* irradiated at 0.014 kGy/min up to 0.2 kGy, 0.1 mg/ml of *C. neoformans* whole or crushed melanin ghosts was added to non-melanized cells, mel – melanized.

protection up to 8 kGy ($P < 0.01$) with a DRF of 1.6 (Figure 2B). Dose reduction factor is a measure of the radioprotective properties of a substance when applied to a biological system. According to its radiobiological definition – DRF range is $1 \leq DRF \leq 2.2$ (Hall, 2000). Thus, both Cn and Hc melanin provided significant protection against very high doses of radiation, especially taking into consideration that the doses were delivered at a very high dose rate of 0.014 kGy/min, and that the damage to the cells is directly proportional to the dose rate at which radiation is being delivered (Hall, 2000). For comparison, in external beam radiation therapy of cancer patients the dose rate is more than 10 times lower.

To elucidate the contribution of the spherical arrangement of melanin in fungal cells to radioprotection, we initially irradiated non-melanized Cn cells with doses of up to 0.2 kGy in the presence of 0.4 mg/ml *Sepia* melanin, which is not arranged in hollow spheres (Figure 1C). *Sepia* melanin conferred no significant protection at any dose (Figure 2C), while 0.1 mg/ml of Cn ghosts afforded significant protection of non-melanized cells (Figure 2C). In a more definitive follow-up experiment non-melanized cells were exposed to radiation in presence of whole and mortar-crushed Cn ghosts (Figure 1B,D). Whole ghosts provided significantly more protection to melanin cells than the crushed ghosts (Figure 2D), an observation consistent with the hypothesis that the spatial arrangement of melanin particles in the ghosts is important in radioprotection.

We then investigated the possible contribution of melanin chemical composition to its radioprotection properties. The structures of melanins are uncertain (reviewed in Meredith and Sarna, 2006) because of the amorphous, heterogeneous, and insoluble nature of these pigments, which preclude their structural solution given the currently available analytical tools. It is generally accepted that there are two major types of melanin: eumelanin and pheomelanin. Eumelanin is a dark-brown to black pigment composed of 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) monomer units with 6–9% nitrogen (Figure 3A), while pheomelanin is a reddish-brown pigment composed of benzothiazine monomer units with 8–11% nitrogen and 9–12% sulfur (Figure 3B) (Ito and Fujita, 1985; Wakamatsu and Ito, 2002). The majority of naturally occurring melanins, however, constitute a mixture of eumelanin and pheomelanin subunits which explains the fact that often up to 1% of sulfur is found in predominantly eumelanin samples. When subjected to acidic permanganate oxidation, DHI converts into pyrrole-2,3-dicarboxylic acid (PDCA), DHICA – into pyrrole-2,3,5-tricarboxylic acid (PTCA), and pheomelanin oxidation results in 1,3-thiazole-2,4,5-tricarboxylic acid (TTCA) and 1,3-thiazole-4,5-dicarboxylic acid (TDCA) (Ito and Fujita, 1985; Wakamatsu and Ito, 2002). We have performed elemental analysis of cryptococcal and *Sepia* melanins used in this study which showed the absence of sulfur in *Sepia* melanin and 0.4% sulfur in cryptococcal melanin with C/N ratio being 7.5 for both of these melanins. We have recently applied high-performance liquid chromatography (HPLC) for analysis of Cn melanin (Frases et al., 2006, 2007; Garcia-Rivera et al., 2005). The HPLC analysis of oxidized Cn revealed a component at the elution time of PTCA and a much smaller one with the elution time of TDCA (Figure 3D); while the chromatogram of Hc melanin was consistent with the presence of slightly more TDCA than PTCA (Figure 3E), and only PTCA was detected in the *Sepia* chromatogram (Figure 3F). The identity of these compounds was confirmed by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF) (Figure 3D,E). Quantification of the chromatographic data revealed a PTCA to TDCA ratio of 0.90 for Hc melanin and 47.7 for Cn melanin. Although these ratios are not quantitative measures of eumelanin and pheomelanin in the cells, they do indicate that benzothiazine subunits predominate in Hc melanin, while DHICA subunits predominate in Cn melanin.

Another important physico-chemical property of melanin that can contribute to its radioprotective properties is the presence of a stable free radical population, which is considered to be a distinguishing characteristics of melanin (Enochs et al., 1993). Analyses by electron spin resonance (ESR) spectroscopy revealed a stable free radical population in all melanins used in our study that is consistent with melanin pigment characteristics (Figure 3G,H). The similarity of the ESR peaks of Hc and Cn melanins is caused by the fact that these natural melanins contain a mixture of ortho-semiquinonimine and ortho-semiquinone radicals (Sarna and Swartz, 1993) which makes it difficult to delineate the contribution of the above radicals to the paramagnetic signal. Besides, earlier work has shown that Cn cells have the same ESR signal as the ghosts (Wang et al., 1995).

To investigate if melanin possesses physical shielding properties that can also contribute to its radioprotective properties in microorganisms, different concentrations of Cn ghosts and *Sepia* melanin were exposed to diagnostic X-rays. For this purpose, suspensions of melanin particles in polylysine solution were placed into the wells of the 96-well microtiter plate with charcoal suspensions in polylysine solution and lead foil of similar weights serving as controls (Figure 4A). The microtiter plate was then exposed to diagnostic X-rays (40 kVp at 10 mA) and the shielding effect was detected with radiographic film placed under the plate during exposure to X-rays (Figure 4B). The attenuation of X-rays by substances in the wells was calculated as the ratio of internal pixel density (IPD) in the wells to IPD of the background wells (Table 1). *Cryptococcus neoformans* ghosts attenuated radiation two times better than the matching amounts of *Sepia* melanin or charcoal and the attenuation was dose-dependent. Interestingly, attenua-

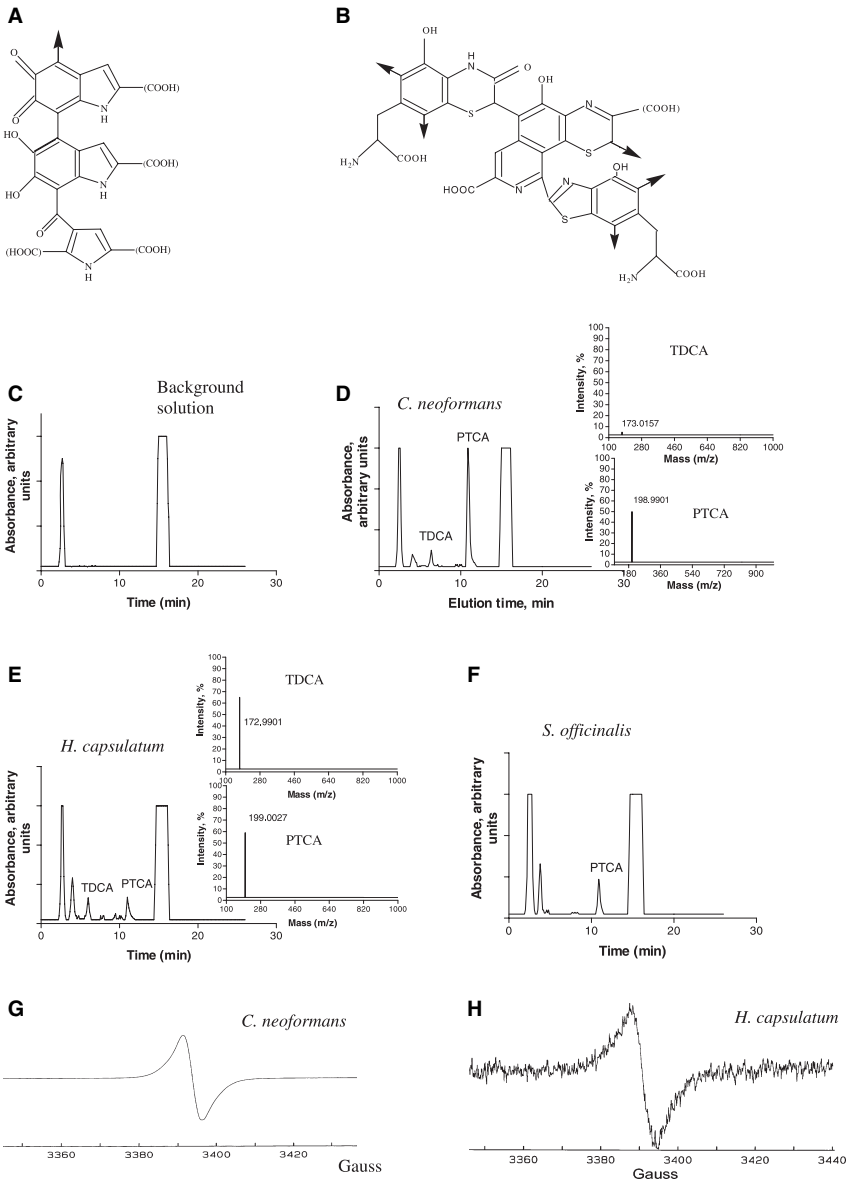

Figure 3. Chemical structure of melanin, high performance liquid chromatography of permanganate-oxidized melanins and electron spin resonance (ESR) spectra of intact melanins: (A) structure of eumelanin oligomer; (B) structure of pheomelanin oligomer; (C) chromatogram of background solution; (D) *Cryptococcus neoformans*. Inserts show matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) of TDCA and PTCA peaks; (E) *Histoplasma capsulatum*. Inserts show MALDI-TOF of TDCA and PTCA peaks; (F) *Sepia officinalis*; (G) ESR spectrum of *C. neoformans*; (H) ESR spectrum of 2 μm *H. capsulatum*. Absorption was monitored at 255 nm and displayed on the linear scale. ESR spectra were obtained by suspending ghosts in water. Ordinate in G, H is the derivative of the ESR absorption in arbitrary units. PDCA – pyrrole-2,3-dicarboxylic acid; PTCA – pyrrole-2,3,5-tricarboxylic acid; TTCA – 1,3-thiazole-2,4,5-tricarboxylic acid; TDCA – 1,3-thiazole-4,5-dicarboxylic acid.

tion by the suspension of ghosts was only two times lower than that of the lead foil which is a solid material. Overall, the results of this experiment showed that melanins can scatter X-ray radiation resulting in a physical shielding effect.

Discussion

We have observed the protective effect of melanin on melanized fungi subjected to sublethal and lethal doses of ionizing radiation and have conducted experiments to establish the relationship between the physico-chemical properties of this pigment and its radioprotective properties in microorganisms. To the best of our knowledge, this is the first systematic attempt to explain melanin radioprotective properties. While the scattering of non-ionizing electromagnetic radiation by melanin can be described by the application of Mie and Rayleigh scattering algorithms for calculation of scattering coefficients for a multilayered sphere (Bhandari, 1985; Riesz et al., 2006), ionizing radiation interacts with melanin to produce profound effects on its electronic properties (Dadachova et al., 2007). This is a fundamentally different process that presumably occurs through the photoelectric effect, Compton effect and/or pair production, depending on the incident photon energy. In our experiments, we used a 137-Cs source emitting 662 keV high-energy photons which interact with low atomic numbers elements C, N, O, and S (Sorenson and Phelps, 1987) found in melanin mainly via Compton scattering. In Compton scattering, the transfer of a photon energy to matter occurs via a cascade of interactions, where the energy of the incident photon is transferred to high-energy electrons, and to secondary

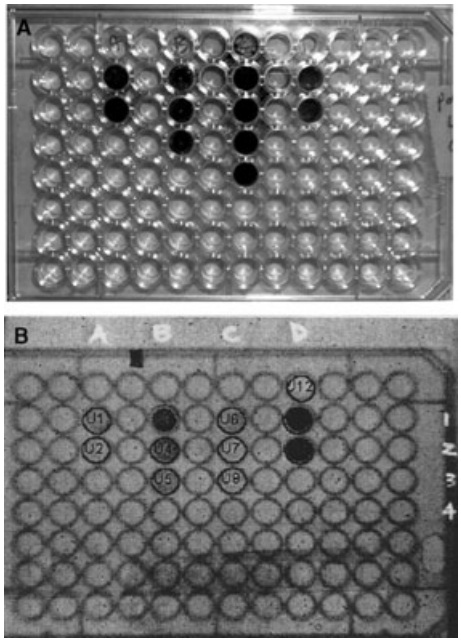

Figure 4. Determination of the shielding properties of melanins: (A) Polysine precoated wells in a 96-well microtiter plate were filled with the suspensions of the following substances in polysine solution: column A – *Sepia* melanin (two amounts decreasing from top to bottom); column B – *Cryptococcus neoformans* ghosts (three amounts decreasing from top to bottom); column C – charcoal (four amounts decreasing from top to bottom); column D – lead foil (two amounts increasing from top to bottom); (B) the image of the radiographic film. The microtiter plate was exposed to diagnostic X-rays (40 kVp at 10 mA) and the shielding effect was detected with Hi-speed Kodak x-omat (xb-1) radiographic film placed under the plate during exposure to X-rays.

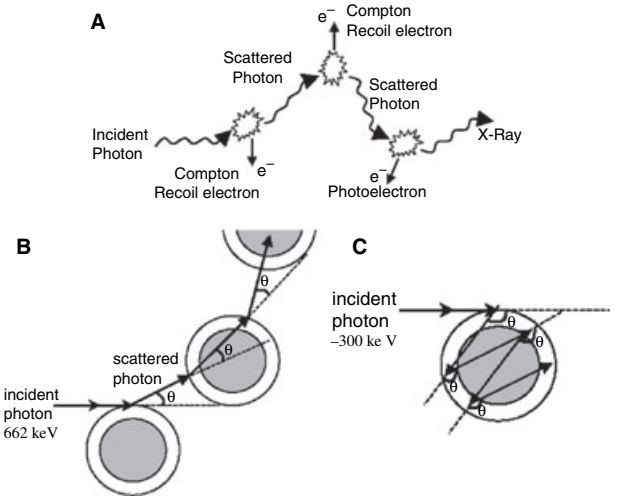

Figure 5. Interaction of ionizing radiation with melanin: (A) diagram illustrating multiple interactions of a photon passing through matter. Incoming photon interacts with an electron on the outer electron shell of the atom which becomes free as a result of this interaction and is called 'recoil electron'. Energy is transferred to recoil electrons in a sequence of photon-energy degrading interactions (adapted from Sorenson and Phelps, 1987). Energy balance of Compton scattering $E_o = E_{re} + E_{sc}$, where E_{re} is the energy of the recoil electron, and E_o and E_{sc} are the energies of the incident and scattered photons, respectively. (B, C) Proposed model of the scatter of ionizing radiation by melanin spheres: (B) forward scatter of initial high energy 662 keV photons incoming at arbitrary angle θ when forward scatter predominates; (C) oscillation within melanin spheres of secondary scattered photons which energies fall below 300 keV and both forward and backward scatter takes place with backward scatter starting to predominate.

Table 1. Attenuation of diagnostic X-ray by suspensions of *Cryptococcus neoformans* and *Sepia* melanin in comparison with non-melanin substances

		Internal pixel density (IPD) per mm^2			
	Amount (mg)	1st	2nd (repeat)	Mean IPD/IPD (bkrd)	Attenuation (%)
(A) *Sepia*	50	1370	1370	1.12	12
melanin	25	1280	1280	1.05	5
(B) *C.*	100	1720	1720	1.41	41
neoformans	50	1470	1470	1.21	21
	30	1346	1346	1.11	11
(C) Charcoal	40	1329	1329	1.09	9
	20	1248	1248	1.02	2
	15	1244	1244	1.02	2
	10	1220	1218	1.01	1
(D) Lead foil	100	2202	2217	1.81	81
	200	2269	2277	1.87	87
Background	–	1218	1218	1	0

bkrd – background.

photons of progressively lower energy (Figure 5A), until the photoelectric effect takes place. The energy of the scattered photon is related to the scattering angle θ by considerations of energy and momentum conservation: The amount of energy transferred to the recoil electron in Compton scattering ranges from nearly zero for $\theta = 0°$ ('grazing' collisions) up to a maximum value E_{re}^{max} that occurs in 180° backscattering events.

$$E_{sc}=E_o/[1+(E_o/511)(1-\cos\theta)],$$

where E_o and E_{sc} are the incident and scattered photon energies in keV, respectively. For high-energy photons, the probability of scattering is highest for the low scattering angles. For example, when a 662 keV photon emitted by a 137-Cs source scatters at 30°, its energy will decrease to 470 keV, and approximately 10 subsequent scattering events at the same angle would reduce the photon energy to a level where the photoelectric effect can occur. Thus, during the first scattering events, when the energy of scattered photons is still high, a photon can be expected to pass through a melanin sphere with forward scattering predominating (Figure 5B). However, when the energy of scattered photon falls below ~300 keV the probability

of backward scattering increases (Sorenson and Phelps, 1987). Consequently, one might theorize that the scattered photons could oscillate within a melanin sphere (Figure 5C) as the wavelength of gamma photons (10^{-12}–10^{-11} m) is 10^5–10^6 times smaller than the diameter of a melanin sphere ($\sim 10^{-5}$ m, Figure 1B). As the absorbance of radiation by matter also depends on the geometric arrangement of the photon source and the absorber, the assembly of melanin into spheres may account for more efficient protection of cells by Cn melanin arranged in spheres than by *Sepia* melanin which has quite similar chemical composition (Figure 2C). In this regard, when the emission properties of eumelanin were studied post-exposure to UV-A radiation, the observed rapid and non-exponential depolarization dynamics was found to be due to energy transfer processes within spherical granules of eumelanin (Forest et al., 2000). We hypothesize that efficient scattering of X-rays by Cn ghosts could explain their superior performance in the shielding experiment (Table 1) when compared with *Sepia* melanin or charcoal both of which are also paramagnetic compounds (Sealy et al., 1980; Sarna and Swartz, 1978).

High-performance liquid chromatography analysis of the different melanins provided important insights into the possible contribution of the intrinsic chemical composition of melanin to its radioprotective properties. Compton scattering involves a collision between a photon and a loosely bound outer shell orbital electron of an atom. The number of outer shell electrons per gram of material is an important contributor to the attenuation properties of a material at the energy levels where the Compton effect predominates (Sorenson and Phelps, 1987). In this regard, the existence of structures composed of π-electron-rich covalently linked aromatic motifs could potentially explain radiation scattering properties of melanins. Eumelanin oligomers contain two fused double ring systems rich in π-electrons, while pheomelanin oligomers contain three with one of them being a triple ring system (Figure 3A,B). Thus, higher number of π-electrons could account for the superior radioprotective properties of Hc melanin, which is a combination of eumelanin and pheomelanin. Pheomelanin also contains divalent sulfur (Figure 2B), which might provide an additional contribution to its radioprotective properties as aminothiols and phosphorothioates are very efficient radioprotective agents (Travis, 1984).

The high-energy electrons generated by Compton scattering are ultimately responsible for the radiobiologic effects caused by gamma radiation through either direct interactions with DNA or radiolysis of water in the cells, a process that results in the formation of reactive short-lived free radicals capable of damaging DNA. By virtue of being a stable free radical (Figure 3G,H), melanin may trap these high-energy electrons and short-lived free radicals and prevent them from entering a cell, thus performing its function as a radioprotector. In this regard, we have observed a dramatic change in ESR signal of Cn melanin ghosts after they were subjected to gamma radiation (Dadachova et al., 2007).

The question of melanin radioprotective function in microorganisms can also be considered from a paleobiological point of view. Large quantities of highly melanized fungal spores have been found in early Cretaceous period deposits when many species of animals and plants died out. This period coincides with Earth's crossing 'magnetic zero' resulting in the loss of its 'shield' against cosmic radiation (Hulot and Gallet, 2003). Additionally, it has been postulated that radiation from a putative passing star called Nemesis contributed to the extinction events (Davis et al., 1985). Melanin is also a virulence factor for fungi and the presence of the polymer may have afforded a survival advantage to fungal species enabling their proliferation to such an extent that they may have contributed to the extinctions at the end of Cretaceous period (Casadevall, 2005). Hence, melanins are ancient pigments that are likely to have been selected in evolution because they enhanced the survival of microbes in both the environment and in various hosts. The emergence of melanin as a non-specific radioprotective material may be a result of the relative ease with which these complicated aromatic structures can be synthesized from a great variety of precursors.

In conclusion, the electronic complexity of melanins might allow them to efficiently scatter/trap photons and electrons, creating an effective radioprotective barrier in diverse microbes. We propose that fungal melanin-mediated radioprotection of living cells results from a combination of Compton scattering by the high number of electrons in its macromolecules, energy attenuation of photons as a consequence of high angular scattering caused by the geometry of spheres, and quenching of free electrons and free radicals generated by radiolysis of water. It may be possible to increase the radiation resistance of cells and tissues through enhanced expression of melanin and to design low-density biopolymers with high radiation shielding properties, which could find uses in a variety of applications by virtue of their low weight.

Materials and methods

Microorganisms

American Type Culture Collection (ATCC, Rockville, MD, USA) strain *C. neoformans* 24067 (serotype D) and CIB strain 1980 of *H. capsulatum* (Hc, a gift from A. Restrepo, Medellin, Colombia) were used in all experiments. *Cryptococcus neoformans* was grown in Sabouraud dextrose broth (Difco Laboratories, Detroit, MI, USA) for 24 h at 30°C with constant shaking at 150 rpm. *Histoplasma capsulatum* was grown with shaking at 37°C in defined media consisting of 29.4 mM KH_2PO_4, 10 mM $MgSO_4 \times 7H_2O$, 13 mM glycine, 15 mM D-glucose, 3 μM thiamine. Melanized Cn and Hc cells were generated by growing the fungi in their respective media with 1 mM 3,4-dihydroxyphenylalanine (L-dopa) for 10 d. The

cells were collected by centrifugation and washed three times with phosphate-buffered saline (PBS), pH 7.2 before radiation exposure.

Other sources of melanin

Melanin from cuttlefish *Sepia officinalis* (*Sepia*) was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Susceptibility of Cn and Hc to external gamma radiation

Approximately 10^5 melanized or non-melanized Cn or Hc cells were placed in microcentrifuge tubes in 0.5 ml PBS and irradiated with a ^{137}Cs source at a dose rate of 0.014 kGy/min. The cells were exposed to doses of up to 0.220 kGy. The exposures of 1–8 kGy were given by irradiating the cells at 0.030 kGy/min. Following the radiation exposure, 10^3 cells from each tube were plated in agar to determine viability as measured by colony forming units (CFUs). Dose reduction factor was calculated according to the formula:

$$\mathrm{DRF} = \frac{(\text{Dose needed to cause effect with protector})}{(\text{Dose needed to cause effect without protector})}.$$

Alternatively, non-melanized Cn cells were irradiated at 0.014 kGy/min in the presence of 0.4 mg/ml of powdered *Sepia* melanin or 0.1 mg/ml of either intact Cn melanin ghosts or agate mortar ground melanin ghosts, followed by determination of viability as measured by CFUs. Their viability was compared with that of melanized and non-melanized Cn irradiated under the same conditions.

Isolation and purification of fungal melanins

The Cn and Hc melanized cells were grown as above. The cells were suspended in 1.0 M sorbitol-0.1 M sodium citrate (pH 5.5). Lysing enzymes from *Trichoderma harzarium* (Sigma Chemical Co.) were added to the suspension at 10 mg/ml and the suspensions were incubated overnight at 30°C. Protoplasts were collected by centrifugation, and incubated in 4.0 M guanidine thiocyanate overnight at room temperature. The resulting particulate material was collected by centrifugation, and digested with Proteinase K (final concentration 1.0 mg/ml) in reaction buffer (10.0 mM tris, 1.0 mM $CaCl_2$, 0.5% SDS) was added to the particles followed by overnight incubation at 37°C. The particles were boiled in 6.0 M HCl for 1 h. Finally, the resulting material (ghosts) was washed with PBS, dialysed against deionized water for 2 d and lyophilized.

Quantitative elemental analysis of melanins

Elemental analysis for carbon, hydrogen, nitrogen and sulfur was performed by Quantitative Technologies Inc. (Whitehouse, NJ, USA) as described in Rosas et al., (2000).

Transmission electron microscopy

Melanin ghosts or *Sepia* melanin were frozen under high pressure using a Leica EMpact High Pressure Freezer (Leica Microsystems, Wetzlar, Germany). Frozen samples were transferred to a Leica EM AFS Freeze Substitution Unit and freeze substituted in 1% osmium tetroxide in acetone. They were brought from –90°C to room temperature over 2–3 d, rinsed in acetone and embedded in Spurrs epoxy resin (Polysciences, Warrington, PA, USA). Ultrathin sections of 70–80 nm were cut on a Reichert Ultracut UCT, Wetzlar, Germany, stained with uranyl acetate followed by lead citrate and viewed on a JEOL (Tokyo, Japan) 1200EX transmission electron microscope at 80 kV. Transmission electron microscopy (TEM) was performed at the Analytical Imaging Facility of Albert Einstein College of Medicine.

Oxidation of melanins and HPLC of oxidized melanins

The melanins were subjected to acidic permanganate oxidation by the modified technique described in Ito and Fujita (1985) and Wakamatsu and Ito (2002). The PTCA, PDCA, TTCA, and TDCA used as standards were a kind gift from Dr K. Wakamatsu of Fujita Health University of the Health Sciences, Toyoake, Japan. The oxidation products were analysed by HPLC using a Shimadzu LC-600 liquid chromatograph, Hamilton PRP-1 C_{18} column (250 × 4.1 mm dimensions, 7 μm particle size), and Shimadzu (Kyoto, Japan) SPD-6AV UV detector. The mobile phase was 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B). At 1.0 ml/min, the elution gradient was (min, %B): 0, 0; 1, 0; 12, 25; 14, 25, 16, 0. The UV detector was set at a 255 nm absorbance.

MALDI mass spectrometry

The major peaks generated during chromatography of oxidized melanins were collected and analysed by MALDI-TOF mass spectrometry in positive pressure mode on PE-Biosystems (Applied Biosystems, Foster City, CA, USA) Mariner mass spectrometer. A peptide mixture with molecular weights of 1059.56, 1296.68, and 1672.95 in 2,5-dihydroxybenzoic acid matrix was used for calibration.

Electron spin resonance spectroscopy

The ESR of melanin ghosts suspended in water was performed on ER 200D EPR/ENDOR spectrometer with ESP 300 upgrade (Brucker Instruments, Inc. Billerica, MA, USA).

Determination of melanins shielding properties

To assess the shielding properties of Cn melanin ghosts, the 96-well microtiter plate was coated with a 0.1% solution of polylysine to make the surface of the wells positively charged to counteract negative charge carried by melanin ghosts. The ghosts were mixed with 0.1% polylysine solution to prepare homogeneous suspension and three different concentrations of ghosts were placed in the wells. For control, different concentrations of *Sepia* melanin and charcoal suspensions in polylysine solution were used as well as lead foil of similar weights. The microtiter plate was exposed to diagnostic X-rays (40 kVp at 10 mA) and the shielding effect was detected with Hi-speed Kodak x-omat (xb-1) radiographic film (Eastman Kodak Company, Rochester, NY, USA) placed under the plate during exposure to X-rays. After the film development the areas of the film located under the wells that were shielded from X-rays with different materials remained under unexposed while the rest of the exposed film became dark. The quantification procedure closely resembled that used in bone densitometry: the film was scanned, and the color was inverted with Adobe Photoshop with the less exposed areas becoming the darkest. This inversion of color allows further quantification of color intensity. The corresponding global intensities were determined by the subtraction method (Biorad Quantity One software, Biorad Laboratories, Hercules, CA, USA) and IPD per mm^2 was calculated. The attenuation of X-rays by substances in the wells was calculated as the ratio of IPD in the wells to IPD of the background wells.

Statistical analysis

The slopes of the fungal cell survival curves were determined by linear regression (GraphPad prism; GraphPad software, San Diego,

CA, USA) and Student's test for unpaired data was performed to analyse the differences in survival. The differences were considered statistically significant when P-values were <0.05.

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References

Bhandari, R. (1985). Scattering coefficients for a multilayered sphere: analytic expressions and algorithms. Appl. Opt. *24*, 1960–1974.

Casadevall, A. (2005). Fungal virulence, vertebrate endothermy, and dinosaur extinction: is there a connection? Fungal Genet. Biol. *42*, 98–106.

Dadachova, E., Howell, R.W., Bryan, R.A., Frenkel, A., Nosanchuk, J.D., and Casadevall, A. (2004). Susceptibility of the human pathogenic fungi *Cryptococcus neoformans* and *Histoplasma capsulatum* to gamma-radiation versus radioimmunotherapy with alpha- and beta-emitting radioisotopes. J. Nucl. Med. *45*, 313–320.

Dadachova, E., Bryan, R.A., Huang, X., Moadel, T., Schweitzer, A.D., Aisen, P., Nosanchuk, J.D., and Casadevall, A. (2007). Ionizing radiation changes the electronic properties of melanin and enhances the growth of melanized fungi. PLoS ONE *5*, e457.

Davis, M., Hut, P., and Muller, R.A. (1985). Terrestrial catastrophism: nemesis or galaxy? Nature *313*, 503.

Eisenman, H.C., Nosanchuk, J.D., Webber, J.B., Emerson, R.J., Camesano, T.A., and Casadevall, A. (2005). Microstructure of cell wall-associated melanin in the human pathogenic fungus *Cryptococcus neoformans*. Biochemistry *44*, 3683–3693.

Enochs, W.S., Nilges, M.J., and Swartz, H.M. (1993). A standardized test for the identification and characterization of melanins using electron paramagnetic resonance (EPR) spectroscopy. Pigment Cell Res. *6*, 91–99.

Forest, S.E., Lam, W.C., Millar, D.P., Nofsinger, J.B., and Simon, J.D. (2000). A model for the activated energy transfer within eumelanin aggregates. J. Phys. Chem. B *104*, 811–814.

Frases, S., Chaskes, S., Dadachova, E., and Casadevall, A. (2006). Induction by Klebsiella aerogenes of a melanin-like pigment in *Cryptococcus neoformans*. Appl. Environ. Microbiol. *72*, 1542–1550.

Frases, S., Salazar, A., Dadachova, E., and Casadevall, A. (2007). *Cryptococcus neoformans* can utilize the bacterial melanin precursor homogentisic acid for fungal melanogenesis. Appl. Environ. Microbiol. *73*, 615–621.

Garcia-Rivera, J., Eisenman, H.C., Nosanchuk, J.D., Aisen, P., Zaragoza, O., Moadel, T., Dadachova, E., and Casadevall, A. (2005). Comparative analysis of *Cryptococcus neoformans* acid-resistant particles generated from pigmented cells grown in different laccase substrates. Fungal Genet. Biol. *42*, 989–998.

Hall, E.J. (2000). Radiobiology for the Radiologist (Philadelphia: Lippincott Williams & Willkins), pp. 91–94.

Hill, H.Z. (1992). The function of melanin or six blind people examine an elephant. Bioessays *14*, 49–56.

Hulot, G., and Gallet, Y. (2003). Do superchrons occur without any palaeomagnetic warning? Earth Planetary Sci. Lett. *210*, 191–201.

Ito, S., and Fujita, K. (1985). Microanalysis of eumelanin and pheomelanin in hair and melanomas by chemical degradation and liquid chromatography. Anal. Biochem. *144*, 527–536.

Jacobson, E.S. (2000). Pathogenic roles for fungal melanins. Clin. Microbiol. Rev. *13*, 708–717.

Meredith, P., and Sarna, T. (2006). The physical and chemical properties of eumelanin. Pigment Cell Res. *19*, 572–594.

Mirchink, T.G., Kashkina, G.B., and Abaturov, I.D. (1972). Resistance of fungi with different pigments to radiation. Mikrobiologiia *41*, 83–86.

Mironenko, N.V., Alekhina, I.A., Zhdanova, N.N., and Bulat, S.A. (2000). Intraspecific variation in gamma-radiation resistance and genomic structure in the filamentous fungus *Alternaria alternata*: a case study of strains inhabiting Chernobyl reactor no. 4. Ecotoxicol. Environ. Saf. *45*, 177–187.

Nosanchuk, J.D., and Casadevall, A. (2003). The contribution of melanin to microbial pathogenesis. Cell Microbiol. *5*, 203–223.

Nosanchuk, J.D., Gomez, B.L., Youngchim, S., Diez, S., Aisen, P., Zancope-Oliveira, R.M., Restrepo, A., Casadevall, A., and Hamilton, A.J. (2002). *Histoplasma capsulatum* synthesizes melanin-like pigments in vitro and during mammalian infection. Infect. Immun. *70*, 5124–5131.

Riesz, J., Gilmore, J., and Meredith, P. (2006). Quantitative scattering of melanin solutions. Biophys. J. *90*, 4137–4144.

Robinson, C.H. (2001). Cold adaptation in Arctic and Antarctic fungi. New Phytol. *151*, 341–353.

Rosas, A.L., Nosanchuk, J.D., Gomez, B.L., Edens, W.A., Henson, J.M., and Casadevall, A. (2000). Isolation and serological analyses of fungal melanins. J. Immunol. Methods *244*, 69–80.

Saleh, Y.G., Mayo, M.S., and Ahearn, D.G. (1988). Resistance of some common fungi to gamma irradiation. Appl. Environ. Microbiol. *54*, 2134–2135.

Sarna, T., and Swartz, H.M. (1978). Identification and characterization of melanin in tissues and body fluids. Folia Histochem. Cytochem. *16*, 275–286.

Sarna, T., and Swartz, H.M. (1993). Interaction of melanin with oxygen (and related species). In Atmospheric Oxidation and Antioxidants, Vol. III, G. Scott, ed. (Amsterdam: Elsevier), pp. 129–169.

Sealy, R.C., Hyde, J.S., Felix, C.C., Menon, I.A., and Prota, G. (1982). Eumelanins and pheomelanins: characterization by electron spin resonance spectroscopy. Science *217*, 545–547.

Sinilova, N.G., Pershina, Z.G., Duplitseva, A.P., and Pavlova, I.B. (1969). A radioresistant pigmented bacterial culture isolated from atomic reactor water. Zh. Mikrobiol. Epidemiol. Immunobiol. *46*, 94–99.

Sorenson, J.A., and Phelps, M.E. (1987). Physics in Nuclear Medicine (Philadelphia: WB Saunders Company).

Travis, E.L. (1984). The oxygen dependence of protection by aminothiols: implications for normal tissues and solid tumors. Int. J. Radiat. Oncol. Biol. Phys. *10*, 1495–1501.

Wakamatsu, K., and Ito, S. (2002). Advanced chemical methods in melanin determination. Pigment Cell Res. *15*, 174–183.

Wang, Y., Aisen, P., and Casadevall, A. (1995). *C. neoformans* melanin and virulence: mechanism of action. Infect. Immun. *63*, 3131–3136.

Wang, Y., Aisen, P., and Casadevall, A. (1996). Melanin, melanin 'ghosts,' and melanin composition in *Cryptococcus neoformans*. Infect. Immun. *64*, 2420–2424.

Zeise, L., Murr, B.L., and Chedekel, M.R. (1992). Melanin standard method: particle description. Pigment Cell Res. *5*, 132–142.

