Introduction

Several fungal species found in different divisions (phyla) within the Kingdom Fungi commonly synthesize melanin pigments [1,2]. Fungal melanin is a complex molecule that has been associated with a variety of functions including protection against free radical fluxes, heavy metal toxicity and electromagnetic radiation [1,3,4]. In addition, fungal melanization is important for pathogenesis because this pigment has been associated with virulence and acquired resistance to antifungal agents [3,5,6]. Melanin was recently proposed to function as an energy transducer in fungi promoting the growth of melanized organisms under gamma radiation fluxes [4]. Melanin is often found in the fungal cell wall where it contributes to cellular structural strength.

Fungal melanins are divided into two major categories known as dihydroxyphenylalanine (DOPA)-melanin and dihydroxynaphthalene (DHN)-melanin depending on their biosynthetic pathway [7]. Like all natural melanins, the structure of fungal melanin remains largely unknown because these pigments are amorphous, insoluble, and are often recovered from natural sources intimately associated with other cellular materials. Consequently, various structural studies have involved synthetic melanins derived from oxidized L-DOPA and DHN but the correspondence of these pigments is largely unknown because these pigments are amorphous, insoluble and often associated with other biological materials. Consequently, there is a dearth of structural techniques to study this enigmatic pigment. Current models of melanin structure envision the stacking of planar structures. X-ray diffraction has historically been used to deduce stacking parameters. In this study we used X-ray diffraction to analyze melanins derived from Cryptococcus neoformans, Aspergillus niger, Wangiella dermatitides and Coprinus comatus. Analysis of melanin in melanized C. neoformans encapsulated cells was precluded by the fortuitous finding that the capsular polysaccharide had a diffraction spectrum that was similar to that of isolated melanin. The capsular polysaccharide spectrum was dominated by a broad non-Bragg feature consistent with origin from a repeating structural motif that may arise from inter-molecular interactions and/or possibly gel organization. Hence, we isolated melanin from each fungal species and compared diffraction parameters. The results show that the inferred stacking distances of fungal melanins differ from that reported for synthetic melanin and neuromelanin, occupying intermediate position between these other melanins. These results suggest that all melanins have a fundamental diffracting unit composed of planar graphitic assemblies that can differ in stacking distance. The stacking peak appears to be a distinguishing universal feature of melanins that may be of use in characterizing these enigmatic pigments.
robust peak similar to that found for eumelans. The position of the peak, however, suggests a difference in the stacking distance of the neuromelans compared to the eumelans. In this study we use X-ray diffraction techniques to compare four different types of fungal melanins. Ours results are consistent with the proposal that melans are composed of planar structures that can differ in stacking distances.

**Materials and Methods**

**Cryptococcus neoformans** (Phylum Basidiomycota)

* C. neoformans* strain ATCC 24067 (serotype D) was obtained from the ATCC (Rockville, MD). Strain H99 (serotype A) was obtained from Dr. John Perfect (Duke University, NC) and strain NIH-191 (serotype C) was obtained from Dr. Ashok Varma (Bethesda, MD). Cells were grown in minimal medium (20 mM MgSO4*7H2O, 58.8 mM KH2PO4, 20 mM Glycine, 15 mM Glucose and 6 uM thiamine) with or without 1 mM of L-dopa at 30°C. Next, cells were washed and incubated overnight at 30°C with 1.0 mg/ml of Proteinase K (Roche Molecular Biochemicals, Indianapolis, IN) in reaction buffer (10 mM Tris, 1 mM CaCl2, 0.05% SDS at pH 7.8) for 4 h at 37°C with shaking for 1 month.

**Aspergillus niger** (Phylum Ascomycota)

*A. niger* strain J9901 was obtained from the ATCC. Conidia from *A. niger* were grown on Sabouraud dextrose Agar at pH 5.6 (Difco Laboratories, Detroit, MI) for 1 month at 25°C. Conidia were removed from the agar with 0.05% of tween 20 in PBS by gentle scraping and shaking.

**Wangiella dermatitides** (Phylum Ascomycota)

*W. dermatitides* strain 8656 was obtained from the ATCC. The strain was grown in YPD media (2% peptone, 1% Bacto yeast extract and 2% dextrose) at 37°C with shaking for 1 month.

**Coprinus comatus** (Phylum Basidiomycota)

These mushrooms ("inky caps") naturally grow along roadsides in the Pacific Northwest. The termination of the mushroom phase is the formation of black-walled spores along the mushroom cap gills followed by a rapid loss of mass by liquefaction (deliquescence) of the mushroom cap into a black inky fluid containing the spores that drop to the ground. Mushroom caps (n = 5) were collected (Discovery Bay, Sequim, Washington) just as they were changing into an inky black fluid. The fluid was stored at room temperature in plastic storage bags and the fluid shipped to New York for further processing.

**Preparation of melanin**

Melanin was isolated by standard protocol as described previously [10]. Briefly, melanized cells were centrifuged at 3,000 rpm for 20 minutes and washed with PBS. Cells were resuspended in a mixture of 1 M sorbitol and 0.1 M of sodium Citrate at pH 5.5, then cell wall lysing enzyme (Sigma Chemical-Aldrich, Inc, St. Louis, MO) was added at 10 mg/ml and incubated overnight at 30°C. Next, cells were washed and incubated in 4.0 M guanidine thiocyanate for 12 h at room temperature with gentle shaking. The melanin preparations were washed with PBS and incubated with 1.0 mg/ml of Proteinase K (Roche Molecular Biochemicals, Indianapolis, IN) in reaction buffer (10 mM Tris, 1 mM CaCl2, 0.05% SDS at pH 7.8) for 4 h at 65°C. After the incubation, samples were washed and resuspended in an aqueous/methanol/chloroform mixture (3:4:8, respectively) and extracted three times. The aqueous and particulate fractions were boiled in 6 M HCl for 1 h and washed with PBS and dialyzed extensively in water. Final samples were lyophilized to dryness. All washes were done three times and dialysis was against deionized distilled water. The protocol described above was applied to all the fungal strains.

**Capsular polysaccharide isolated by DMSO extraction**

Capsular PS was isolated by a modified protocol described previously [15]. Briefly, yeast cells were grown for six days in minimum medium then centrifuged at 6K RPM and washed three times with Milli-Q water. The cell suspension was mixed with DMSO at a ratio of 1:2, respectively and incubated for 1.0 hr at room temperature. Cells were removed by centrifugation, and the supernatant was then dialyzed against water (change 2–3 times daily) for at least 72 hrs. Then, the sample was lyophilized to dryness.

**X-ray Diffraction**

Samples of lyophilized melanin powders were pressed into discs, 1 cm in diameter and 1 mm thick. The samples were scanned using a Philips diffractometer operating with a 1.54 Angstrom X-ray beam. Scattering intensity was recorded as a function of the scattering angle. The data were converted from angle coordinates to Q scale with Q being the magnitude of the momentum change of the X-ray photon elastically scattered through an angle Θ. The parameter Q is related to the scattering angle by the equation: 

\[ Q = 4\pi \sin(\theta/2)/\lambda \]

where \( \lambda \) is the X-ray photon wavelength (1.54 Angstroms in this case). It is useful to relate the Q peak maximum to the distance between the molecular sheets, R, where \( R = 2\pi/Q \). However, it should be borne in mind that R represents an average value for the distance, the uncertainty being related to the width of the stacking feature in Q space [11–14].

**Electron Spin resonance (ESR)**

ESR measurement of melanin where done using the same methods as in prior studies [16].

**Results**

**Diffraction studies on whole cells**

Our initial studies were designed to ascertain whether we could gain insight into melanin structure from pigment in the cell wall. Hence, we compared the X-ray diffraction features observed with melanized and non-melanized whole *C. neoformans* cells using 1.54 Angstrom source. The scattering of X-rays by crystalline structures produces sharp peaks in the diffraction spectrum that serve as a signature for the crystal analyzed. In contrast, amorphous compounds like melans and polysaccharide produce broad features in the diffraction spectrum known as non-Bragg features resulting from the absence of coherent scattering from regular and repeating structures (e.g., crystals). When whole cells were analyzed the essential feature of these measurements was the presence of a broad non-Bragg feature, whose location was identified in units of Q (reciprocal angstroms). The parameter Q provides a measure of the spacing for the diffracting structure (Figure 1). To explore the origin of this signal we compared the diffraction spectrum of encapsulated and acapsular *C. neoformans* cells in their melanized and non-melanized states (Figure 1). The acapsular strain samples, whether melanized or non-melanized produced a spectrum that lacked the amorphous peak but instead showed several crystalline Bragg peaks (Figure 1). Since the position of these peaks in the melanized and non-melanized strains was the same we conclude that they do not come from melanin. Next we compared the diffraction pattern of whole encapsulated cells, polysaccharide (PS) naturally released from such cells (Exo-PS) and polysaccharide extracted from the capsules (Cap-PS) using dimethylethyl sulfoxide (DMSO) from four different cryptococcal
strains differing in serotype and polysaccharide structure [Table 1].
Whole cells produced spectra with two Q values, referred to as Q1
and Q2, in the range of 1.38–1.39 and 1.48–1.51, respectively (see
Figure 2 for representative spectra). In contrast, the diffraction
spectra of isolated exopolysaccharide and polysaccharides released
from the capsule was in the range 1.46–1.51, a value consistent
with the range for Q2. Numerous Bragg-type diffraction peaks
were apparent in all samples studied. Since these peaks occur in
the same position irrespective of the presence of capsule or
melanin we attribute their origin from microcrystalline impurities.
The presence of melanin in the polysaccharide preparation was
ruled out by the absence of any pigmentation or ESR signal
indicative of a free radical population (data not shown).

Fungal melanins. The results are given as the Q peaks of the X-ray
diffraction measurements of the melanins derived from the four
different fungal species [Table 2]. The fungal melanins appear to
fall into two groups. The melanins of *Aspergillus niger* and *C.
neoformans* group into one category with Q peaks of 1.41 and 1.43
and calculated R spacing of 4.39 and 4.45 respectively. The
second group was composed of melanin from *Wangiella dermatitides*
and the *Coprinus comatus*, which manifested Q peaks of 1.51 and
1.53 corresponding to R spacing of 4.15 and 4.10 angstroms,
respectively. Representative spectra are shown in Figure 3.

These values can be compared to those obtained for two
synthetic melanins derived from oxidized dihydroxynaphthelene
and from oxidized L-Dopa and to eumelanins derived from squid
ink (*Sepia officinalis*) and from various pigmented centers in the
human brain [Table 2]. The melanins of *Wangiella dermatitides*,
*Coprinus comatus* and *Aspergillus niger* have Q peaks and R spacing
similar to the synthetic DHN melanin. *C. neoformans* melanin is
known to be a DOPA melanin but the Q peak for melanin was
smaller than that of autopolymerized L-dopa.

**Discussion**

The object of the investigation was to use X-ray diffraction to
gain insight into the structure of fungal melanins. Given their
amorphous nature melanins are not amenable to study by
crystallography and their insolubility precludes the use of solution
structural analytic techniques such as NMR. The utility of X-ray
diffraction techniques for the analysis of the structure of
amorphous materials is limited. The spectra are dominated by

![Figure 1. X ray diffraction spectra of whole encapsulated and acapsular cells in their melanized and non-melanized states. Panels: A) Acapsular melanized; B) Acapsular non-melanized; C) encapsulated melanized; and D) encapsulated non-melanized. The spectrum of encapsulated strains is dominated by a broad non-Bragg diffraction feature that is not present in non-encapsulated strains. The sharp Bragg-type diffraction peaks are found in all the spectra and these are likely to originate from microcrystalline elements found in all samples.](https://doi.org/10.1371/journal.pone.0030299.g001)
the incoherent background that obscures the coherent constructive interference component that carries the structural information. If the atomic composition is known, the incoherent component can be calculated and subtracted. However, in amorphous materials, the orientations of the structural elements are random and the resulting spectrum represents an integrated spatial average. Such diffraction spectra can provide a template for iterative testing of proposed structures by rejecting those structures for which the calculated coherent scattering spectra are in significant disagreement with the measured spectrum after the background is removed.

Melanin pigments are common in the fungal kingdom and their presence has been associated with numerous functions ranging from cellular strength to virulence and energy transduction [2–4]. Our initial approach was to study melanin in situ in fungal cell walls by comparing the diffraction from melanized and non-melanized cells grown with and without L-DOPA, respectively. We initially hoped that by using whole cells that we could gain structural insights of melanin in its native cell wall state. Unfortunately, the diffraction spectrum of encapsulated melanized and non-melanized cells was very similar and we could not assign any spectral features to melanin. Consequently, we switched to comparing melanized and non-melanized acapsular cells but again failed to assign melanin spectral features in the diffraction spectrum. Our inability to detect a clear melanin signature from the comparison of melanized and non-melanized cells is perhaps not surprising if one considers that the amount of melanin per cell is likely to be only a small percentage of the total mass [10]. Of interest, we noted the absence of the broad monotonic band in acapsular cells and proceeded to investigate whether this signal was originating from the polysaccharide capsule. Analysis of isolated polysaccharide confirmed that it produced a diffraction spectrum dominated by a broad monotonic band which was intriguingly similar to that of melanin.

To further investigate the nature of the contribution of polysaccharide to the diffraction spectra from whole cells we removed the polysaccharide using DMSO extraction and analyzed it separately. Given the scant information about the conformational structure of cryptococcal polysaccharide we also hoped that the analysis would provide additional information. We noted that for most strains the isolated polysaccharides provided two peaks denoted as $Q_1$ and $Q_2$, suggesting the presence of at least two types of structures in the polysaccharide. Since these values of $Q$ imply an inter-scatter distance that is much larger than any interatomic distance we infer that it is likely to originate from a repeating structural motif that arises from inter-molecular interactions and/or possibly gel organization. Consequently, we suggest that the broad non-Bragg structural feature arises from an electron dense larger repeating structure that may be altered by organic solvent extraction such as inter-molecular interactions or possibly helical parameters, as have been suggested for this polysaccharide [17]. Although this data does not allow us to propose a model to account for that structure within the context of polysaccharide chemical structures the feature is possibly related to

### Table 1. Summary of staking peak for the main non-Bragg diffraction feature observed with cells, exopolysaccharide (Exo-PS) and capsule extracted polysaccharide (CAP-PS) of four strains of *C. neoformans.*

| Strain | Serotype | Cells Q1 | Exo-PS Q1 | CAP-PS Q1 |
|--------|----------|---------|-----------|-----------|
| H99    | A        | 1.37    | 1.48      | 1.46      |
| 24067  | D        | 1.38    | 1.51      | 1.50      |
| NIH 191| C        | 1.39    | -         | -         |
| NIH 198| B        | 1.38    | 1.49      | 1.51      | 1.46      |

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![Figure 2. X-ray diffraction spectra of 24067 whole encapsulated cells, 24067 exo-polysaccharide and 24067 capsular polysaccharide.](doi:10.1371/journal.pone.0030299.g002)
non-covalent associations of polymeric strands, something akin to tangles or knots. Nevertheless, the finding of the capsular non-crystalline feature is interesting and could provide an avenue for new line of investigations to determine the dependence of the feature on experimentally controllable factors.

The detection of signal originating from capsular polysaccharide in the region where the melanin signal was expected suggested that was very difficult to obtain structural insights from cell wall associated melanin by comparing melanized and non-melanized cells. Consequently, we turned our attention to analyzing melanin recovered from fungal cells and comparing their diffraction data to literature studies. Our goal was to analyze Cryptococcus neoformans melanin formed when the fungi were grown in a medium which included the melanin precursor L-Dopa. Furthermore, we sought to compare C. neoformans melanin (DOPA-melanin) to Wangiella dermatitidis melanin (DHN-melanin). Previous studies had established that melanins presented a diffraction feature whose peak position appeared to vary depending on the process of formation of the various melanins. Although the fungal melanin preparations used exhibit free radical signals characteristic of melanins, these natural melanins almost certainly contain other components, possibly in covalent linkages [8,9].

The diffraction spectrum revealed that C. neoformans melanin produced a peak at approximately \( Q = 1.4 \), which is significantly smaller different from that of polymerized L-Dopa and other eumelanins \( Q = 1.6 \) reported previously [11]. Furthermore, diffraction pattern for Wangiella dermatitidis, Aspergillus niger and Coprinus comatus melanins had \( Q \) values that differed from both cryptococcal melanin and other eumelanins. The finding that all the fungal melanins yielded \( Q \) values suggesting stacking distances in the 4.1–4.5 Å range is

Table 2. Summary of staking peak and distances for various melanins obtained from this study and literature sources.

| Melanin Source                  | Stacking peak \( Q \) | Stacking Distance (Å) | Reference        |
|---------------------------------|------------------------|------------------------|------------------|
| **Fungal melanins**             |                        |                        |                  |
| C. neoformans melanin ghosts    | 1.43                   | 4.39                   | This study       |
| Aspergillus niger               | 1.41                   | 4.45                   | This study       |
| Wangiella dermatitidis          | 1.51                   | 4.15                   | This study       |
| Coprinus comatus                | 1.53                   | 4.10                   | This study       |
| **Other melanins**              |                        |                        |                  |
| Auto oxidized L-dopa            | 1.64                   | 3.45                   | [11]             |
| Sepia officinalis               | 1.6                    | 3.46                   | [11]             |
| Synthetic oxidized dihydroxynapthene | 1.53               | 4.10                   | Unpublished      |
| Neuro putamen                   | 1.34                   | 4.67                   | [14]             |
| Neuro pre-temporal cortex       | 1.35                   | 4.65                   | [14]             |
| Neuro cerebellum                | 1.35                   | 4.65                   | [14]             |
| Neuro substantia nigra          | 1.33                   | 4.72                   | [14]             |

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Figure 3. X ray diffraction spectra of melanin isolated from Wangiella dermatitidis (A) and C. neoformans (B). The spectrum of each melanin is dominated by a broad non-Bragg diffraction pattern.

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consistent with the model that melanin from this kingdom also consists of stacked discs that differ in distance based on their composition. In fact, the stacking peak may be a universal feature for melanin that could be used in classifying this enigmatic pigment. Hence, it is conceivable that all melanins share a similar organizational structure but that individual pigments differ in stacking distance details as a result of local differences in composition and possibly associated components, such as the aliphatic material detected in C. neoformans melanin by solid NMR analysis [9].

In summary, we conclude that the X-ray diffraction is a useful technique for comparing melanins from various sources and could have usefulness in studying capsular polysaccharides. For instance, we serendipitously observed a diffraction signal from cryptococcal polysaccharide indicative of a repeating structure that could be exploited in future studies to gain new structural insights. Our results provide the first evidence that natural fungal melanins share the basic stacked planar sheet structure proposed for other melanins and indicate that fungal melanins can be identified by their stacking peak parameters and appear to fall into groups subclasses, perhaps related to their biosynthetic pathway differences. Although this alone does not provide information on details of the structure we conclude that the concept of a unique structure for all melanins cannot be supported. While our results are consistent with a general stacking model, it is important to note that there were significant differences between the various melanins. More structural information might be obtained by carrying out a systematic analysis of the diffraction spectra including the subtraction of the incoherent background and testing various proposed models against the experimental data. The much simpler task of measuring the location of the stacking peak on many melanins from the raw data could provide some interesting insights into the richness of melanins structure and functions.

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Author Contributions
Conceived and designed the experiments: AC. Performed the experiments: AN. Analyzed the data: PC. Contributed reagents/materials/analysis tools: ME. Wrote the paper: AC.

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