Solid-state NMR Reveals the Carbon-based Molecular Architecture of Cryptococcus neoformans Fungal Eumelanins in the Cell Wall*

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Subhasish Chatterjee, Rafael Prados-Rosales, Boris Itin, Arturo Casadevall, and Ruth E. Stark

From the Department of Chemistry, City College of New York, Graduate Center and Institute for Macromolecular Assemblies, City University of New York, New York, New York 10031-9101, the Department of Microbiology and Immunology, Albert Einstein College of Medicine, Yeshiva University, Bronx, New York 10461, and the New York Structural Biology Center, New York, New York 10027

Background: Melanin is a poorly understood fungal virulence factor.

Results: 2D $^{13}$C-$^{13}$C correlation solid-state nuclear magnetic resonance reveals the carbon-based molecular architecture of intact melanin pigment assemblies in Cryptococcus neoformans.

Conclusion: Polysaccharide cell-wall components form a scaffold for layered deposition of aromatic-based pigment assemblies.

Significance: Deciphering macromolecular interactions that drive melanin pigment assembly in fungal cell walls facilitates the development of drug delivery materials.

Melanin pigments protect against both ionizing radiation and free radicals and have potential soil remediation capabilities. Eumelanins produced by pathogenic Cryptococcus neoformans fungi are virulence factors that render the fungal cells resistant to host defenses and certain antifungal drugs. Because of their insoluble and amorphous characteristics, neither the pigment bonding framework nor the cellular interactions underlying melanization of C. neoformans have yielded to comprehensive molecular-scale investigation. This study used the C. neoformans requirement of exogenous obligatory catecholamine precursors for melanization to produce isotopically enriched pigment “ghosts” and applied 2D $^{13}$C-$^{13}$C correlation solid-state NMR to reveal the carbon-based architecture of intact natural eumelanin assemblies in fungal cells. We demonstrated that the aliphatic moieties of solid C. neoformans melanin ghosts include cell-wall components derived from polysaccharides and/or chitin that are associated proximally with lipid membrane constituents. Prior to development of the mature aromatic fungal pigment, these aliphatic moieties form a chemically resistant framework that could serve as the scaffold for melanin synthesis. The indole-based core aromatic moieties show interconnections that are consistent with proposed melanin structures consisting of stacked planar assemblies, which are associated spatially with the aliphatic scaffold. The pyrrole aromatic carbons of the pigments bind covalently to the aliphatic framework via glycoside or glyceride functional groups. These findings establish that the structure of the pigment assembly changes with time and provide the first biophysical information on the mechanism by which melanin is assembled in the fungal cell wall, offering vital insights that can advance the design of bioinspired conductive nanomaterials and novel therapeutics.

Among the natural pigments that are used increasingly to guide the design of therapeutic and “smart” energy conversion materials (1–3), black or brown fungal eumelanins have attracted particular interest because of their versatile roles as virulence factors, in drug resistance, and in protection from UV radiation (4, 5). Nonetheless, elucidating the molecular-scale basis for these important properties has been challenging because the materials are insoluble, heterogeneous, and amorphous in structure. Despite spectroscopic and structural reports on melanins from diverse biological sources (2, 3, 6–10), the detailed molecular architecture of these natural pigments within their cellular milieu has remained unresolved.

The pathogenic Cryptococcus neoformans fungus has provided a unique investigative system for melanin biopolymer structure because this organism uses obligatory exogenous catecholamine precursors to produce the natural pigment. Hence, in contrast to other sources of natural melanins (6, 7, 9, 10), the starting materials and corresponding metabolic products for C. neoformans melanization can be well defined. Furthermore, we can selectively isolate for investigation those cellular constituents that are closely associated with the pigment and thereby protected from both environmental effects and chemical degradation. Finally, high resolution solid-state nuclear magnetic resonance (NMR) approaches can yield direct insights into the atomic level structure, dynamics, and action mechanisms of noncrystalline bioassemblies, including plant and microbial complexes that have polysaccharide or lipid constituents (11–
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16). Thus, it is feasible to circumvent the difficulties of solubilization or crystallization to access macromolecular structure for pigments derived from known small molecule isotopically enriched precursors by using the *C. neoformans* fungal melanin and solid-state NMR.

For instance, we have produced *C. neoformans* “ghosts” consisting exclusively of melanin and the cell-wall remnants from melanized fungal cells (17, 18), which enable us to monitor the metabolic fate of both t-dopa and mannose or glucose “feedstocks,” to track the molecular development of precursors containing $^{13}$C isotopic labels at defined molecular sites, and to test mechanistic hypotheses for *C. neoformans* melanin biosynthesis by systematically varying the catecholamine precursors (8, 19, 20). To date, the eumelanin structural arrangements for indole-based aromatics, cell wall-derived polysaccharide components, and associated “lipid-like” aliphatic moieties have been deduced partially and indirectly from chemical shift trends observed in cross-polarization magic-angle spinning (CPMAS) $^3$ NMR experiments on intact solid samples or bonded spin-spin interactions observed by high resolution MAS of the aliphatic fraction of the pigment-cell wall assembly that is capable of swelling (8, 19–21). Although the major 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid building blocks have been deduced by chemical analysis of degraded melanins (1, 22, 23), key architectural questions regarding the aromatic core of the intact pigment produced in cell-free or fungal systems have just begun to be addressed (24). The pigment associations and covalent connections to the cell-wall constituents remain uncertain, despite their essential functional roles for *C. neoformans* melanin cellular protection, treatment of infections, and energy trapping (4, 5, 25).

A major unsolved question in fungal cell biology focuses on the process by which melanin is incorporated into cell walls to generate structures that enhance structural hardness and diminish susceptibility to immune defense mechanisms. For eumelanin embedded in the innermost layer of polysaccharide cell walls of the *C. neoformans* fungus and proximal to the phospholipid cell membrane (26, 27), this study focuses on analyzing the structural framework of the pigmented assemblies formed from obligatory catecholamine and glucose starting materials and then isolated from the fungal cells as chemically resistant melanin ghosts. A suite of 1- and 2D $^{13}$C solid-state NMR experiments was used to address several important open structural questions as follows. 1) What are the developmental time frames that characterize the formation of indole-based aromatics, oxygenated carbons from cell-wall polysaccharides, and fatty acyl-based cell membrane constituents within the melanizing fungal cells? 2) What kinds of polysaccharide and acylglyceride molecular frameworks, or co-organized scaffolds, are formed by *C. neoformans* glucose metabolism in the presence of t-dopa? 3) Which indole-derived moieties from the obligatory t-dopa precursor are spatially close and/or covalently linked to the glucose-derived constituents of the cell wall in melanized *C. neoformans* cells? 4) Which side chain-derived functional moieties from the obligatory t-dopa precursor are spatially close and/or covalently connected to the glucose-derived constituents of the cell wall in melanized *C. neoformans* cells?

Hence, in addition to probing the (supra)molecular architectures of the cell-wall polysaccharides and indole-based polymers individually at atomic scale, we have examined their intercomponent spatial proximities and covalent linkages within the melanin assembly of *C. neoformans*. Given the ubiquity of melanin pigments in all biological kingdoms, our fundamental studies can have important practical consequences for medical therapeutics, environmental remediation agents, protective coatings, and drug carriers (2 –4).

Experimental Procedures

*Melanin Biosynthesis in C. neoformans*—The serotype D 24067 strain of the *C. neoformans* fungus (American Type Culture Collection 208821) was incubated with 1 mM solutions of t-dopa or dopamine substrates in chemically defined media (29.4 mM KH$_2$PO$_4$, 10 mM MgSO$_4$, 13 mM glycerine, 15 mM D-glucose, and 3 $\mu$m thiamine, all from Sigma), as described previously (17, 18, 28); in designated experiments, these materials were supplied as t-[ ring-U-1$^{13}$C]dopa, l-[ 2,3-$^{13}$C]dopa, and/or [U-$^{13}$C]glucose (from Cambridge Isotope Labs, Andover, MA). The cells were grown at 30 °C for periods of 4 –14 days in separate experiments, using a rotatory shaker operating at 150 rpm.

Fungal cell pellets were obtained by centrifugation at 2000 rpm and washed with phosphate-buffered saline (PBS) to isolate melanin ghosts for biophysical study. Cell walls were removed by suspending the cells in 1.0 M sorbitol, 0.1 mM sodium citrate, pH 5.5, and incubating for 24 h at 30 °C with 10 mg/ml lysozyme enzymes from *Trichoderma harzianum*. Centrifugation at 2000 rpm for 10 min yielded a pellet of melanized protoplasts that was washed several times with PBS to obtain a nearly clear supernatant. To denature proteinaceous materials, the melanized cell suspension was incubated with 4 M guanidine thiocyanate for 12 h at room temperature in a rocker (Shaker 35, Labnet, Woodbridge, NJ). The recovered cell debris was collected, washed 2–3 times with ~20 ml of PBS, and then incubated for 4 h at 65 °C in 5 ml of buffer (10 mM Tris-HCl, pH 8.0, 5 mM CaCl$_2$, 5% SDS) containing 1 mg/ml protease K (Roche Applied Science). The cell debris was recovered, washed 2–3 times with ~20 ml of PBS, and then subjected to three successive Folch lipid extractions (29) while maintaining the proportions of chloroform, methanol, and saline solution in the final mixture as 8:4:3. To hydrolyze cellular contaminants associated with melanin, the final product was suspended in 20 ml of 6 M HCl and boiled for 1 h. The black particles that survived HCl treatment retain the cellular shape of melanized *C. neoformans* cells and are known as melanin ghosts; they correspond to melanin pigments and pigment-bound cellular components. These particles were dialyzed against distilled water for 14 days with daily water changes and then lyophilized. The reproducibility of the protocol was tested by repeating the extraction process with two different batches of *C. neoformans* pigments produced with each of the catecholamine precursors.

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$^3$ The abbreviations used are: CPMAS, cross-polarization magic-angle spinning NMR; DARR, dipolar assisted rotational resonance; SAR-COSY, sensitive absorptive refocused scalar correlation spectroscopy; CP, cross-polarization; MAS, magic-angle spinning.
**Solid-state NMR—** Solid-state NMR measurements were carried out using either of two instruments as follows: a Varian (Agilent) DirectDrive 1 NMR spectrometer operating at a \( ^1H \) frequency of 600 MHz and equipped with a 1.6-mm HXY fast-MAS probe filled with 2–6 mg of powdered sample and spinning typically at 15 kHz (± 20 Hz) (Agilent Technologies, Santa Clara, CA); or a Bruker Avance I spectrometer (Bruker BioSpin Corp., Billerica, MA) operating at a \( ^1H \) frequency of 750 MHz and equipped with 4-mm HX, 3.2-mm HCN, or 3.2 mm HCN E-free probes containing 6–18 mg of powdered sample and spinning typically at 15 kHz (± 5 Hz). All spectra were acquired at spectrometer-set temperatures of 25 °C.

Typical 90° pulse lengths for \( ^1H \) were ~2.5 μs for the Bruker HCN probe and ~3 μs for the HCN E-free probe; \(^{13}C\) 90° pulse lengths were ~5 μs for both the 4-mm HX and 3.2-mm HCN probes. For the 1.6-mm Varian HXY fastMAS probe, typical 90° pulse lengths were ~1.2 μs for \(^1H\) and ~1.3 μs for \(^{13}C\). The 1D \(^{13}C\) spectral datasets were processed with 50–200 Hz of line broadening; chemical shifts were referenced externally to the methylene (–CH2–) group of adamantane (Sigma) at \( \delta_\text{C} = 38.48 \text{ ppm} \) (30).

For 1D \(^{13}C\) NMR using the 3.2-mm Bruker probes, ~20–50% linearly ramped radiofrequency (rf) field strengths (31) for \(^1H\) and a 50 kHz constant rf field for \(^{13}C\) were applied with typical 1–3-ms CP times to transfer magnetization from \(^1H\) to \(^{13}C\) nuclear spin baths; 80–100 kHz high power heteronuclear proton decoupling was applied using the two-pulse phase-modulated pulse sequence (32, 33); 3-s recycle delays were inserted between successive scans. Typical experimental parameters on the Bruker spectrometer included ~100 kHz sweep width, ~10–15-ms acquisition time, and 128–1024 transients for \(^{13}C\)-enriched samples.

For low yield samples (<5 mg), 1D \(^{13}C\) CPMAS spectra were recorded with the 1.6-mm Varian probe using typical 1–3-ms cross-polarization times with ramped field strengths as described above. High power heteronuclear \(^1H\) decoupling (175–185 kHz) was achieved using the small phase incremental alternation pulse sequence (33), and acquisition was carried out with a 3-s recycle delay. Typical experimental parameters (16, 20, 24) on the Varian spectrometer included 46 kHz sweep width, ~25-ms acquisition time, and 128–1024 transients for \(^{13}C\)-enriched pigments. \(^{13}C\) multiple CP experiments (34) were validated against traditional direct polarization measurements and used to obtain high throughput quantification of the pigment composition. The recycle delays at the beginning of the multiple CP experiments were 3 s, and the duration of the polarization period was 0.8 s for the natural abundance pigments. Cross-polarization times of 1.0 ms with 10 recursive cycles were used for the multiple CP measurement, and 5000–6000 transients were acquired for natural abundance samples.

The 2D \(^{13}C\)-\(^{13}C\) through-space correlation spectra were collected on \(^{13}C\)-enriched melanin samples using radiofrequency field-assisted diffusion mixing implemented in a dipolar assisted rotational resonance (DARR) mixing experiment (35, 36) with the Bruker HCN and HX probes or the Varian HXY probe. The 2D \(^{13}C\)-\(^{13}C\) correlation spectra were collected with 25–500-ms mixing times, typical MAS rates of 15 kHz, and 80–100 kHz two-pulse phase-modulated \(^1H\) decoupling during acquisition in separate experiments. The small phase incremental alternation pulse sequence was implemented to achieve 175–185 kHz \(^1H\) decoupling during acquisition with the Varian HXY probe. \(^1H\)-\(^{13}C\) cross-polarization was accomplished with a \(^{13}C\) field of ~50 kHz and a proton field strength that was ramped up to 90 kHz during 1–3-ms mixing times. Proton irradiation with a field strength corresponding to 15 kHz was applied during the DARR mixing period for both uniformly and selectively \(^{13}C\)-enriched samples. Spectral widths of ~46–100 kHz in each \(^{13}C\) dimension were used in separate experiments, defined by 1024–2048 points in the direct dimension, 128–1024 scans (direct dimension), and 96–360 points in the indirect dimension. The time proportional phase incrementation method (37) or the small phase incremental alternation pulse sequence (33) were utilized for phase-sensitive detection of the 2D spectra. For some \( C. neoformans \) melanin pigments, two or three identical data sets were added together to produce final DARR spectra with increased sensitivity.

Through-bond \(^{13}C\)-\(^{13}C\) interactions were measured for \(^{13}C\)-enriched melanins with 2D sensitive absorptive refocused scalar correlation spectroscopy (SAR-COSY) experiments (38) using the Bruker spectrometer operating at a \(^1H\) frequency of 750 MHz. The samples were spun in a 3.2-mm HCN E-free probe with 15 kHz MAS, and ~80–90 kHz of two-pulse phase-modulated heteronuclear decoupling was applied. Typical delays for refocusing (4 ms), z-filtering (6 ms), and spin lattice relaxation (3 s) were used in these experiments. Spectral widths of ~100 kHz were used in both direct and indirect dimensions, defined by 890–2048 and 40–136 points, respectively, and 512–1024 scans (direct dimension). For some \( C. neoformans \) samples, two identical data sets were added together to produce the final SAR-COSY spectra. Pure phase 2D line shapes were obtained using the States time proportional phase incrementation method (37). For 2D \(^{13}C\)-\(^{13}C\) spectral data, an exponential apodization function with 100–200 Hz line broadening was used for both direct and indirect detected dimensions. Identical 1D \(^{13}C\) spectra were recorded before and after lengthy 2D NMR experiments to confirm sample stability.

**Results**

*Time Course of C. neoformans Melanin Development—* Solid-state NMR spectra of the melanin ghosts were examined at two stages of cellular melanization, 4 and 14 days after the start of cell growth, to probe the temporal progression of molecular events involved in \( C. neoformans \) melanin biosynthesis. As noted above, our isolation treatments ensured that the spectroscopic characterization pertained exclusively to those cellular components that are bound to the pigment. These temporal comparisons included eumelanins derived from laccase-catalyzed polymerization of both \( l \)-dopa and dopamine exogenous precursors.

Fig. 1 illustrates a structural comparison between the quantitatively reliable 1D \(^{13}C\) multiple cross-polarization magic-angle spinning (multi-CPMAS) spectra of natural abundance \( l \)-dopa \( C. neoformans \) melanins at these two growth stages (Fig. 1); aliphatic frameworks displaying similar resonances were present at both early and late times, but the typically broad envelope of prominent aromatic signals does not appear prom-
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FIGURE 1. Left, 1D solid-state multi-CPMAS $^{13}$C NMR spectra of natural abundance C. neoformans (CN) strain 24067 l-dopa melanins obtained at a $^1$H frequency of 600 MHz and 15 kHz MAS. Top, mature melanin ghosts isolated after 14 days of cellular biosynthesis. Bottom, developing melanin ghosts isolated after 4 days of cell culture. The largest peak of each spectrum was set to full scale; spinning sidebands were ruled out by examining the spectral regions 15 kHz from each major resonance. Assignments of the major resonances to molecular moieties are described in the text. Right, 1D multi-CPMAS $^{13}$C NMR spectra of natural abundance C. neoformans strain 24067 dopamine melanins obtained at a $^1$H frequency of 600 MHz and 15 kHz MAS. Top, mature melanin ghosts isolated after 14 days of cellular biosynthesis. Bottom, developing melanin ghosts isolated after 4 days of cell culture. The largest peak of each spectrum was set to full scale; spinning sidebands were ruled out by examining spectral regions 20 and 30 kHz from each major resonance. The spectral resolution was improved modestly at a spinning rate of 30 kHz but did not benefit from use of higher speeds.

In contrast to the above findings, resistant aliphatic molecular framework takes place prior to significant deposition of acid-resistant oligomeric or polymeric aromatic pigments in the melanized fungal cells, indicating that the aliphatic moieties could form a supporting scaffold for the biosynthesized eumelanins.

Aliphatic Molecular Scaffolds for C. neoformans Melanin Pigment Deposition—The discovery of early developing insoluble aliphatic constituents, which could nonetheless survive the degradative treatments used to isolate fungal melanin ghosts, prompted a detailed spectroscopic examination of the carbon-based molecular frameworks formed in two C. neoformans cell lines. Once again, we isolated only cellular components that were bound to the melanin pigment. A d-$^3$-[U-$^{13}$C$_6$]glucose sugar source along with a natural abundance l-dopa catecholamine precursor were first used to focus on the carbon skeleton(s) involving the alkyl, alkoxy, alkene, carboxylate, and amide groups (Fig. 2). For mature 14-day l-dopa pigments in C. neoformans prepared in d-$^3$-[U-$^{13}$C$_6$]glucose, our 2D $^{13}$C-$^{13}$C DARR (35, 36) and SAR-COSY (38) measurements (Fig. 3) resolved many overlapping NMR signals and identified both through-space and through-bond pairwise spin connectivities, respectively, in the intact solid melanin ghosts. These $^{13}$C-$^{13}$C interactions established molecular-level structural constraints for $^{13}$C nuclei of the melanized polysaccharide cell walls and/or cell membranes, either within a single molecular species or between pairs of constituents within a defined architectural composite.

Many of the resonances observed in the 2D NMR experiments were in accord with reported spectra for the major cell-wall glucan, chitin, mannan, mannoprotein, and phospholipid constituents (39–43), allowing us to make structural assignments and propose molecular building blocks corresponding to the major C. neoformans melanin resonances of the $^{13}$C-enriched constituents (Table 1). For instance, solid-state $^{13}$C NMR spectra of insect chitins similar to the N-acetylglucosamine-based polysaccharides implicated in fungal melanization (44, 45) have been assigned to 56–105 ppm (for the ring carbons) and to 24 and 172 ppm (for the acetamido side chain) (39, 43). Through-space and through-bond interactions that support our assignments are detailed below.
We also observed chemical shift discrepancies, which were attributed to cell-wall structural alterations during melanization (4) and an isolation protocol selecting pigment-bound aliphatic constituents that could withstand exhaustive chemical treatments (17, 28). The network of through-space cross-correlated carbon pairs became more extended, as expected, with increasing DARR mixing time from 50 to 250 to 500 ms (Fig. 3). Cross-peaks observed with a 50-ms DARR mixing time reflect primarily short carbon-carbon distances (e.g. 171 × 22 ppm within a chitin N-acetylglucosamine unit) (39, 43). By contrast, data from the 250- and 500-ms mixing periods displayed additional cross-peaks corresponding to pairwise 13C-13C distances as long as ~6 Å (46) (e.g. 126 × 31 ppm between alkene and chain methylene groups and 171 × 72 ppm between carboxylates/amides and oxymethines). These latter aliphatic proximal 13C pairs could arise from acylglycerides, glycosides, or distinct molecular precursors that combine to form a new covalently bonded structure. Notably, our long mixing time DARR spectra include carbon pairs attributable to different C. neoformans aliphatic cell-wall constituents found in closely associated but nonbonded arrangements (45) within the melanin ghost assembly, e.g. carbons derived from β-glucans paired with chitin acetyl-amido methyl groups ([74 × 22], [82 × 22], and [104 × 22] ppm), glyceride esters, or chitin acetylcarboxyl paired

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**TABLE 1**

| Observed shift (ppm) | Reference shift (ppm) | Chemical grouping | Reference material(s) | Literature Refs. |
|----------------------|-----------------------|-------------------|-----------------------|------------------|
| 15–17                | 14                    | –CH2CH3           | Egg PC               | 40               |
| 22                   | 24                    | –CO2H13            | Chitin, N-acetylglucosamine | 39, 43       |
| 20–26, 30–35         | 23–32                 | –(CH3)2, –OOCCH(CH2)NH3 | Egg PC, polydopamine, l-dopa side chain | 3, 8, 19, 20, 40 |
| 40–44                | 43                    | –OCH2CH2–, –H2NCH– | Polypolyamine, l-dopa side chain | 3               |
| 52–56                | 56                    | –OCH2 Ar–OCH2, –OOCCH (CH2) NH3 | Chitin, suberin, l-dopa side chain | 9, 19, 39, 43, 61, 62 |
| 60–65                | 62                    | –CH2O–             | Cellulose, chitin     | 39, 43, 63, 64   |
| 72–75                | 72, 75                | –CHOH, carbohydrate C2,C3,C5 | Cellulose, chitin | 39, 43, 63, 64   |
| 80–85                | 85                    | –CHOH, carbohydrate C4–CHOAr- | Chitin, cellulose | 9, 39, 43, 63, 64 |
| 101–105              | 105                   | Carbohydrate C1, aromatic –CH–, | Cellulose, chitin | 39, 43, 53, 63, 64 |
| 110–118              | 115, 117, 114         | Aliphatic and aromatic –CH- | Polypolyamine, pyrrole diester | 3, 9, 53         |
| 125–130              | 130                   | –CH = CH-, indole or alkene | Polypolyamine, dimethoxyindole, egg PC | 3, 9, 40, 53     |
| 144–156              | 142, 145, 148         | Aromatic –CH = CHCO–, aromatic –C– | Polypolyamine, poly-l-dopa | 3, 24, 53         |
| 157–165              | 174, 172, 170         | Aromatic –C–, O-aryl | Polypolyamine         | 3, 53             |
| 168–175              | 174, 172, 170         | –COO–, –CONH        | Egg PC, chitin, polypolyamine, poly-l-dopa | 3, 24, 39, 40, 43 |

a Observed values for broad resonances in several spectra are consistent within 1–2 ppm. Assignments for l-dopa melanins reflect reported cell wall composition, one-dimensional chemical shifts for related compounds, and two-dimensional 13C-13C connectivities in isotopically enriched compounds.

b Examples are as follows: egg yolk phosphatidylcholine (egg PC), a model biological membrane containing 1-palmitoyl-2-oleoyl-3-phosphoglycerol-3-phosphocholine as the principal phospholipid constituent (40); chitin from tomato hornworm cuticle, the corresponding extracted chitin, and N-acetylglucosamine (2-acetamido-2-deoxy-D-glucopyranose) carbohydrates (39, 43); synthetic polydopamine and poly-l-dopa with cyclized indole and uncyclized catecholamine units (3, 24); suberin polyester in potato and cork periderm tissues (61, 62); sepia melanin model compounds such as ethyl 5,6-dimethoxyindole-2-carboxylate and 2-methoxycarbonyl-3-ethoxycarbonyl-4-methylpyrrole (9); solid commercial cellulose (63, 64).

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with β-glucan and alkene moieties ((171 × 101), (171 × 82), and (171 × 126) ppm).

FIGURE 3. Solid-state NMR results, obtained at a 1H frequency of 750 MHz and 15 kHz MAS, for C. neoformans melanins produced with natural-abundance L-dopa and d-[U-13C6]glucose in the 24067 fungal cell line. Left to right, 2D contour plots from DARR experiments (35, 36) conducted with 50-, 250-, and 500-ms mixing times, showing 13C-13C cross-peaks corresponding to lengthening pairwise through-space proximities between glycosidic ring carbons, within acylglyceride structures, and between these cellular constituents. Far right, 2D contour plot from SAR-COSY experiment (38), showing through-bond cross-peaks that match many features of the 50-ms DARR spectrum. All experiments were conducted at ambient temperature with 15 kHz MAS; ridges parallel to the diagonal in the DARR plots arise from spinning sidebands. The purple color of the traces is assigned according to the d-[U-13C6]glucose isotopically enriched precursor.

Taken together, the current analyses of intact solid samples using higher magnetic field strengths and 2D spectroscopy yield a multicarbon structural network of chemically resistant aliphatic moieties resulting from glucose metabolism. Our through-space connectivity evidence for both glycoside and acyl chain structures in l-dopa C. neoformans melanin ghosts augments prior through-bond structural information deduced from 1D 13C CPMAS and solvent-mediated 1H high resolution MAS experiments (19). Moreover, unlike prior efforts to identify bonded 1H-13C chemical fragments within single molecular constituents that are capable of swelling, 2D NMR experiments on solid melanin ghost samples can directly reveal interactions of spatially close pairs of different polysaccharide- and/or acylglyceride-based structural moieties. Figs. 1–3 show the formation of chemically resistant fungal cell-wall molecular architectures after 4 days of incubation with catecholamine precursors, composed of multiple proximal aliphatic molecular precursors and thus providing a scaffolding support upon which melanin pigments can be deposited as development proceeds. As illustrated under “Spatial Proximity and Covalent Bonding of C. neoformans Melanin Molecular Structures Derived from Glucose and L-Dopa Benzenoid Moieties” and “Spatial Proximity and Covalent Bonding of C. neoformans Melanin Pigment Deposition” (27).

Spatial Proximity and Covalent Bonding of C. neoformans Melanin Molecular Structures Derived from Glucose and L-Dopa Benzenoid Moieties—To investigate the macromolecular architecture of the fungal melanin assembly, we began by comparing DARR experiments that displayed through-space 13C-13C connectivities for melanized ghosts with three patterns of isotope enrichment as follows: (a) d-[U-13C6]glucose and l-[12C]dopa (described under “Aliphatic Molecular Scaffolds for C. neoformans Melanin Pigment Deposition”); (b) d-[13C]-glucose and l-[ring-13C6]dopa; and (c) dual-labeled d-[U-13C6]glucose and l-[ring-13C6]dopa. As shown in the spectra and contour diagrams of Figs. 3 and 4, the purple spectra of sample a are dominated by 13C-enriched aliphatic and carboxyl moieties, whereas the blue spectra of sample b are dominated by 13C-enriched indole-based aromatic resonances, and the red spectra of sample c display contributions from both isotopically enriched constituents. Provisional chemical shift assignments are again summarized in Table 1.

By accounting for the aliphatic cross-peak proximities identified in the DARR spectra of aliphatic-enriched sample a (Fig. 3), as well as the DARR cross-peaks shown in Fig. 4 for the l-[ring-13C6]dopa melanin sample b, we can identify the functional groups in the dual-labeled sample c that exhibit proximal intercomponent spatial arrangements. Comparable cross-peak features and spectral resolution are observed at 600- and 750-
MHz operating frequencies, supporting the overlap of resonances from structurally similar carbons as a prime contributor to the NMR linewidths.

Single-component cross-peaks are attributed to $^{13}$C-$^{13}$C proximities within or between indole rings (no glucose enrichment, blue) and to aliphatic constituents derived from D-[U-$^{13}$C$_6$]glucose (red traces) showing $^{13}$C-$^{13}$C cross-peaks corresponding to lengthening pairwise through-space proximities. The cross-peaks within particular indole and aliphatic moieties but no correlations between these two structural classes. The colors of the traces are assigned according to the isotopic enrichment schemes of the precursors as follows: L-[ring-$^{13}$C$_6$]dopa, blue; D-[U-$^{13}$C$_6$]glucose, red.

The corresponding SAR-COSY plot displays no cross-peaks with chemical shifts corresponding to the inter-component through-space $^{13}$C-$^{13}$C interactions, we can rule out direct covalent bonding between the L-[ring-$^{13}$C$_6$]dopa- and D-[U-$^{13}$C$_6$]glucose-derived $^{13}$C-enriched sites in this C. neoformans melanin sample. Thus, these three sets of 2D NMR data support an architecture in which the benzenoid aromatic ring of the melanin pigment can be associated hydrophobically with the acylglyceride chains of the aliphatic scaffold.
Spatial Proximity and Covalent Bonding of C. neoformans Melanin Moieties Derived from Glucose and L-Dopa Side Chain—The strategy used to deduce relative spatial relationships of benzenoid and aliphatic moieties in the fungal melanin ghosts was adapted to evaluate the proximity and covalent bonding patterns involving macromolecular structures originating from d-glucose and primarily pyrrole (8, 20, 24) pigment moieties that were formed from the l-dopa side chain. Thus through-space and through-bond 13C-13C connectivities were evaluated for C. neoformans melanins derived from the following isotopically enriched feedstocks: (a) d-[U-13C6]glucose and l-[2,3,13C3]dopa (described under “Aliphatic Molecular Scaffolds for C. neoformans Melanin Pigment Deposition”); (b) d-[13C15]glucose and l-[2,3,13C3]dopa reported previously (8) and re-examined herein using current experimental methodologies; and (c) dual-labeled d-[U-13C6]glucose and l-[2,3,13C3]dopa. As noted in conjunction with the experiments using ring-labeled l-dopa (see under “Spatial Proximity and Covalent Bonding of C. neoformans Melanin Molecular Structures Derived from Glucose and l-Dopa Benzenoid Moieties”), Fig. 3 shows that the purple 2D contours of sample a are dominated by 13C-enriched (oxy)alkane, alkene, carboxylate, and amide moieties. By comparison, Figs. 5 and 6 show that our 2D 13C-13C DARR for l-[2,3,13C3]dopa C. neoformans melanin with either d-[13C15]glucose (sample b, blue contours) or d-[U-13C6]glucose (sample c, red contours) display a variety of cross-peaks from isotopically enriched aromatic and aliphatic groups. Table 1 again provides a guide to the chemical shift assignments that underlie our structural interpretations.

The high field DARR experiments on C. neoformans melanins from d-[13C15]glucose and l-[2,3,13C3]dopa showed many through-space proximities within the aromatic pigment core as well as cross-peaks between resonances assigned to ester, pyrrole-in-indole, oxymethylene, and alkyl chain pairs of moieties (170 × 125 and 55 × 30 ppm, Fig. 5) (9). In addition to supporting prior low-field proton-driven 13C-13C spin diffusion experiments showing connections within the aromatic core (8), the 55 × 30 ppm pairwise spatial proximity revealed in these high sensitivity 750 MHz 2D spectra offered particular support for proposed unicycled or cleaved ring aliphatic molecular fragments associated with catecholamine-derived melanin pigments (3, 8, 20, 24).

As noted above, the d-[U-13C6]glucose-enriched sample a displayed similarly located DARR cross-peaks (171 × 126 ppm, Fig. 3), indicating ~6 Å spatial proximity between chitin amide and alkene groups. By comparison, DARR results for the dual-labeled sample c included spatial interactions between two pairs of carbons in these respective spectral regions, 171 × 127 and 169 × 117 ppm (Fig. 6). Whereas the first pair of cross-peaks coincided with features observed for the singly labeled C. neoformans melanins, the second pair was observed only for the sample with 13C-enriched glucose and l-dopa feedstocks. A provisional structural identification could be made using prior knowledge of the pigment and fungal cell-wall constituents (45), NMR of model compounds, and empirically based spectral predictions of various aromatic fragments (3, 9, 53). Thus, we attributed the 169 × 117 ppm feature to a through-space proximity between a glyceride carboxylate and/or a chitin amide and a free pyrrole, i.e. to distinct molecular constituents of the C. neoformans melanin assembly.

Our 2D through-bond SAR-COSY results (Figs. 5 and 6) provided a means to confirm the two-component assignment of the 169 × 117 ppm DARR cross-peak and to test whether the connections between chemical constituents of the dual-labeled C. neoformans melanin sample correspond to covalently bonded pairs. Significant through-bond SAR-COSY connections in l-[2,3,13C3]dopa C. neoformans melanin were observed only between rigid aromatic carbons (128 × 111 ppm, Fig. 5), whereas d-[U-13C6]glucose-enriched C. neoformans melanin produced no SAR-COSY signals at all in this spectral region (Fig. 3). Therefore, the bonded interactions involving amide/carboxylate carbons at ~168 ppm with aromatic and alkene groups resonating between 110 and 130 ppm (Fig. 6, green ovals) in the dual-labeled sample were taken to originate from different macromolecular components.

In terms of molecular architecture, the SAR-COSY results revealed covalent bonds between different pyrrole ring carbons (130 × 115 ppm) and between amide/carboxylate groups and free pyroles ~168 × 120 ppm). Thus, whereas the pyrrole SAR-COSY and DARR data of Fig. 6 bolstered the aromatic ring stacking proposal introduced under “Spatial Proximity and Covalent Bonding of C. neoformans Melanin Molecular Structures Derived from Glucose and l-Dopa Benzenoid Moieties,” the 2D NMR results also suggested carbon-containing covalent linkages between N-acetylglucosamines of chitin or membrane
glycerides and the pigment pyrroles (Fig. 7). Among the possible interacting fungal cell-wall constituents that are reported to impact melanization are chitin and chitosan (44), which display amide resonances with the requisite \(^{13}\text{C}\) chemical shift values (39) and are thus strong candidates for bonded partners with the pigment pyrroles.

**Discussion**

This study reveals several key design attributes of the *C. neoformans* melanin assembly and allows us to test previously proposed structural models (1, 3, 48–52) in related macromolecular systems. First, our \(^{13}\text{C}\) NMR spectra establish a sequence involving early (4 days) deposition of an aliphatic framework followed by later (14 days) appearance of an aromatic component (Fig. 1), suggesting that the cellular materials serve as a scaffolding for the developing pigment. As the pigment granules are deposited on this structural support, the aliphatic framework becomes chemically functionalized, permitting it to survive degradative procedures used to isolate the melanin ghosts and remain available for physical scrutiny. The increasing aromatic pigment buildup that is apparent in the spectroscopic data provides a molecular rationale for the augmented negative charge and cell hydrophobicity associated with *C. neoformans* and *Aspergillus fumigatus* melanization (28, 54). Moreover, the NMR-monitored growth in the proportion of aromatic pigment constituents with respect to the aliphatic scaffold in L-dopa and dopamine *C. neoformans* melanin preparations offers a structural explanation for previously reported increases in wall thickness and decreases in porosity in *C. neoformans* ghosts after 4, 7, and 10 days of growth, respectively (55).

Importantly, this study sheds new light on both the fungal scaffolding architecture and the pigment organization. Our evidence for proximal and bonded \(^{13}\text{C}\)-\(^{13}\text{C}\) pairs supports an acid-resistant aliphatic scaffold involving interacting glucan, chitin, and acylglyceride architectural elements (Fig. 7). Previous scanning, transmission, and atomic force microscopy studies of melanized *C. neoformans* ghosts have been accommodated by a model in which pigment granules 50–80 nm in diameter are assembled into ~200-nm thick concentric layers that form a barrier against animal hosts and environmental challenges (28, 55). Whereas the formation of small granules rather than a contiguous melanin layer would demand a scaffold to anchor the particles, the layered pigment arrangement also underscores the prospect of both radial and axial particle growth during *C. neoformans* melanization. Here, we addressed the question of whether the granules are held together by e.g. cross-links or nonpigment scaffolding using 2D solid-state NMR experiments on isotopically enriched fungal eumelanins.

The isotopically labeled benzenoid aromatic rings of the pigment produced from L-\(\text{[ring-}^{13}\text{C}_6\text{]}\)dopa and [U-\(^{13}\text{C}_6\)]glucose exhibit a network of short range spatial proximities (up to ~6 Å) (46), including several spin connectivities to the aliphatic framework. This *C. neoformans* melanin exhibits several overlapping aromatic-aromatic interactions observed among the longer distance cross-peaks in the 500-ms DARR spectrum.
The benzenoid-mediated interactions revealed by 2D NMR also support the energetically favored π-stacking of aromatic structures predicted computationally (51, 52) and account for magnetically distinct but overlapping aromatic resonances in the solid-state $^{13}$C (8, 9, 19, 20) and 2D $^{13}$C-$^{15}$N (24) NMR spectra. Such aromatic interactions can produce the layered architecture observed by transmission electron microscopy (28, 55), also providing a rationale for the observation of negative particle charge and hydrophobic character associated with melanization (28, 54). The increasing dispersion of indole-based NMR spectral features observed at day 14 also suggests a range of similar aromatic environments that could reflect imperfect alignment of successive layers of stacked aromatic ring structures (Fig. 7). Such heterogeneous organization could result in a material that is locally ordered but globally amorphous in structure and thus unsuitable for crystallographic analysis. Furthermore, progressive melanization could account in turn for the observed constriction of pores in melanin ghosts, which has been proposed to prevent infusion of echinocandin antifungal drugs while still permitting nutrient passage into the fungal cells (4). Our solid-state NMR data thus show that the indole-based aromatic rings are interconnected predominantly as postulated previously (48), and additionally that their benzenoid aromatic structures can be proposed as associating “hydrophobically” rather than being linked covalently to the long-chain acylglycerides of the underlying aliphatic scaffolding.

By contrast, the labeled pyrrole aromatic structures (often within indole units in C. neoformans melanins) that are derived from 1-[2,3-$^{13}$C$_2$]dopa exhibit both spatial and bonded connections to aliphatics originating from [U-$^{13}$C$_6$]glucose. Different pyrrole ring sites (130 × 115 ppm) are linked to each other and also to carbohydrate- or amide-containing structures from other cellular constituents. The former linkage is in accord with a close $^{13}$C-$^{15}$N spatial relationship reported recently in cell-free L-dopa melanin (24). The latter molecular pair ~169 ppm is attributable to cellular constituents such as chitin or acylglycerides and to free pyrrole pigment units, respectively (Fig. 7). As described for the 1-[ring-$^{13}$C$_6$]dopa melanins, the pyrrole-in-indole aromatic ring connections in solid-state NMR of 1-[2,3-$^{13}$C$_2$]dopa C. neoformans melanin support the postulated stacking of indole-based aromatic units and covalent cross-linking between such aromatic moieties (48). Moreover, the intercomponent pyrrole attachment to amides in N-acetylglycosamines of chitin or carboxylates in membrane glycerides suggests covalent modification of the cell membrane, chitin, or chitosan components (44) to form a progressively more complex macromolecular assembly during C. neoformans melanization (Figs. 1 and 5). Thus, whereas the pyrrole SARCOSY and DARR data of Figs. 5 and 6 bolstered the aromatic ring stacking proposal introduced under “Spatial Proximity and Covalent Bonding of C. neoformans Melanin Molecular Structures Derived From Glucose and L-Dopa Benzenoid Mieties,” the 2D NMR data also suggested carbon-containing covalent linkages between the pigment pyroles and either chitin-derived N-acetylglycosamines or membrane glycerides.

Chitin, for instance, is a major hydrophilic cell-wall constituent that forms hydrogen-bonded microfibrils to confer mechanical strength and is reported to interact strongly with melanin in A. nidulans (45). In Candida albicans, chitin is essential for externalization of melanin (56); in C. neoformans, both chitin synthase (57) and chitosan in the cell wall (44) are reported to modulate melanization. Chitin and melanin have also been reported to interact closely in marine invertebrates (58) and insect wings (59). Hence, the covalent connection between chitin and melanin suggested by this spectroscopic study is supported by, and consistent with, independent observations in animals and fungi showing these components to be closely linked. The versatility of the C. neoformans melanin pigment assembly that allows for both stacking and scaffold binding reflects its dual hydrophobic-hydrophilic character; our conceptual model posits that the hydrophobic aromatics assemble in multiple layers, and the hydrophilic moieties “reach
out” to bind covalently with cellular glycerides or polysaccharides such as chitin or chitosan. Given the commonality of aromatic core structures demonstrated recently for synthetic and \textit{C. neofor mans} melanins (24), these defining arrangements also help to establish the architectural capabilities of designed melanin-based soft materials.

In conclusion, the current solid-state NMR findings offer the first biophysical view of the mechanism by which melanin is assembled in the fungal cell wall, providing a structural rationale for the major pigment-associated changes in melanized \textit{C. neofor mans} cell-wall architecture probed previously using diverse physical characterization methods (4). The 1D $^{13}$C and 2D $^{13}$C-$^{13}$C solid-state NMR results support the following picture: an early developing aliphatic scaffold consisting of several proximal polysaccharides and acylglycerides; a late-developing aromatic pigment, including indole and pyrrole structural moieties; and architectural networks that include close indole-in-dole associations and pyrrole-chitin covalent bonds. The progressive development of multicomponent melanized assemblies, wherein aromatic rings are nevertheless predominantly proximal to each other, supports proposed interlayer stacking (48–52) or lateral interactions and argues for a striking degree of regulation accompanying this particular free radical polymerization process. Although this work focuses on the formation and assembly of intact melanin pigments in fungal cells, our insights into the structural requisites of melanin-cell wall assemblies should have broader practical potential as follows: for enhancing the efficacy of melanoma treatments; bioremediation of radioactively contaminated soils or reactors, and design of bioinspired materials for protective coatings and therapeutic drug delivery (2–4).

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