ABSTRACT The fungus *Cryptococcus neoformans* possesses a polysaccharide capsule and can form biofilms on medical devices. The increasing use of ventriculoperitoneal shunts to manage intracranial hypertension associated with cryptococcal meningoencephalitis highlights the importance of investigating the biofilm-forming properties of this organism. Like other microbe-forming biofilms, *C. neoformans* biofilms are resistant to antimicrobial agents and host defense mechanisms, causing significant morbidity and mortality. This chapter discusses the recent advances in the understanding of cryptococcal biofilms, including the role of its polysaccharide capsule in adherence, gene expression, and quorum sensing in biofilm formation. We describe novel strategies for the prevention or eradication of cryptococcal colonization of medical prosthetic devices. Finally, we provide fresh thoughts on the diverse but interesting directions of research in this field that may result in new insights into *C. neoformans* biology.

INTRODUCTION Historically, microbiologists have studied microbes that cause infectious diseases by analyzing microbial cells grown in suspension (planktonic) in the laboratory. This tradition derives in great part from the early influences of Koch’s postulates, which emphasized working with pure cultures. Unfortunately, this growth in pure cultures has little to do with the growth of microbes in “natural” or host environments. Advances in confocal microscopy and molecular genetics in the last two decades have provided evidence that biofilm formation represents the most common mode of growth of microorganisms in nature. This growth form presumably allows microbial cells to survive in hostile environments, enhances their resistance to physical and chemical pressures, and promotes metabolic cooperation (1). In fact, it is estimated that approximately 80% of all bacteria in the environment exist in biofilm communities, and more than 65% of human microbial infections involve biofilm formation (2). Microbial biofilms are dynamic communities of microorganisms strongly attached to biological and nonbiological substrata that are enclosed in a self-produced protective exopolymeric matrix (EPM) (3).

Researchers’ interest in studying the role of microbial biofilms in human disease stems from the observation that microbes within biofilms display unique phenotypic characteristics that increase resistance to host immune mechanisms and antimicrobial therapy (4–7). For instance, the successful eradication of biofilms in vivo usually requires concentrations of antimicrobial drugs that are usually toxic to the host (8). Similarly, microbial biofilms are more resistant to antimicrobial molecules produced by the host immune system compared to their planktonic counterparts (5). Although bacterial biofilms have been extensively studied since the mid-1980s, little attention was paid to medically relevant fungal biofilms until the past decade.
Cryptococcus neoformans is an encapsulated, environmental fungus that frequently causes life-threatening meningoencephalitis in immunocompromised patients and also occasionally causes disease in apparently normal individuals. C. neoformans capsular polysaccharide is mainly composed of glucuronoxylomannan (GXM), and the capsule is a critical virulence phenotype since acapsular strains are not pathogenic (9). Copious amounts of GXM are released during cryptococcal infection, causing deleterious effects for the host immune response (9). In addition, C. neoformans forms biofilms on polystyrene plates and medical devices including ventriculostrial shunt catheters (10, 11). Similarly, there are several reports of C. neoformans infection of polytetrafluoroethylene peritoneal dialysis fistula (12) and prosthetic cardiac valves (13) that highlight the ability of this organism to adhere to medical devices. The increasing use of ventriculoperitoneal shunts to manage intracranial hypertension associated with cryptococcal meningoencephalitis suggests the importance of investigating the biofilm-forming properties of this organism (11). This chapter describes the current knowledge of the biology of C. neoformans biofilms, the role of the polysaccharide capsule in biofilm formation, development of therapeutic strategies in preventing and treating cryptococcal biofilms, and recent advances in the field.

C. neoformans BIOFILM FORMATION

C. neoformans biofilm-related infections were reported clinically in the mid-1980s (10), but until we visited this problem approximately a decade ago there was no information on the dynamics of this process (14). The characteristics of C. neoformans biofilm development were described using a microtiter plate model, microscopic examinations, and a colorimetric XTT reduction assay to observe the metabolic activity of cryptococci within a biofilm. Biofilm formation by this fungus exhibited coordinated phases such as surface attachment, microcolony formation, EPM production, and maturation (8).

During the adhesion period (2 to 4 h) or early stages of biofilm formation, the cryptococcal cells were metabolically active and became firmly attached to the plastic surface of the microtiter plate in a monolayer arrangement. However, adhesion of fungi to a surface can also be facilitated by formation of an organic conditioning layer, which may include compounds released by the host inflammatory response in serum, saliva, or vaginal excretions (15). For instance, cerebrospinal fluid surrounding a ventriculoperitoneal shunt contains high concentrations of cations that may promote interactions of the microbe with the support surface. Furthermore, constant motion of cerebrospinal fluid across the solid surface influences the adhesion of microorganisms to biomaterials. These variables may affect the rate and the extent of fungal attachment (16). The cryptococcal cells adherent to the plastic support consisted of growing cells as indicated by the presence of many budding cells. At the intermediate stage (4 to 16 h), the fungal population had increased significantly and consisted of yeast cells spread uniformly throughout the plastic support, forming microcolonies. The proximity of cells within the microcolony could present a formidable environment for the establishment of nutrient gradients, genetic exchange, and quorum sensing. During the maturation stage (24 to 48 h), the metabolic activity of the cryptococcal cells on the biofilms remained high and steady. Scanning electron and confocal microscopy examinations have demonstrated that the microarchitecture of mature C. neoformans biofilms became more complex due to an increasing amount of extracellular material surrounding the cells and producing compact structures that tenaciously adhered to the plastic support (14, 16, 17) (Fig. 1). The structural organization of biofilms and the presence of flowing water channels (18) may allow nutrient and gas exchange while providing the fungal cells with a sheltered niche for protection against environmental predators, immune cells, shear forces, and antimicrobial drugs.

Because C. neoformans is an environmental fungus found ubiquitously in association with pigeon excreta in urban environments, and only an accidental pathogen, it is not surprising that biofilm formation constitutes an important survival strategy in hostile environmental conditions (e.g., ultraviolet light) and against predation (16, 19). Conditions that mimic the external environment have been suggested to be permissive for biofilm formation (16). Perhaps biofilm formation is a survival strategy used by C. neoformans that emerged and developed through environmental interactions due to the constant selection by predation (20). In this scenario, cryptococcal survival strategies are the result of environmental selection, suggesting that the fungus undergoes accidental adaptation to the host. Additional evidence for this theory comes from the finding that during nonlytic exocytosis from macrophages (21), fungal cells that were internalized by antibody (Ab)-opsonization emerge in biofilm-like microcolonies (22).

The addition of conditioned medium also stimulated the cryptococcal polysaccharide capsule and melanin production, mechanisms needed by this eukaryotic
pathogen to thrive in the environment and cause disease in the host. These investigators provided evidence that *C. neoformans* cells can act in concert when expressing the necessary genes to survive in the host and cross-talk to other fungal species in the environment. Likewise, Cfl1, an adhesion protein, was identified to be responsible for the paracrine communication in *C. neoformans* (24). Consistent with its role in communication, Cfl1 is highly induced during mating colony differentiation, and some of the Cfl1 proteins undergo shedding and are released from the cell wall. However, its role in cryptococcal biofilm formation and pathogenesis remain to be elucidated.

**POLYSACCHARIDE CAPSULE IN BIOFILM FORMATION**

The composition of the microbial cell surface, which may exhibit fimbriae (25), flagella (26), or a capsule (14), greatly influences the rate and extent of attachment. The role of *C. neoformans* polysaccharide capsule in biofilm formation was elucidated (14). Studies with the acapsular *cap59* mutant C536 revealed no biofilm formation relative to the parental strain 3501 or the encapsulated complemented mutant 538 (14). This indicated that capsular polysaccharide was necessary for biofilm formation and implied a critical role for capsular polysaccharide in this process. Since the *C. neoformans* capsule is composed primarily of GXM (16), which is a constituent of the cryptococcal biofilm EPM, it is reasonable to conclude that the inability of the acapsular mutant strain C536 to form a biofilm reflects a failure to shed polysaccharide and form a matrix. Furthermore, the addition of exogenous polysaccharide to acapsular cell cultures was not sufficient to compensate for a lack of capsular production in biofilm formation (14).

The importance of *C. neoformans* capsular polysaccharide release in biofilm formation was also investigated (14). Using enzyme-linked immunosorbent assay (ELISA) spot assays, it was shown that *C. neoformans* biofilm is established via the local release of capsular polysaccharide by attached cryptococcal cells. In addition, the binding of shed polysaccharide to the solid surface created an EPM. However, it was also established that biofilm formation was dependant on environmental conditions such as surface support differences, conditioning films on the surface, characteristics of the medium, and properties of the microbial cell (16).
**C. neoformans** copiously releases capsular polysaccharide in the supernatant of liquid cultures (27) and in tissues (28). Previous studies have shown that monoclonal antibodies (mAbs) to **C. neoformans** GXM significantly reduced serum GXM levels in vitro (29) and in vivo (30). GXM-specific Ab inhibits polysaccharide release from encapsulated cells by cross-linking the carbohydrate molecules in the capsule (29). Since **C. neoformans** can form biofilms on medical devices that presumably contain polysaccharide components, this finding raised the intriguing possibility that Ab to GXM would also interfere with cryptococcal biofilm formation. Addition of GXM-binding mAbs to **C. neoformans** cultures in microtiter plates did not affect fungal cell adhesion but prevented biofilm formation by interference with release of GXM (14) (Fig. 2). This function had not been previously described for specific Abs to polysaccharides, and it suggests a new role for humoral immunity in defense against biofilm-forming microbes. Moreover, Ab-mediated inhibition of biofilm formation was associated with protective Abs, suggesting the tantalizing possibility that this effect is involved in Ab protective efficacy against **C. neoformans** (14). Subsequent studies established that Ab binding to the capsule triggered changes in gene expression (31), suggesting the possibility that the inhibition of biofilm formation was also a reflection of Ab-mediated alterations in fungal physiology.

Preparations of cryptococcal biofilm matrix material were analyzed for carbohydrate composition by combined gas chromatography/mass spectrometry (16). The glycosyl composition of EPM isolated from biofilms was consistent with the presence of GXM. However, significant quantities of sugars not found in GXM, such as glucose, ribose, and fucose, were also detected, implying that the EPM of **C. neoformans** biofilms includes polysaccharides other than GXM. Based on the observation that GXM may be the main component of the extracellular polysaccharide comprising the matrix enclosing cryptococci within a mature biofilm is shed GXM and can be stained by specific mAbs. Therefore, the use of specific mAbs as a simple and effective method to study microbial biofilm by microscopy was introduced.

**RESISTANCE TO HOST IMMUNE MECHANISMS AND ANTI FUNGAL THERAPY**

Biofilm formation is associated with persistent infection because biofilms increase resistance to host immune mechanisms and antimicrobial therapy. Given that microbial cells within biofilms are highly resistant to standard concentrations of antimicrobial agents, the antifungal activity of commonly used and newly developed drugs against **C. neoformans** biofilms has been evaluated. Biofilms were more resistant than planktonic cells to amphotericin B and caspofungin and completely resistant to the two azole compounds, fluconazole and voriconazole (5). These results correlate with other reports suggesting that biofilm phenotype confers resistance against antifungal drug therapy (32, 33). Amphotericin B and caspofungin mediated a significant reduction of the metabolic activity of **C. neoformans** cells consistent with their fungicidal properties, but the concentrations used were high and above achievable levels in vivo after systemic administration and their susceptibility to these drugs was further reduced if cryptococcal cells contained melanin. Moreover, an antifungal surfactant-like lipopeptide, kannurin, produced by **Bacillus cereus** showed moderate effects against cryptococcal biofilms (34).

Although biofilms are known to be less susceptible to antimicrobial drugs, little is known about their susceptibility to antimicrobial molecules produced by the innate immune system. Lactoferrin, a component of the innate immune system, was unable to prevent fungal biofilm formation (14), despite its reported efficacy against bacterial biofilms (35). **C. neoformans** cells within biofilms were more resistant than planktonic cells to oxidative stress but remained vulnerable to cationic antimicrobial peptides (4). Nevertheless, melanin production protected fungal biofilms against antimicrobial peptides (5).

Numerous studies have established that specific Abs can enhance the efficacy of antifungal therapy in animal models of fungal infection (36, 37) and in vitro (38). However, none of these models involved the formation of fungal biofilms. Since biofilm formation results in the
formation of a physical barrier against host immune mechanisms and antimicrobial therapy, the efficacy of combining GXM-binding mAbs and antifungal drugs against *C. neoformans* biofilms was investigated (39). The presence of GXM-specific IgG1 protected cryptococcal biofilms from amphotericin B or caspofungin, presumably by creating a protein layer upon binding to extracellular polysaccharides. This effect was not observed with an irrelevant or nonspecific IgG1. Confocal microscopy revealed that GXM-specific IgG1 binds through all the EPM surrounding metabolically active yeast cells within *C. neoformans* biofilms. Given that amphotericin B and caspofungin are relatively large molecules of 924 and 1,213 Da, respectively, the antagonism observed may be a result of Ab-mediated interference with drug penetration. These findings suggest
the possibility of antagonistic effects when combining Ab and drug therapy for those clinical situations where the presence of established biofilms can be expected, such as with infected prosthetic devices. Hence, Abs may be useful in preventing biofilm formation, but once the biofilms are formed, the binding of additional protein to the matrix could produce antagonistic effects with antimicrobial drugs. Furthermore, these results raised the possibility that products of the immune response contribute to drug resistance for biofilms formed in vivo. One can anticipate that microbial biofilms formed on prosthetic devices in tissues contain Abs and other molecules such as complement that may contribute to acquired drug resistance in vivo.

Ab-mediated agglutination and biofilm formation can also mediate resistance to and escape from phagocytosis inside macrophages (22). Both Cryptococcus gattii and C. neoformans cells exited macrophages in biofilm-like microcolonies where the yeast cells were aggregated in a polysaccharide matrix that contained bound Ab. In contrast, complement-opsonized C. neoformans was released from macrophages dispersed as individual cells. Hence, both Ab- and complement-mediated phagocytosis resulted in intracellular replication, but the mode of opsonization affected the outcome of exocytosis. The biofilm-like microcolony exit strategy of cryptococcal species following Ab opsonization reduced fungal cell dispersion. This finding suggests that Ab effects inside phagocytic cells might mediate physiological effects on fungal cells.

These are examples of biofilm phenotype increasing resistance against host immune mechanisms, a phenomenon that could contribute to the ability of biofilm-forming microbes to establish persistent infections. Various mechanisms of biofilm resistance to antimicrobial agents and molecules have been proposed, including the presence of physical barriers that prevent the penetration of the antimicrobial compounds into the biofilm (40), slow growth or regulation of the metabolic activity of the biofilm due to nutrient limitation (41), phenotypic switching (42), activation of the general stress response (43), changes in temperature (44), and the existence of a subpopulation of cells within the biofilm (known as persisters) that are preserved by antimicrobial pressure (45).

**THERAPEUTIC APPROACHES**

Currently, strategies to prevent microbial colonization of catheters have included impregnation of the catheter material with antimicrobial drugs, altering the chemical composition of the polymer, use of nanotechnology, and changing the physical surface properties. Unfortunately, these approaches have not been very effective in reduc-
ing the problem of biofilm formation by microbes. In fact, some antibiotics may contribute to the problem (46). For instance, in certain organisms, aminoglycoside antibiotics can induce bacterial biofilm formation.

Based on the urgent need to develop novel therapeutic strategies to combat microbial biofilms in patients, it was demonstrated using confocal microscopy that a capsular polysaccharide-binding IgG1 penetrated the EPM of a biofilm and bound to metabolically active C. neoformans cells, which were susceptible to alpha-radiation (47)(Fig. 4). Unlabeled IgG1, alpha-radiation-labeled nonspecific IgG1, and gamma and beta types of radiation did not have any effect on biofilms. The lack of efficacy of gamma and beta radiation probably reflects the radioprotective properties of EPM. In contrast, when GXM-specific IgM labeled with alpha-radiation was used, there was no penetration of the fungal biofilm and no damage, most likely due to the large size of the pentameric molecule. These results suggested a novel option for the prevention or treatment of microbial biofilms on indwelling medical devices. Since removing certain types of indwelling devices is difficult, one can imagine situations where it may be possible to treat infected devices in situ with radioimmunotherapy by local administration of radiolabeled mAbs in close proximity to the infected device. Alternatively, since mAbs may have a role in preventing biofilm formation, a prophylactic dose of unlabeled and radiolabeled Ab may be administered immediately after insertion of the device. In this regard, successful clinical experience has

**FIGURE 4** Schematic of radioimmunotherapy of a biofilm with an antibody labeled with alpha-emitting radionuclide. The “direct hit” effect is the killing of a cell by radiation emanating from a radiolabeled antibody molecule bound to this cell. “Cross-fire” is the killing of a cell by radiation emanating from a radiolabeled antibody bound to an adjacent or a distant cell. “Bystander” denotes the death of an unirradiated cell through the signaling from irradiated cells. doi:10.1128/microbiolspec.MB-0006-2014.f4
been accumulated in oncology in locoregional administration of radiolabeled mAbs. Novel therapeutic strategies against biofilm-related microbial infections may also be designed by combining radioimmunotherapy and conventional antimicrobial therapy.

Chitosan, a polymer isolated from crustacean exoskeletons, may offer a flexible, biocompatible platform for designing coatings to protect surfaces from infection. As recently demonstrated, chitosan in biofilms significantly reduced the metabolic activity and the cell viability of *C. neoformans* (17). Notably, melanization, an important virulence determinant of *C. neoformans*, did not protect cryptococcal biofilms against chitosan. This phenomenon was attributed to the ability of cationic chitosan to disrupt negatively charged cell membranes as microbes settle on the surface (48). Chitosan has a profound effect on the negative charge of the fungal cellular membrane and thus may interfere with surface colonization or adhesion and cell-cell interactions during biofilm formation (49). Charging the fungal surfaces may keep yeast cells in suspension, preventing biofilm formation (50), or may increase phagocytosis and killing of fungal cells by macrophages. Binding of chitosan to DNA and inhibition of messenger RNA synthesis occurs through enhanced chitosan penetration (51). Thus, it is likely that the interaction between positively charged chitosan molecules and negatively charged microbial cell membranes leads to the leakage of proteinaceous and other intracellular constituents, causing cell death (52). The chitosan concentrations used in these studies to evaluate the effect on biofilm formation were not toxic to human endothelial cells, suggesting an option for preventing or treating fungal biofilms on indwelling medical devices.

To improve the efficacy of antifungal drugs against fungal biofilms and biofilm-associated infections, treatment of early biofilm stages, novel formulations of antifungal drugs, and antifungal drug combinations have been recently developed and successfully applied. One of these examples is that the inhibitory effect of the antifolate combinations sulfamethoxazole–trimethoprim (SMX/TMP) and sulfadiazine–pyrimethamine (SDZ/ PYR) against planktonic cells and biofilms of multiple environmental or clinical *C. neoformans* or *C. gattii* strains was recently evaluated (53). SMX/TMP and SDZ/PYR showed antifungal activity against free living cells and sessile cells of *Cryptococcus* spp. The drug combinations SMX/TMP and SDZ/PYR were able to prevent biofilm formation and showed an inhibitory effect against mature biofilms of both species. Additionally, the study showed that antifolate drugs reduced the ergosterol content in *C. neoformans* and *C. gattii* planktonic cells, highlighting the antifungal potential of these drug combinations.

Several groups are assessing the modulatory and synergistic effects of chemical compounds with traditional antimicrobials. For example, EDTA inhibits biofilm growth by *C. neoformans*, and the inhibition could be reversed by the addition of magnesium or calcium, implying that the inhibitory effect is by divalent cation starvation (54). EDTA also reduces GXM released into the EPM, thus providing a potential mechanism for the inhibitory effect of this cation-chelating compound. Unfortunately, the addition of EDTA does not make biofilms more susceptible to antifungal drugs such as fluconazole and voriconazole. Similarly, butyrate is a short-chain fatty acid that is produced by several human commensal bacteria, such as *Clostridium* and *Lactobacillus* species, and acts by inhibiting histone deacetylase (55). In contrast, sodium butyrate strongly inhibited yeast growth in a concentration-dependent manner, interfered with virulence traits such as melanization and capsule formation in *C. neoformans* and, importantly, significantly decreased yeast biofilm formation (56). This fatty acid also enhanced the antifungal activity of azole drugs. Additionally, sodium butyrate augmented the antifungal activity of macrophages by enhancing the production of reactive oxygen species.

Although there have been important advances for combating fungal biofilms, the majority of these prospective strategies are in preclinical development. There is hope that these promising approaches will progress into single or innovative combinatorial therapies to combat microbial biofilms in industrial and medical settings.

**CONCLUSIONS AND FUTURE PERSPECTIVES**

We have reviewed the available information and synthesized it to provide evidence that biofilm formation by *C. neoformans* may play an important role in fungal pathogenesis. However, the understanding of fungal biofilms is still in its infancy. New technological advances not existent before have made available better tools to study microbial cells within biofilms. Hence, the continuing study of *C. neoformans* biofilms would emphasize the elucidation of the genes expressed during biofilm development. For instance, recent proteomic data comparing cryptococcal biofilms and planktonic cells suggest general changes in metabolism, protein turnover, and global stress responses (43). Many changes in metabolic enzymes were identified in studies...
of bacterial biofilm, potentially revealing a conserved strategy in biofilm lifestyle. There seems to be a striking parallel between biofilm formation in bacteria, which results in chronic dormant infection with the potential for acute outbreaks, and the dormant state of primary infection followed by secondary outbreaks in *C. neoformans*. In this regard, Moranova et al. proposed a provocative hypothesis that the cryptococcal response to hypoxia might be the driving force for developing a biofilm-like state of dormant infection which is characterized by slowed proliferation and extensive changes in transcriptome and phenotype (57). This dormant state might facilitate *C. neoformans* survival in the host and possibly develop life-threatening acute outbreaks later.

An observation that supports this school of thought was recently documented in a murine model of methamphetamine abuse and *C. neoformans* infection (58). This drug of abuse stimulated colonization and biofilm formation in the lungs, followed by dissemination of the fungus to the central nervous system, where cryptococcomas harbored fungal cells surrounded by vast amounts of capsular material in a biofilm-like structure. It is particularly interesting that *C. neoformans* modifies its capsular polysaccharide after methamphetamine exposure, highlighting the fungus’s ability to adapt to environmental stimuli, a possible explanation for its pathogenesis. In contrast, Lei et al. discovered that the NLRP3 inflammasome preferably responds to *C. neoformans* biofilms over planktonic cells, suggesting that this multiprotein oligomer is an important arm for host defense against *C. neoformans* infection (59). This group proposed that manipulating NLRP3 signaling may help to oppose *C. neoformans* infection in patients. Perhaps it is unproductive to begin a debate of whether cryptococcal biofilms are either a dormant infection or an infection that induces a strong immune response; the important point is that these thoughts opened novel avenues for investigation and expansion of this exciting field.

Additional studies would focus on understanding how microbial cells in a mature biofilm communicate by the isolation and characterization of the molecules and mechanisms involved in quorum sensing. Although a quorum sensing system (23) and putative molecules such as Cfl1 (24) have been identified, the use of genomic and proteomic techniques should help to elucidate the phenotypes associated with quorum sensing and the mechanisms by which these pathways work in cryptococcal biofilms. Therapeutics that antagonize quorum sensing molecules may potentially be useful in inhibiting fungal growth, capsular production, and biofilm formation by this opportunistic fungus.

Finally, major efforts should be concentrated on the development of strategies for either preventing or eradicating microbial colonization of medical prosthetic devices and development of new methods for assessing the efficacy of these treatments. In particular, studies of biofilms from *in vivo* and clinical sources, by using genome sequencing, comparative genomics, proteomics, and other multidimensional technologies, might open the door to a new frontier in dissecting the molecular mechanisms of cryptococcal biofilm formation and of designing and developing novel antibiofilm agents.

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