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Fungal Biofilms: Relevance in the Setting of Human Disease

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Abstract

The use of indwelling medical devices is rapidly growing and is often complicated by infections with biofilm-forming microbes that are resistant to antimicrobial agents and host defense mechanisms. Fungal biofilms have emerged as a clinical problem associated with these medical device infections, causing significant morbidity and mortality. This review discusses the recent advances in the understanding of fungal biofilms, including the role of fungal surface components in adherence, gene expression, and quorum sensing in biofilm formation. We propose novel strategies for the prevention or eradication of microbial colonization of medical prosthetic devices.

Keywords

Fungi; Biofilms; Antifungal drugs; Immune response; Gene expression; Quorum sensing; Therapy

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 pattern has little to do with the growth of microbes in "natural" or host environments. Recent advances in confocal microscopy and molecular genetics 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 biofilms [2]. Microbial biofilms are dynamic communities of micro-organisms strongly attached to biologic and nonbiologic substrata that are enclosed in a self-produced protective exopolymeric matrix (EPM) [3]. Furthermore, mixed poly-microbial biofilms are common and can be considered even more complex communities of microbes that cooperatively interact in an altruistic manner [4].

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 [5-8]. The successful eradication of biofilms in vivo usually requires toxic concentrations of antibiotics [9]. Although bacterial biofilms have been extensively studied since the mid 1980s, little

attention was paid to medically relevant fungal biofilms until the past decade. To date, a wide variety of fungi have been shown to colonize surfaces and form biofilms. This review focuses on recent information about fungal biofilms and their relevance in the setting of human disease.

Clinical Importance of Fungal Biofilms

The use of broad spectrum antibiotics and corticosteroids, invasive medical procedures, and the AIDS epidemic are associated with a dramatically increased incidence of invasive fungal diseases, which are inherently difficult to treat. The persistence of fungal infections is promoted by the ability of fungi to form biofilms on a wide variety of implanted medical devices [10]. Consequently, fungal biofilms have become a significant clinical and economic problem.

Much of our knowledge about fungal biofilm formation has been gained through the studies of biofilms formed by *Candida* species on artificial surfaces [11-13]. Furthermore, the increasing awareness of the importance of fungal biofilms is reflected by reports on other yeasts and filamentous fungi, including *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Fusarium species*, *Pneumocystis species*, *Trichosporon asahii*, *Blastoschizomyces capitatus*, *Malassezia pachydermatis*, *Saccharomyces cerevisiae*, and *Coccidioides immitis* [8,14-21].

Candida albicans, a commensal of human mucosal surfaces and an opportunistic pathogen in immunocompromised individuals, is commonly associated with biofilm formation [22]. *C. albicans* is the third leading cause of infections related to intravascular catheters, with the second highest rate of colonization to infection and the overall highest crude mortality [23,24]. This fungus can colonize prosthetic devices either endogenously or exogenously (Fig. 1). Yeast cells can detach from adherent biofilms on the devices and cause fungemia and systemic infection. Treatment usually requires removal of the device and a prolonged course of antimicrobial therapy [25], resulting in costs exceeding \$50,000 per patient. The number of non-*albicans* *Candida* species that exhibit biofilm formation and cause device-related infections is rising steadily and thus is of great concern. *Candida* species that cause nosocomial infections include *C. glabrata*, *C. parapsilosis*, *C. krusei*, and *C. tropicalis* [26-30].

C. neoformans is an encapsulated fungus that causes life-threatening meningoencephalitis in immunocompromised individuals. It colonizes and subsequently forms biofilms on diverse prosthetic devices, including ventricular shunts, peritoneal dialysis fistulas, and cardiac valves [14,31-33]. Such biofilms comprise yeast cells surrounded by vast amounts of polysaccharide (Fig. 2), likely preventing successful eradication. Because *C. neoformans* is an environmental fungus and only an accidental pathogen, it is not surprising that biofilm formation constitutes an important survival strategy in hostile environmental conditions (eg, ultraviolet light) and against predation [34,35].

Various *Trichosporon* species can cause disseminated life-threatening infections in immunocompromised hosts; they have been associated with infections related to devices such as catheters, breast implants, and cardiac grafts [36-38]. In addition, *B. capitatus* has been associated with catheter-related fungemia, and *M. pachydermatis* has been isolated from patients undergoing parenteral nutrition. The baker's yeast, *S. cerevisiae*, has been found in association with dentures of stomatitis patients [15,16,27], and persistent meningitis was associated with a *C. immitis* biofilm at the tip of ventriculoperitoneal shunt tubing in a patient [17].

A filamentous mold, *A. fumigatus*, has also caused infections of medical implant devices, including cardiac pacemakers, joint replacements, and breast implants [39,40]. Furthermore,

Fusarium solani and *Fusarium oxysporum* were the causative organisms of a recent outbreak of contact lens-associated fungal keratitis [20].

In general, many of the clinically relevant fungi have been reported to form biofilms in human infection. Given that most of these infections occur in immunocompromised hosts who often have indwelling devices, biofilm formation is highly relevant for the pathogenesis of fungal infections and needs to be studied. Moreover, the thick fungal cell wall and the formation of adherent hyphal phenotypes may enhance drug resistance and adhesion and thus contribute to the resilience of fungal biofilms.

Characteristics of Fungal Biofilm Development

Biofilm formation in fungi is a well-organized process that progresses through coordinated early, intermediate, and maturation stages [41-43]. It begins with attachment of a microorganism to a surface, followed by a cascade of differential gene expression resulting in biofilm formation. 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 [44-46]. 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 [34]. These variables may affect the rate and the extent of fungal attachment.

The composition of the microbial cell surface, which may exhibit fimbriae [47], flagella [48], or a capsule [49], greatly influences the rate and extent of attachment. In *C. neoformans*, the polysaccharide capsule promotes the attachment process [49]; accordingly, hypovirulent, acapsular mutants reveal no biofilm formation relative to the parental or the encapsulated complemented strains. The dynamic nature of the capsule could allow *C. neoformans* cells to vary binding to a variety of substrata and promote fungal attachment. In addition, nonencapsulated yeasts express adhesins that facilitate stronger adhesion. Among these specific adhesion molecules are cell-surface glycoproteins that are encoded by the agglutinin-like sequence (ALS) gene family [50] in *C. albicans* and EPA genes in *C. glabrata* [51].

The intermediate phase of fungal biofilm formation is characterized by a homogeneous aggregation of free-floating cells in a microcolony arrangement on a surface. Proximity of cells within the microcolony may present a formidable environment for the establishment of nutrient gradients [52], genetic exchange [53,54], and quorum sensing (discussed below) [55]. Confocal laser scanning microscopy shows that mature biofilms consist of a dense arrangement of cells embedded within large amounts of exopolymeric material, which can be flat or mushroom-shaped depending on the nutrient source [4,56]. A mature *C. albicans* biofilm consists of yeasts, pseudohyphal forms, and true hyphae. Similarly, *T. asahii* biofilms were identified as containing yeast cells and hyphal elements [18]. Other fungi, including *C. neoformans* [49], undergo similar development but are restricted to their yeast morphology and rely specifically on adhesion via a polysaccharide capsule or cell wall glycoproteins and the production of matrix material [19,28,57]. Biofilm formation involving molds such as *A. fumigatus* has been shown to include phase-dependent developmental stages similar to those of *C. albicans* [58]. The structural organization of biofilms and the presence of flowing water channels [59] may allow nutrient exchange [60] and gas exchange [59] while providing the fungal cells with a sheltered niche for protection against environmental predators [61,62], immune cells [63], shear forces [60,64], and antimicrobial drugs [4].

Cells within a mature biofilm can slowly detach or disperse as single cells or aggregates of cells into the surrounding environment, leading to acute systemic illness. This process appears to be regulated by either external forces [65] or intercellular messengers [66]. Recently, studies showed that *C. albicans* biofilm dispersion is dependent on growing conditions, including the carbon source and the pH of the media used for biofilm development [67]. Dispersed *C. albicans* cells exhibit a yeast morphology but display greater adherence and filamentation than their planktonic counterparts. Furthermore, these cells showed increased pathogenicity in a murine model of infection, indicating that dispersed cells are armed with a complete arsenal of “virulence factors” important for seeding and establishing new foci of infection.

Candidal biofilm formation in in vivo models seems to follow the same sequence [68], but in vivo biofilms mature faster and are thicker than those grown in vitro. The thickness of a biofilm grown in vitro can range from 25 µm to 450 µm [41,69,70], whereas in vivo biofilms usually exceed 100 µm [68].

Fungal Biofilm Exopolymeric Matrix Composition

The EPM surrounds a microbial biofilm and acts as a protective barrier by preventing penetration of host immune factors or antifungal drugs and impeding physical disruption of underlying cells. Mature *C. albicans* biofilms are complex three-dimensional structures between 50 µm and 350 µm thick [71,72]. *C. albicans* EPM consists of proteins, chitins, DNA, and β-1,3 glucan carbohydrates [73]. *C. neoformans* EPM consists of xylose, mannose, glucose, and several minor sugars not found in either of the *C. neoformans* major capsular polysaccharides, glucurinoxylomannan (GXM) or galactoxylomannan (GalXM) [34]. Furthermore, analysis of the *A. fumigatus* EPM produced in vivo showed that galactosaminogalactan and galactomannan were the major polysaccharide components [74••]. This area of study is highly relevant, as EPM or its components constitute potential drug targets.

Molecular Studies of Fungal Biofilms

Molecular studies (especially of *C. albicans* biofilms) have provided insights into the mechanisms governing biofilm formation. These studies include systematic searches using collections of mutants [75], microarray analyses [76,77], and individual transcription factor analysis [78].

Biofilm-associated *C. albicans* differs from its planktonic counterpart with respect to gene expression profiles, as early as within 30 min of surface contact [77]. Most of the *Candida* genes identified to date, which are critical for biofilm formation, are at least indirectly linked to hyphal development [79]. A gene encoding a major regulator of hyphal development is *EFG1*. This gene affects biofilm formation on various solid surfaces including polystyrene, polyurethane, and glass [76,78,80]. Another important gene for regulating hyphae-specific genes is *TEC1* [81], which regulates the transcription of *BCR1*, which in turn regulates genes downstream of hyphal differentiation [72]. A *tec1* mutant strain is unable to undergo hyphal morpho-genesis and shows a severe biofilm defect. Taken together, *TEC1* and *EFG1* data strongly support the hypothesis that hyphal formation is required for robust biofilm development.

Adhesion genes, including those belonging to the *ALS* family, have long been considered excellent candidates for biofilm adhesins. Several *ALS* genes are upregulated during biofilm formation, facilitating co-aggregation with other yeasts and bacteria [82,83]. A *bcr1* mutant that produces hyphae still has a severe biofilm defect in *C. albicans* and *C. parapsilosis* [84,85]. *BCR1* controls the expression of *ALS1*, *ALS3*, and other proteins found in the cell

wall, including *HWP1* (hyphal wall protein) [84]. Mutant analysis shows that *ALS3* is important for biofilm formation in vitro, but is not necessary for biofilm formation in vivo [86], whereas *HWP1* has a role in biofilm formation both in vitro and in vivo [87]. Overexpression of either gene can partially rescue the biofilm defect of a *bcr1/bcr1* mutant strain in vitro and in vivo [72], providing further evidence that these genes work downstream of *BCR1* in promoting biofilm formation. Mutants lacking *TEC1*, *BCR1*, *ALS3*, or *HWP1* demonstrate severe biofilm defects, which suggest that these genes are all important for early events in biofilm formation. The fact that these mutants all result in thin, rudimentary biofilm formation suggests that these proteins may not play a role in surface adherence. Their role is probably in early cell-cell adherence events and, because *ALS3* and *HWP1* are expressed only in hyphae [88,89], they probably mediate hypha-hypha or hypha-yeast adherence.

For *C. glabrata*, the biofilm proteome is also distinct when compared with the planktonic proteome. Specifically, upregulation of stress response proteins and downregulation of glycolysis enzymes can be observed also at the transcriptomic level. This pattern potentially contributes to the higher antifungal resistance seen in *C. glabrata* biofilms [90]. *C. glabrata* contains a large number of genes encoding adhesin-like GPI proteins, which include the lectin-like *EPA* family. *EPA* genes confer adhesion to mammalian host tissues. The *EPA1* gene has 23 paralogs in the genome. Most of these *EPA* genes are located in subtelomeric positions, where they are transcriptionally silenced [51]. Transcription of some subtelomeric *EPA* genes can be de-repressed by limitation of NAD⁺ precursors [91]. Null mutations in *SIR3*, *SIR4*, and *RIF1* lead to simultaneous expression of many *EPA* genes, resulting in hyperadherent phenotypes [51,92]. *AWP1/2/3/4* and *EPA6* are involved in adherence to human epithelia and biofilm formation [93]. Culture of *C. glabrata* with a variety of vaginal products induced expression of *EPA6*, leading to increased adherence to cultured human cells as well as primary human vaginal epithelial cells. Activation of *EPA6* transcription depends on the *FLO8* and *MSS11* transcription factors [46].

For *C. neoformans*, the presence of the polysaccharide capsule affects the ability of cells to adhere [49]. The capsule can be altered by phenotypic switching, which can also change the ability of *C. neoformans* to generate biofilms [94]. Conditions that mimic the host environment have been suggested to be permissive for biofilm formation [95]. Genetic pathways in *C. neoformans* have not yet been examined with a focus on biofilm formation.

Matrix production is closely tied to biofilm formation, yet little is known about its regulation or mechanisms. Studies recently showed that the *C. albicans* zinc-response transcription factor *ZAP1* is a negative regulator of a major matrix component, soluble β -1,3 glucan, in both in vitro and in vivo biofilm models [96]. It was demonstrated that two glucoamylases, *GCA1* and *GCA2*, have positive roles in matrix production and may function through hydrolysis of insoluble β -1,3 glucan chains. Additionally, the alcohol dehydrogenases *ADH5*, *CSH1*, and *IFD6* affected matrix production: *ADH5* acted positively, and *CSH1* and *IFD6* acted negatively. It was proposed that these alcohol dehydrogenases generate quorum-sensing aryl and acyl alcohols that in turn govern multiple events in biofilm maturation [96].

Quorum sensing is the ability of microorganisms to communicate and coordinate their behavior via the secretion of signaling molecules in a population-dependent manner. Quorum sensing in eukaryotes was first described in *C. albicans* with the identification of farnesol as a component in high-density cultures that inhibits hyphal formation [97]. Microarray analysis of farnesol-treated biofilms indicates that the molecule controls expression of genes important for hyphal development (*TUP1* and *CRK1*), drug resistance (*FCR1* and *PDR16*), and cell wall integrity (*CHT2* and *CHT3*) [98]. A second study confirmed that farnesol represses the expression of hypha-related genes and induces the

expression of drug-resistance genes [99]. Genetic studies suggest that the histidine kinase *CHK1*, a member of a two-component signal transduction pathway, may be part of the farnesol signaling pathway. A *chk1* mutant strain will form a biofilm in the presence of farnesol, suggesting that this kinase is a key mediator of the cellular response to quorum sensing [100]. In *C. albicans*, it has been speculated (but not directly tested) that, through the mediation of quorum sensing, nutrient limitation in a mature biofilm may lead to dispersion of daughter cells less adherent to the biofilm matrix [101]. An interesting possibility is that yeast cells may possess mechanisms that enable them to regulate the activity of their adhesins. Granger et al. [102] described a yeast cell wall-specific protein, *YWP1*, which may act as an anti-adhesin. *YWP1* deletion leads to greatly enhanced adherence of yeast cells to several surfaces. A *YWP1* propeptide is released into the culture medium of *YWP1*-expressing cells, and the extracts from cultures of *YWP1*-expressing stationary-phase cells inhibit adherence of strains that do not express *YWP1*, suggesting that the *YWP1* propeptide can inhibit adherence in surrounding cells. Quorum sensing is also described for *C. neoformans* [103], but its impact on biofilm formation is still unknown. There is evidence that certain cell variants exhibit higher switching rates when grown as biofilm-associated cells rather than planktonic cells [94].

Molecular mechanisms conferring antifungal resistance in fungal biofilms have not been fully elucidated. Genes encoding for drug efflux pumps and ergosterol biosynthesis, implicated in azole resistance, have been reported to be differentially regulated upon exposure to antimicrobial agents; these genes include *MDR1*, *CDR1*, *CDR2*, and *ERG11*, which have previously been implicated within the biofilm developmental phase [78,104,105]. Animal studies have also shown that biofilms formed on catheters express *CDR* pumps [68]. In *C. albicans* and *C. glabrata*, mRNA transcripts indicated that these genes were rapidly upregulated and may play a defined role in resistance. Similarly, *A. fumigatus* has also shown that *MDR4* is upregulated as the biofilm becomes more complex, and that azole pretreatment induced further upregulation [58]. More sophisticated in vitro and in vivo genome-wide expression analyses are needed to elucidate the role of genes that confer higher antifungal resistance in fungal biofilms.

Resistance of Fungal Biofilms to Host Immune Mechanisms and Antifungal Therapy

Biofilm formation is associated with persistent infection because biofilms increase resistance to host immune mechanisms and antimicrobial therapy. Limited information is available on the role of the host immune response in relation to biofilm formation. A recent report suggests that epithelial surfaces can be protected from the development of mucosal infections through a TLR4-mediated protective mechanism that is dependent on polymorphonuclear leukocytes (PMNs) [106,107]. *C. albicans* biofilms' interaction with human PMNs induced significantly more IL-1 β , IL-10, and MCP-1 than interaction with planktonic cells [107]. Cells within a biofilm are protected against phagocytosis [108] and are relatively resistant to host immune mechanisms [109]. For instance, *HYR1* is an important virulence factor for *C. albicans*, mediating resistance to phagocyte killing. As for *C. albicans*, heterologous expression of *HYR1* in *C. glabrata* rendered the organism more resistant to neutrophil killing activity. In fact, *HYR1p* is a promising target for vaccine or other immunologic or small-molecule intervention to improve the outcomes of disseminated candidiasis.

In *C. neoformans*, biofilm formation depends on the presence of a polysaccharide capsule composed primarily of GXM, and specific protective antibodies against this component inhibited biofilm formation [49]. In contrast, lactoferrin, a component of the innate immune system, was unable to prevent fungal biofilm formation despite its previously reported

efficacy against bacterial biofilms [49]. Under certain circumstances, however, biofilm-specific antibodies can antagonize the action of antifungal drugs and reduce their activity against *C. neoformans* biofilms [110]. Moreover, *C. neoformans* cells within biofilms were more resistant than planktonic cells to oxidative stress but remained vulnerable to cationic antimicrobial peptides [6]. Interestingly, antibody-mediated agglutination and biofilm formation can also mediate resistance to and escape from phagocytosis inside macrophages [111].

Consequently, biofilms constitute a physical barrier preventing efficient penetration of antimicrobial drugs, conferring microorganisms forming biofilms with higher resistance to antimicrobial activity than their planktonic counterparts [57,112]. *Candida* biofilms are 30 to 2,000 times more resistant than planktonic cells to antifungal agents, including amphotericin B, fluconazole, itraconazole, and ketoconazole [28]. Biofilm formation and drug resistance of the biofilms can differ among clinical strains, although the degree of biofilm formation is stable after passage through the infected human host. Some *Candida* biofilms remain sensitive to higher doses of amphotericin B [113]. *C. neoformans* biofilms are also significantly more resistant to amphotericin B and caspofungin than planktonic cells [7]. Susceptibility to these drugs can be further reduced if cryptococcal cells are melanized [7]. However, the exposure of *C. neoformans* cells to fluconazole or voriconazole did not inhibit yeast growth, and the exposure of preformed cryptococcal biofilms to the same drugs did not affect the metabolic activities of the biofilms [7].

Various mechanisms of biofilm resistance to antimicrobial agents have been proposed, including the presence of physical barriers that prevent the penetration of the antimicrobial compounds into the biofilm, slow growth or regulation of the metabolic activity of the biofilm due to nutrient limitation, activation of the general stress response, and the existence of a subpopulation of cells within the biofilm (known as *persisters*) that are preserved by antimicrobial pressure [114].

The structure of biofilms and the presence of EPM may reduce the diffusion of antifungal drug. Reports have demonstrated that EPM production is greater in biofilms grown under shaking conditions, compared with static cultures [115]. Also, *C. albicans* biofilm developed under shaking conditions demonstrated higher resistance to amphotericin B than resuspended cells [116]. However, the biofilm ultrastructure and the presence of EPM is not entirely attributable to drug resistance, as biofilms that have been detached and dispersed were at least eight times more susceptible to fluconazole than intact sessile structures [78]. In studies of biofilms formed by *Aspergillus* species, several groups correlated the production of EPM and reduced susceptibility to antifungal drugs [58,117-119].

One mechanism of resistance that has gathered some attention recently is the persister cell theory [120,121]. A small subset of yeast cells in a *C. albicans* biofilm has been described as highly resistant to amphotericin B following adhesion, and this resistance is independent of upregulation of efflux pumps and cell membrane composition [120,121]. More recently, persisters have been found in biofilms of *C. krusei* and *C. parapsilosis* [122].

Furthermore, Perumal and colleagues [123] demonstrated the contribution of cell density to antifungal drug resistance in *C. albicans* biofilms and concluded that azole drug tolerance at high cell density differs mechanistically from tolerance at low cell density. This observation is important, considering that cell density is a key aspect of quorum sensing, which play an essential role in biofilm regulation.

Therapeutic Approaches for Treatment of Fungal Biofilms

The use of indwelling medical devices such as pacemakers, prosthetic joints, and catheters continues to increase, yet these devices are all too often complicated by infections with biofilm-forming microbes with increased resistance to antimicrobial agents and host defense mechanisms. Treatment of infectious diseases associated with indwelling medical devices usually requires surgical intervention combined with a prolonged course of antimicrobial therapy, with high costs. There is also significant morbidity and mortality associated with such treatments.

Work in *C. neoformans* biofilms suggested that monoclonal antibodies (MAbs) are potentially useful in the clinical management of disease caused by biofilm-forming microbes [110]. In addition, radioimmunotherapy can kill microorganisms quickly and efficiently; it might be delivered through biofilm-specific MAbs. A recent report demonstrated that MAb-guided alpha radiation effectively damages fungal biofilms [124]. In clinical cases in which removing indwelling devices is difficult, treatment of infected devices in situ by local administration of radio-labeled biofilm-specific MAbs may constitute a feasible alternative approach. Furthermore, biofilm-specific MAbs could be exploited to prevent biofilm formation [49], and a prophylactic dose of biofilm-specific antibodies could be administered immediately after insertion of the device. Novel therapeutic strategies against biofilm-related microbial infections may also be designed by combining radio-immunotherapy 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 [125••] in a central venous catheter model [68], chitosan catheters inhibit *C. albicans* biofilm formation in vivo. It was shown that chitosan in biofilms significantly reduced the metabolic activity of *C. parapsilosis* [125••] and the cell viability of *C. neoformans* [126]. Notably, melanization, an important virulence determinant of *C. neoformans*, did not protect cryptococcal biofilms against chitosan [126]. This phenomenon has been attributed to the ability of cationic chitosan to disrupt negatively charged cell membranes as microbes settle on the surface [127]. 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.

Charge of the fungal surfaces may keep yeast cells in suspension, preventing biofilm formation, or may increase phagocytosis and killing of fungal cells by macrophages [128]. Binding of chitosan to DNA and inhibition of messenger RNA synthesis occurs through enhanced chitosan penetration [127]. 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 [129]. The chitosan concentrations used in 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 [58], novel formulations of antifungal drugs [130], and antifungal combinations [131] have been successfully applied. For instance, treatment of actively growing *A. fumigatus* biofilms with amphotericin B was more efficacious in vitro than treating mature structures [58], the use of amphotericin B lipid complex was effective in treating *C. albicans* biofilms in vivo [130], and *C. albicans* biofilms showed higher susceptibility when treated with a combination of amphotericin B and posaconazole than when treated with single drugs [131].

Conclusions

Biofilm formation by fungi may play an important role in pathogenesis. New technological advances have made available better tools to study microbial cells within biofilms. Future studies of fungal biofilms should emphasize the elucidation of genes expressed during biofilm development and drug resistance. These studies should also incorporate non-*albicans* species, which are very prevalent in nosocomial infections, and more clinical isolates, and they should focus on biofilms formed in vivo. More investigations are required to further our understanding of how microbial cells communicate in a mature biofilm, which is pivotal for the pathogenesis of dissemination. We propose that an interdisciplinary approach will be required to develop novel strategies to prevent or eradicate microbial colonization of medical prosthetic devices.

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Fig. 1. Scanning electron microscopy image of mature (24-h) *Candida albicans* biofilms formed on a rat central venous catheter model showed a network comprising yeast cells and hyphae surrounded by moderate amounts of exopolymeric matrix. Bar, 5 μ m

Fig. 2. Scanning electron microscopy image of mature (48-h) *Cryptococcus neoformans* biofilms formed on glass coverslips revealed that cryptococcal cells are surrounded by copious amounts of polysaccharide. Bar, 10 μ m