Biofilm formation by *Aspergillus fumigatus*

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**Abstract**

*Aspergillus fumigatus* is a well adapted, opportunistic fungus that causes a severe and commonly fatal disease, invasive pulmonary aspergillosis (IPA), in highly immunocompromised patients, aspergilloma in patients with lung cavities and allergic bronchopulmonary aspergillosis (ABPA) in hypersensitive individuals. Recent studies have suggested that biofilm formation by *A. fumigatus* may be one of the most important virulence factors in IPA and aspergilloma. Several fungal constituents may contribute to the formation of biofilm structures on host cells, including cell wall components, secondary metabolites and drug transporters. The biofilm phenotype of the fungus is refractory to most conventional antifungal treatment options. Thus, an in-depth analysis and understanding of *A. fumigatus* biofilms is necessary to devise newer and better antifungal targets for treating complex *A. fumigatus* biofilm-associated diseases.

**Key words:** *Aspergillus fumigatus*, aspergilloma, biofilms, invasive pulmonary aspergillosis.

**Introduction**

Most microorganisms preferably grow in a complex milieu, in the form of structured polymicrobial biofilms on both natural and inanimate surfaces. Microorganisms growing together in composite biofilms produce an extracellular matrix (ECM) that binds them to one another and to the *in vitro* or *in vivo* substrate. These biofilms are well protected against hostile environments of the host immune system and also resist antimicrobial killing. The transition of a planktonic form of a microbe to a biofilm or a sessile structure (surface attached and heterogeneous cells) is the result of interactions between different pathogen and host factors. The formation of these microbial biofilms is a pre-requisite event towards the development of invasive disease and it has been reported that these biofilms are involved in about 80% of non-acute infections in humans [1]. However, only recently have studies been initiated to investigate the complicated biofilm phenotype during diseases, especially fungal diseases. Among the fungal species, biofilms formed by *Candida albicans* on catheter material and human cell surfaces are the most well-studied fungal systems [2,3]. The formation of biofilms by another opportunistic fungus, *Aspergillus fumigatus* has just begun to be understood. The major players and molecular mechanisms underlying biofilm formation in *A. fumigatus*-mediated invasive diseases remain largely elusive. In the present review, we discuss the current knowledge of biofilms formed by *A. fumigatus* under *in vitro* and *in vivo* conditions and also list some of the important pathogen factors that contribute to their formation.

**A. fumigatus** biofilms: *in vitro* studies

*A. fumigatus* is a saprophytic fungus that survives and grows on organic debris. It is one of the most ubiquitous fungal pathogens that release thousands of conidia into the
Figure 1. Different phases of Aspergillus fumigatus biofilm formation. The formation of mature biofilms embedded in a thick extracellular matrix (ECM) takes place after 48–72 h, after adhesion and germination of the fungal conidia. Different fungal factors including cell wall components and secondary metabolites are activated and contribute to the biofilm formation. A. fumigatus biofilm has a heterogeneous architecture, in terms of distribution of fungal cells and ECM. This Figure is reproduced in color in the online version of Medical Mycology.

environment [4]. A. fumigatus is the second most common cause of fungal infection found in hospitalized patients, after C. albicans [5]. In immunocompetent hosts, Aspergillus conidia are efficiently cleared by pulmonary macrophages, neutrophils and pattern recognition molecules such as surfactant proteins and toll-like receptors [6,7]. However, in others, their inhalation causes a spectrum of different diseases like aspergilloma, invasive aspergillosis (IPA) and allergic bronchopulmonary aspergillosis (ABPA), depending on the extent of mycelial colonization, which is further dependent on the immunological status of the host.

The initial establishment of chronic A. fumigatus infection involves the germination of conidia into mycelia and then subsequent invasion of the mycelial structure into pulmonary epithelial and endothelial cells [8]. The first analysis of the three-dimensional structure of a mycelial colony of A. fumigatus was performed by Beauvais et al. in 2007 [9]. The study implicated that under static aerial conditions, mycelial growth is greater than in shaken, submerged conditions. Furthermore, static A. fumigatus colonies were shown to be more resistant to polynes than the submerged mycelium in liquid shaken conditions. The report also documented that the hydrophobic extracellular matrix (ECM) of A. fumigatus that binds the fungal hyphae into a contiguous sheath on the colony surface of A. fumigatus cultures is composed of galactomannan, α-1,3-glucans, galactosaminogalactan, monosaccharides and polylols, melanin, and proteins [9]. After the initial report, a few other studies have reported the formation of Aspergillus biofilms under in vitro conditions on polystyrene microtitre plates seeded with human bronchial epithelial cells and cystic fibrosis (CF) human bronchial epithelial cells [10–12]. These studies depict that all the filamentous multicellular biofilm structures of A. fumigatus exhibit acute-angle dichotomous branching to varying extent. Further, the conidial seeding density plays an important role in the overall structural integrity of the biofilm structure. Use of a low density inoculum (1 × 10⁴ conidia/ml) produces thick and easily disruptable biofilms, and a very high density inoculum (1 × 10⁶ conidia/ml) gives rise to relatively thinner biofilms with less biomass. A concentration of 1 × 10⁵ conidia/ml is known to produce the most robust filamentous structures that are also resistant to mechanical disruption [10]. In terms of kinetics, the development of Aspergillus biofilm has been shown to be slower than that of C. albicans biofilms. In A. fumigatus, following initial conidial seeding, there is a lag phase of approximately 10 h (conidial adhesion and germination) before the hyphae begin to intertwine forming a monolayer (10–16 h), followed by increased structural complexity over the subsequent 4–8 h. The hydrophobic ECM that cohesively binds the hyphae together increases with maturity of the developing biofilm structure and the depth of the biofilm increases from 10–200 μm [10]. These phase-dependant growth characteristics of A. fumigatus are known to play a key role in the outcome of antifungal treatment.

An in vitro co-culture study of A. fumigatus biofilms on lung epithelial cells has also shown that A. fumigatus biofilms consisting of both parallel and crossing hyphae with a heterogeneous structure of ECM are established on human bronchial epithelial cells [11,12]. Similar to that observed on microtitre plates, the study has demonstrated that A. fumigatus biofilm formation on epithelial cells proceeds through early (12 h), intermediate (48 h) and maturation phases (72 h). Further, the A. fumigatus biofilm on epithelial cells in polystyrene plates has a heterogeneous architecture, in terms of distribution of fungal cells and ECM (Fig. 1). More details about the morphological and physiological aspects of A. fumigatus biofilms have been discussed elsewhere [13].

A. fumigatus biofilms: in vivo studies

Although convincing studies of A. fumigatus biofilm development in vivo are lacking, evidence such as high mortality in neutropenic cancer patients suffering from invasive aspergillosis and resistance of chronic Aspergillus infections to potent antifungal drugs in vitro clearly implicate the
formation of *A. fumigatus* biofilms *in vivo*. Histological and microscopic examination of bronchopulmonary lavage samples from the lungs has revealed the presence of numerous *A. fumigatus* hyphae in the form of dense intertwined mycelial balls or grains, referred to as mycetoma, which is similar to the biofilms formed by *Candida* species *in vivo* [14]. Studies in patients have reported that *A. fumigatus* forms different types of biofilms in aspergilloma and IPA with varied ECM composition [15,16]. Electron microscopy of ultrathin sections of aspergilloma samples from different patients has shown that biofilms formed in aspergilloma consist of fungal hyphae embedded in a thicker ECM, in contrast to IPA, and in aspergilloma the hyphae show degradation and vacuolated cells after 48 h of infection. ECM composition analysis by immunocytochemistry has further revealed that in aspergilloma it consists of the same polysaccharides found in ECM produced *in vitro* in static and aerial cultures, i.e., α-1,3 glucan, galactomannan and galactosaminogalactan [15,16].

In contrast, in lungs with IPA *A. fumigatus* do not develop a typical biofilm as occurs *in vitro* in static culture or *in vivo* in aspergilloma. The hyphae are not agglutinated together but are separate. Also, unlike in aspergillomas, all the hyphal cells are viable in an IPA biofilm even after 4–5 days of *A. fumigatus* infection [16]. These hyphae are embedded in an ECM that is less electron-dense and thinner than in aspergilloma. ECM composition analysis show the presence of galactosaminogalactan and galactomannan as major components of ECM in IPA biofilms, but an absence of alpha-1,3-glucans [15]. This is in accordance with the hyphae-hyphae adhesion role of alpha-1,3-glucans observed in static culture *in vitro* and in aspergilloma *in vivo* [16].

The main clinical manifestation of *A. fumigatus* in CF patients, as described earlier, is in the form of ABPA. In fact, *A. fumigatus* is one of the predominant fungal species that colonize damaged lung parenchyma, and is found in about 60% of patients with CF [17]. It has been proposed that abnormal mucus in the lungs promotes the trapping of *A. fumigatus* conidia within the bronchial airway, presumably promoting growth of *A. fumigatus* mycelia, thus contributing to an increased *A. fumigatus* colonization of the lung, which further stimulates T-helper cell type (Th) 2-biased responses favoring the development of ABPA [18]. As discussed earlier, the formation of *Aspergillus* biofilm has been shown in CF bronchial epithelial cells *in vitro*; however, its formation has not been reported in CF patients *in vivo* [11]. It may be postulated that the dynamics of *A. fumigatus* biofilm formation may be largely modified in ABPA patients due to the coexistence of *Pseudomonas aeruginosa* in these patients. A recent study has reported that *P. aeruginosa* inhibits *A. fumigatus* biofilm formation when cultured together, but has no effect on preformed *Aspergillus* biofilms. The study has also demonstrated that this antagonistic relationship between *A. fumigatus* and *P. aeruginosa* is influenced through the release of small diffusible extracellular molecules, plausibly quorum sensing molecules, by the bacteria [19]. Clinical studies have however reported contrasting results. A case-control study has indicated that CF-patients colonized with *A. fumigatus* have a higher risk of developing chronic *P. aeruginosa* infections despite receiving antimicrobial therapy [20]. Another study has instead hypothesized that *P. aeruginosa* colonization favors the process of *A. fumigatus* sensitization, as the bacterial colonization takes place much earlier than *Aspergillus* infection [21]. Another study by Skov et al. has reported that *A. fumigatus*-mediated ABPA occurs independently of *P. aeruginosa* infection [22]. It may be presumed that the initial interaction between the fungus and *P. aeruginosa* is synergistic; however, later during the development of the biofilm, this relationship becomes competitive or antagonistic, possibly depending on host immune factors (Fig. 2). It would be extremely worthwhile to characterize the complex relationship between *A. fumigatus* and *P. aeruginosa* in mixed biofilms in CF.

### A. fumigatus biofilms and antifungal agents

As compared to the planktonic forms, sessile biofilm forms of *A. fumigatus* are considerably different in respect to their response to antifungal agents *in vitro*, and a number of currently prescribed antifungal agents may be futile in the treatment of established biofilm-associated infections. It has been clearly demonstrated that the minimum inhibitory concentration of antifungal agents required to kill biofilm structures of *A. fumigatus* is much higher than that required to kill the planktonic forms of the fungus, both on polystyrene and epithelial cells [11,12]. *A. fumigatus* becomes increasingly resistant to polyene, azole, and echinocandin antifungal agents throughout its morphological differentiation to a biofilm phenotype [23]. It is hypothesized that ECM plays a significant role in antifungal resistance by adsorbing antifungal drug molecules and preventing their diffusion. Studies in *C. albicans* have demonstrated that ECM expression (specifically beta-glucans) sequesters antifungal agents and reduces susceptibility [24]. It is also reported that multidrug resistance (MDR) pumps, which are involved in the active extrusion of antimicrobial molecules, are activated as the biofilm develops. A recent study has shown that *AfuMDR4* is significantly induced by treatment with voriconazole after 24 h, both *in vitro* in *A. fumigatus* biofilm phenotype and *in vivo* in a mouse biofilm model [25].
Among the various antifungals used, Amphotericin B (ampB) has been reported to be the most effective against all phases of *A. fumigatus* growth, followed by voriconazole, caspofungin and itraconazole. AmpB binds directly to membrane sterols causing the formation of channels or pores, resulting in cytoplasmic and ion leakage leading to cell death. Therefore, its ability to kill cells rapidly and reduce metabolic activity is not directly influenced by the growth phase *per se*. However, as structural complexity increases, a reduction in ampB activity has been observed, suggesting that the ECM associated with this growth phase plays a role in impeding drug action [26]. Persister cells may also exist in fungal biofilms, which may be an alternative explanation to the effect observed in *A. fumigatus* cells following amphotericin B challenge. Persister cells are ‘quiescent variants of regular cells in microbial populations that are highly tolerant to antibiotics’. For example, in *C. albicans* biofilms, a small subset of yeast cells has been known to be highly resistant to ampB following adhesion, which is independent of upregulation of efflux pumps and cell membrane composition [27]. Voriconazole is also effective, but primarily against actively growing hyphae associated with early cell populations, although overall significantly less than amp B. Both voriconazole and AmpB have demonstrated a proven ability to reduce cellular viability of *A. fumigatus* biofilms by about 50%, suggesting that these antifungals may prevent the establishment of a mature biofilm *in vivo* [11]. The observations of antifungal drug susceptibility testing in *A. fumigatus* biofilm formation in various studies suggest that the best antifungal therapy should not be selected solely on the basis of its efficiency to kill fungal cells *in vitro* but also its ability to penetrate the ECM of the fungal cells in a biofilm structure.

Pathogen factors in the formation of *A. fumigatus* biofilms

In addition to providing structural integrity, the cell wall represents the major interface between the internal physiology of the fungus and the hostile environment of human tissue. As discussed above, the *A. fumigatus* cell wall is a dynamic, complex structure composed of galactomannan, galactosaminogalactan, alpha-1,3 glucans, monosaccharides, polysols, melanin, proteins and extracellular DNA. All these constituents along with other some other elements including secondary metabolites and drug transporters significantly contribute to the formation and development of *A. fumigatus* biofilms at various stages. Some of these are discussed briefly in the following section.

**Cell wall carbohydrates.** The carbohydrate component of the *A. fumigatus* cell wall consist of branched and linear β-(1,3) and β-(1,4) glucan, α-(1,3) glucan, chitin, chitosan and galactomannan and galactosaminogalactan. β-(1–3) glucan branched with b(1–6)-glucan forms the skeleton of the wall, and these are covalently bound to chitin and β-(1–3/ 1–4)-glucan [28].

α 1,3-glucans play a predominant role *in vitro* in the aggregation of hyphae in biofilms in both aerial conditions and in patients with aspergilloma [16]. There are three known α-(1,3) glucan synthase genes in the *A. fumigatus* genome, *ags 1, 2 and 3. All three glucan synthase genes have shown
to be upregulated in *A. fumigatus* biofilms, providing evidence for their function in hyphal adhesion to host components and hyphal aggregation [29]. Other polysaccharides of ECM including galactomannan and galactosaminogalactan are also known to have a role in protecting the fungus, and in adhesion of its biofilm structures to biotic or abiotic surfaces [16].

**Hydrophobins.** The outer cell wall layer of *A. fumigatus* conidia contains two hydrophobins, RodAp and RodBp, which are found as highly insoluble complexes. RodA encodes a cysteine-containing polypeptide that is assembled into a regular array of rodlets on the surface of conidia to render the surface highly hydrophobic [30]. The disruption of RodA gene has been shown to be associated with a decrease in adherence of conidia to collagen and albumin, though not the basement membrane glycoprotein, laminin, fibrinogen or pulmonary epithelial cells [31]. RodB is known to play a role in the structure of the conidial cell wall. Both RodA and RodB genes have been reported to be upregulated in *A. fumigatus* biofilms relative to planktonic forms [29]. These rodletless mutants are more sensitive to killing by alveolar macrophages, suggesting that the rodlet structure is also involved in the resistance to host cells.

**Glycophosphatidylinositol (GPI)-lated and glycosylated proteins.** The addition of glycosylphosphatidylinositol (GPI) anchor or GPI-lation and glycosylation of proteins are the two major post-translational modifications that are observed in *A. fumigatus*. Some cell surface proteins of the fungus are modified at their C-terminus by the addition of a GPI anchor and transported to the plasma membrane and cell wall, where they are directly or indirectly involved in cell wall organization. Deletion of *cpsA* gene in, that encodes a repeat-rich glycophosphatidylinositol (GPI)-anchored cell wall protein results in weakening of the conidial cell wall, whereas its overexpression increases conidial resistance to cell wall-degrading enzymes and inhibits conidial germination in *A. fumigatus* [32]. Along with GPI-anchored proteins, GPI anchoring and/or synthesis are also essential for cell viability in yeasts [33]. In *A. fumigatus*, GPI anchor is not only essential for viability and cell wall integrity but does seem to be required for its morphogenesis and virulence as complete blocking of the GPI anchor synthesis in the fungus leads to abnormal hyphal growth, rapid conidial germination and aberrant conidiation [34]. Another novel first-in-class broad-spectrum antifungal agent, E1210, that inhibits the inositol acylation step in glycophosphatidylinositol (GPI) biosynthesis itself, has shown to result in the suppression of hyphal growth, adherence, and biofilm formation in *C. albicans* [35]. E1210 has also been shown to be a potent antifungal agent in *A. fumigatus*, however whether it also has an inhibitory effect on *A. fumigatus* biofilms needs to be established [36].

Among glycosylated proteins, glycosylated adhesins play an important role in structuring the colony of *A. fumigatus*. Adhesins have been characterized in bacteria and yeasts, and their role in cell-to-cell adhesion and biofilm formation is well established [37,38]. Recently, Upadhayay and colleagues have used a bioinformatic approach for the identification of *A. fumigatus* adhesins. In this study, a whole proteome analysis has been conducted using the software SPAAN, a program for protein sequence-based identification of adhesion proteins. The authors have identified 82 proteins with a 90% or higher probability of encoding an adhesion and reported seven novel fibrinogen-binding proteins of *A. fumigatus* [39]. From this list, a serine threonine-rich protein, CalA, has been selected for further study. Recombinant CalAp has been found to bind to laminin and murine lung and spleen cells in *vitro*. However, the role of adhesins in *A. fumigatus* biofilm formation needs to be established.

**Melanin.** The wall of *A. fumigatus* conidia is distinguished by the presence of melanin, a pigment that is thought to guard its genome from the adverse effects of ionizing radiation in the environment. Melanin pigment on the surface of resting *A. fumigatus* conidia also serve to mask pathogen-associated molecular patterns (PAMPs)-induced cytokine responses. It has been shown that albino conidia without melanin induce significantly more proinflammatory cytokines in human peripheral blood mononuclear cells (PBMC), as compared to melanized wild-type conidia, indicating an immune-modulatory role of melanin [40]. Melanin biosynthesis is regulated by a cluster of six genes, *pksP/alb1, ayg1, arp1, arp2, abr1* and *abr2*. Of these, *pksP/alb1*, that encodes a polyketide synthase, has been examined most closely. *PksP*, encoding polyketide synthase, represents the key enzyme for the biosynthesis of dihydroxyxnaphthalene (DHN) melanin, which confers the gray-green pigmentation to *A. fumigatus* conidia. Mutants lacking this enzyme have white conidia and attenuated virulence when inoculated intravenously into mice [41]. It has also been documented that in contrast to pigmentless *pksP* mutant conidia of *A. fumigatus*, the gray-green wild-type conidia inhibit the acidification of phagolysosomes of alveolar macrophages, monocyte-derived macrophages, and human neutrophil granulocytes, implicating that the DHN melanin inhibits the host endocytosis pathway and enables the fungus to survive in phagocytes [42,43]. With regard to biofilm formation, *alb1* gene mutants that are deficient in melanin synthesis have been studied in *A. niger*. The mutants have an altered surface structure and changed physicochemical surface properties. The mutants show considerable difference
in conidial adhesion and biofilm formation as compared to wild type strains [44]. Given an important role of melanin in impeding the host protective mechanisms, it would be extremely worthwhile to study the role of pks1/alb1 in biofilm formation in A. fumigatus under in vivo conditions.

Extracellular DNA. Recent studies have shown that extracellular DNA (eDNA) is another important component of biofilm ECM [45]. eDNA is a key component of both fungal and bacterial biofilms and is proposed to improve overall structural integrity [46,47]. In C. albicans, eDNA has also been shown to be a regulator of biofilm cell antifungal resistance [48]. Studies by Rajendran et al. have now also demonstrated that eDNA is an important structural constituent of A. fumigatus ECM, and plays an important functional role in maintaining structural and architectural integrity of its biofilms. Furthermore, the study has also shown that the release of this eDNA by autolysis in biofilms is significantly associated with the levels of antifungal resistance, suggesting that eDNA plays an important role in biofilm resistance to antifungals [49].

Secondary metabolites. A. fumigatus secretes numerous secretory products including degradative enzymes such as proteases, phospholipases, mycotoxins and damaging secondary metabolites into its vicinity, which facilitate its colonization in the lung tissue and modulate epithelial function and viability. Phospholipases are key enzymes in pathogenic fungi that cleave host phospholipids, resulting in membrane destabilization and host cell penetration. The disruption of the fungal pld gene, encoding phospholipase D has been reported to significantly suppress the internalization of A. fumigatus into A549 epithelial cells without affecting conidial adhesion to epithelial cells. Loss of the pld gene has also been shown to attenuate the virulence of A. fumigatus in immunosuppressed mice, suggesting that PLD may represent an important virulence factor for A. fumigatus infection [50].

Among the myotoxins, gliotoxin has attracted the most attention in A. fumigatus because of its potent immunosuppressive and cytotoxic properties and the fact that it can be readily detected during experimental infection and in sera from patients with aspergillosis [51]. Gliotoxin is an epipolythiodioxopiperazine (ETP) molecule with a molecular weight of 326 Da. The toxicity of gliotoxin is mediated by the presence of an internal disulphide bond which forms mixed disulphides with mixed thiol groups. A study by Bruns et al. shows that the proteins of the gliotoxin secondary metabolite gene cluster are induced in biofilm cultures of A. fumigatus [52]. Studies have reported that gliotoxin mainly affects the contribution of neutrophils to host defense, likely via the induction of neutrophil apoptosis by preventing the formation of the NADPH oxidase complex in neutrophils [53].

MedA. MedA is a developmentally regulated protein that was first identified in the related model organism A. nidulans, where it is expressed in competent hyphae [54]. In A. nidulans, MedA was characterized as a temporal modifier of the expression of the core conidiation genes. A. fumigatus mutants deficient in MedA are impaired in biofilm formation on glass and plastic, as well as in adherence to pulmonary epithelial cells, vascular endothelial cells, and fibronectin [55]. Thus, it may be an important antifungal target for A. fumigatus-mediated invasive infections.

ABC transporters. Reduced susceptibility to drugs in A. fumigatus biofilms could also result from an increased activity of efflux pumps and transporter proteins. The genes mdr1, mdr2, atrF and mdr4 encode ATP-binding cassette (ABC)-type transporters and are involved in antifungal resistance. They also detoxify the components of the host immune system in the fungus [56,57]. The members of the MDR1 family are significantly upregulated in the A. fumigatus biofilms in comparison to planktonic forms [29]. A similar gene that codes for a protein of the major facilitator superfamily (MFS) related to azole resistance has also been documented to be upregulated in A. fumigatus biofilms [58].

Conclusions

With the increasing number of immuno-compromised individuals such as AIDS and cancer patients or transplant recipients, systemic opportunistic A. fumigatus biofilm-associated infections are emerging as life-threatening infections with a very high mortality rate even after antifungal therapy. Since they are difficult to treat with conventional antifungal agents, there is a need to study the characteristics of this sessile fungal phenotype and understand the molecular mechanisms that lead to its antifungal drug resistance. Such studies would help to ascertain the best and most effective antifungal treatment for A. fumigatus biofilm-associated diseases. Furthermore, it is essential to identify and characterize the major molecular players that are involved in the transition of a planktonic form of A. fumigatus to its biofilm form. The above review lists a few important pathogen factors that contribute to the biofilm phenotype of the fungus. A variety of host components are also activated during aspergillosis which enhance the in vivo colonization and biofilm formation by A. fumigatus. For example, one of the serum components, fetuin A, has been recently reported to accelerate the growth of A. fumigatus and facilitate the formation of a thick biofilm [59]. To further identify such factors, elaborate transcriptome,
proteome and mutant studies on in vitro and in vivo-grown fungal biofilms in different host disease models of A. fumigatus are imperative. These comprehensive studies would lead to the identification of potential fungal targets for inhibition of biofilms and the development of novel therapeutic strategies for modification and/or impeding A. fumigatus colonization and hence the advancement to subsequent invasive diseases.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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