Review
Special Focus Issue – Antifungal Drug Discovery

Future Medicinal Chemistry

Fungal biofilm composition and opportunities in drug discovery

Biofilm infections are exceptionally recalcitrant to antimicrobial treatment or clearance by host immune responses. Within biofilms, microbes form adherent multicellular communities that are embedded in an extracellular matrix. Many prescribed antifungal drugs are not effective against biofilm infections owing to several protective factors including poor diffusion of drugs through biofilms as well as specific drug–matrix interactions. Despite the key roles that biofilms play in infections, there is little quantitative information about their composition and structural complexity because of the analytical challenge of studying these dense networks using traditional techniques. Within this review, recent work to elucidate fungal biofilm composition is discussed, with particular attention given to the challenges of annotation and quantification of matrix composition.

Courtney Reichhardt[1], David A Stevens[2,3] & Lynette Cegelski*[,1]
[1]Department of Chemistry, Stanford University, Stanford, CA 94305, USA
[2]Department of Medicine, Division of Infectious Diseases and Geographic Medicine, Stanford University, Stanford, CA 94305, USA
[3]California Institute for Medical Research, San Jose, CA 95128, USA
*Author for correspondence: cegelski@stanford.edu

First draft submitted: 19 February 2016; Accepted for publication: 27 May 2016; Published online: 3 August 2016

Keywords: *Aspergillus* • biofilm • *Candida* • efflux pumps • fungal • immunoassay • NMR • persister cells • solid-state nuclear magnetic resonance

Aim

The majority of micro-organisms, including fungi, exist in nature as multicellular communities called biofilms [1]. Traditionally, most microbes have been studied as planktonic organisms. However, research into biofilm communities and biofilm composition and physiology is expanding given the recognition of biofilms as major contributors to microbial persistence and virulence in infectious diseases. Indeed, it is estimated that up to 80% of human microbial infections are biofilm-related [2]. Biofilms can be recalcitrant to antimicrobial treatment and can avoid clearance by host immune responses [1–5]. Both *Candida albicans* and *Aspergillus fumigatus* form biofilms, and these biofilms are involved in a range of human infections, including lethal fungal infections among immunosuppressed individuals [6–9]. *C. albicans* contributes to hospital-acquired infections and fouling of medical devices including catheters [5,6,10]. Biofilms formed by the opportunistic filamentous fungus *A. fumigatus* are also implicated in aspergilloma and invasive pulmonary aspergillosis (IPA) [11,12]. Additionally, both *C. albicans* and *A. fumigatus* have been found to co-infect with *Pseudomonas aeruginosa* in lung infections of patients with cystic fibrosis [13,14]. In a study of the prevalence of fungal species in the sputum of adult patients with cystic fibrosis, *A. fumigatus* was isolated from the sputum from 45.7% of patients in the study and *C. albicans* from 75.5% of patients [13]. Several additional fungal species are known to form biofilms and contribute to human infections [15–18].

A defining feature of a biofilm is the extracellular matrix (ECM), which is a self-produced, typically noncrystalline material that encases microbial cells [19,20]. The ECM is rich in biopolymers and can contain proteins, polysaccharides, lipids, nucleic acids, and other molecules [21] that can interact with one another and the cellular surface to form a robust, protective network [3,22,23]. ECM composition varies across species and even growth conditions [19,22], yet the ECM composition of many biofilms remains

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unknown [19,21]. Functionally, the ECM can serve as a protective barrier against chemical and biological antimicrobial agents including many prescribed antifungal drugs (Figure 1A) [5,19–21]. In some instances, ECM can contribute to antifungal resistance by binding to antifungals, thereby preventing access to their intended target at the surface or within fungal cells [16,24–29]. As such, a better understanding of ECM composition is key to understanding the specific mechanisms of antifungal resistance exhibited by biofilm formers. Antifungal resistance has been studied extensively for *C. albicans*, and in some cases *Candida* biofilms have been found to be capable of withstanding antifungal concentrations that are 1000-fold higher than those that inhibit planktonic cells [5,30–32]. The presence of β-1,3-glucans in the ECM partially can explain this remarkable level of resistance as β-1,3-glucans can interact with a number of antimicrobials [33–37]. For example, radio-labeled fluconazole was found to be sequestered by ECM, which was correlated with the quantity of β-1,3-glucans in the matrix [30]. β-1,3-glucans can also interact with amphotericin B, flucytosine and echinocandins [30]. Interestingly, biofilm resistance to echinocandins is lower as compared with other antifungals, and it has been hypothesized that this may be because echinocandins impair β-1,3-glucan synthesis [30]. Indeed, mutants unable to produce the enzymes that transfer glucan from the cell to the extracellular milieu demonstrated reduced ECM glucan levels and decreased resistance to antifungal therapy [27]. Other ECM components, including extracellular DNA, also modulate drug resistance in biofilms produced by *C. albicans* and *A. fumigatus*, and in some instances, treatment of biofilms with DNase can reduce antifungal resistance [30,38–42]. *A. fumigatus* biofilms similarly exhibit decreased susceptibility to azoles, echinocandins and polyenes [43–45], including to amphotericin B. In addition, the co-administration of amphotericin B with alginate lyase, an enzyme that can degrade some exopolysaccharides within biofilms, was found to enhance the antifungal activity of amphotericin B against *A. fumigatus* biofilms, possibly by disrupting the polysaccharide component of the ECM and thus permitting drug access to hyphae [46]. These examples help to demonstrate the importance of having knowledge of ECM composition to provide insight into inhibitor modes of action and, ultimately, for developing strategies to combat the antifungal resistance exhibited by biofilms.

The ECM is often noted as a permeability barrier to antifungals that contributes to decreased drug susceptibility, although exceptions have been reported. One study utilized a disk diffusion assay to determine the *C. albicans* biofilm permeability of four antimicrobials: flucytosine, fluconazole, amphotericin B, and voriconazole [25]. Drug penetration through the biofilm failed to kill the cells, which suggested the existence of drug resistance mechanisms in addition to poor antifungal penetration for *Candida* biofilms. However, a previous study showed that the growth conditions (shaking above 30 rpm) used can inhibit ECM formation for *C. albicans*, as documented by scanning electron microscopy (SEM). Thus, further analysis is needed to probe effects on drug penetration [21,25,48]. Resistance can also be conferred to biofilm cells through the community's harboring of persister cells (Figure 1B) [24,29,49–51]. Persister cells are metabolically dormant cells that exhibit tolerance to multiple antifungals including amphotericin B, azoles, and chlorhexidine [30,49,50]. Finally, biofilm microbes also upregulate the production of efflux pumps to confer enhanced antibiotic resistance relative to microbes not associated with biofilms (Figure 1C) [28,29,52]. Increased expression of efflux pumps during biofilm growth has been observed for several *Candida spp.* and *A. fumigatus*, and resistance to azoles, drugs that disrupt ergosterol synthesis, is frequently due to increased expression of efflux pumps [30,53,54].

The formidable survival traits of biofilms combined with our dwindling pipeline of effective antifungals make it imperative that we undertake in-depth analyses to improve upon our understanding of biofilms and ECM composition and function [55–57]. Indeed, standard antimicrobial susceptibility tests are still performed to examine the efficacy of single drugs using planktonically grown cultures, yet the biofilm state is often more relevant [3,24]. Early descriptions of biofilms were often limited to 'slime', and this simplistic description masked the complexity of biofilms and slowed the design of biofilm inhibitors and interventions [20,58]. The poor solubility and lack of crystallinity of biofilms and ECM render these materials especially challenging to examine using many traditional biochemical and biophysical techniques [19,20,59,60]. Despite these challenges, recent studies have developed the necessary tools to measure and define quantitative parameters of biofilms and the ECM [3,61,62]. Here we review two approaches to annotate ECM composition with specific attention given to *C. albicans* and *A. fumigatus*, the most well-studied fungal systems and common causative agents of fungal infections in hospitalized patients. The first approach implements an arsenal of traditional biochemical methods to identify important ECM components, including identification via immunoassays [63–66]; and the second approach relies upon solid-state nuclear magnetic resonance (NMR) analysis of the intact ECM to profile the atomic-level contributions of unique classes of

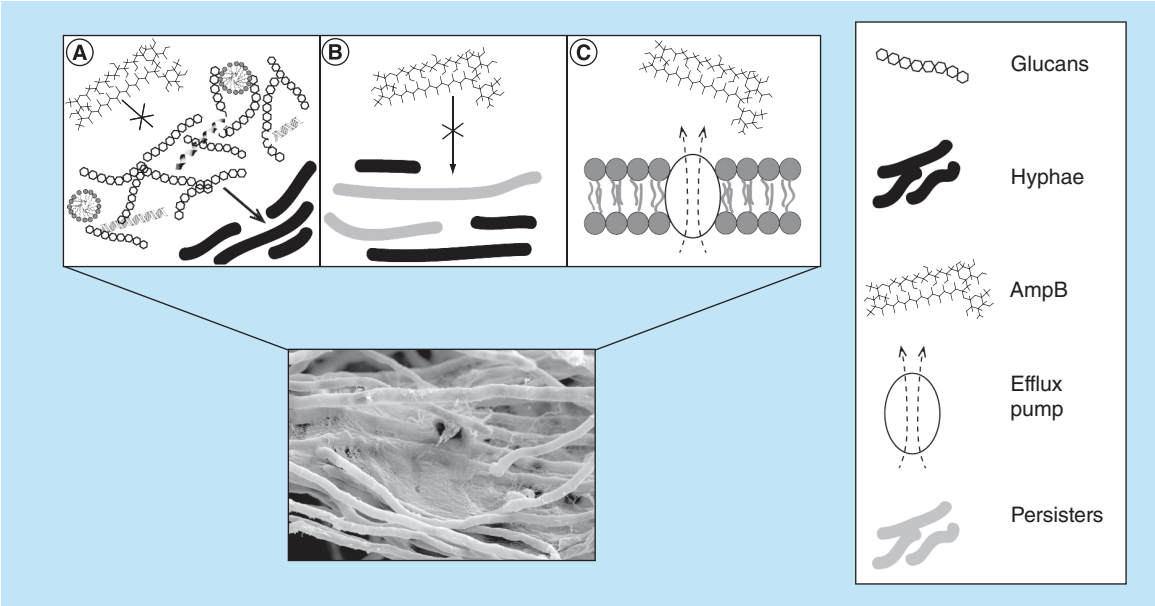

Figure 1. A scanning electron micrograph of *Aspergillus fumigatus* biofilm reveals a network of extracellular matrix that surrounds agglutinated hyphae. Biofilms are recalcitrant to antifungal treatment, and several mechanisms have been proposed for this increased tolerance. **(A)** The extracellular matrix can serve as a physical barrier that blocks antifungals from accessing the fungal cells. An example is the binding of amphotericin B by β-1,3-glucans that is associated with decreased antifungal susceptibility. **(B)** Biofilms can harbor persister cells, which are metabolically dormant cells that exhibit increased antifungal tolerance. **(C)** Biofilms often have a relative upregulation of efflux pumps compared to their planktonic counterparts. The efflux pumps can expunge antifungals from the cells.
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biomolecules to the ECM [47]. Solid-state NMR uses the nuclear spin property to obtain information about chemical composition and structure, and can be used to examine any heterogeneous and insoluble materials, including whole cells and biofilm communities, and is not restricted to the study of soluble materials as many conventional methods are. The strengths and limitations of the two methods will be compared, and at the conclusion of this review, we discuss how these approaches might be used in the context of drug discovery to provide improved opportunities to effectively treat fungal infections.

Immunoassays to identify *A. fumigatus* ECM constituents

Specific biomolecules can be identified using a range of indicator dyes, antibodies, and lectins [67]. Alternatively, the ECM constituents within each macromolecular class can be annotated through systematic biochemical analysis without prerequisite knowledge of putative ECM composition [65]. The first approach, using indicator dyes and antibody- and lectin-conjugated dyes, has been useful in fluorescence microscopy studies to identify and spatially resolve biofilm constituents [23,32,68–70], and recent advances in super-resolution microscopy have permitted the tracking of single proteins and polysaccharides during biofilm growth [71]. These studies have taught us that many ECM constituents appear to play complementary architectural roles and that localization of components within a biofilm can vary over time. It is important to note, however, that an inherent limitation of these studies is that they require prior knowledge of the biofilm molecules of interest.

Immunoassays, which use antibodies that recognize specific molecules in order to identify or track the presence of that molecule, were useful to validate the presence of specific ECM polysaccharides and proteins in *A. fumigatus* biofilm, including biofilms formed *in vitro* and *in vivo* [63,64]. Early work by Beauvais *et al.* revealed that the colony surface of *A. fumigatus* grown under aerial static conditions contained a hydrophobic ECM that bound hyphae in a manner that closely resembled *in vivo* 'fungal balls' [63]. Additionally, as predicted for biofilm, cultures grown under these conditions exhibited enhanced resistance to polyene antifungals relative to those grown under non-ECM-producing conditions (shaken, submerged). Interestingly, gentle mechanical removal of the ECM did not modify resistance to nystatin, which suggested that the *A. fumigatus* ECM may only reduce mycelial accessibility of some polyene antifungals, and in some cases additional biofilm-specific mechanisms may be responsible for antifungal resistance. Immunoassays and subsequent gas chroma-

tography identified galactomannan and α-1,3-glucans as *A. fumigatus* ECM components.

Glucose was identified as a major ECM component of *in vitro A. fumigatus* biofilms by monosaccharide analysis of digested ECM. Although the authors controlled for the possibility of contaminating glucose from the growth medium by culturing in glucose-free medium, it is important to recognize the general limitations of conditions used for monosaccharide analysis in many applications. To emphasize this, we highlight an example from work with the opportunistic human pathogen *Pseudomonas aeruginosa*. Until recently, Pel, which is an important extracellular polysaccharide of *P. aeruginosa*, was mistakenly believed to be a glucose-rich molecule. This misidentification was due in part to the recalcitrance of Pel to isolation and standard preparatory hydrolysis conditions for monosaccharide compositional analysis [23]. Using harsher hydrolysis conditions for monosaccharide compositional analysis, Pel was determined to be a polymer of N-acetylglucosamine and N-acetylgalactosamine [23]. This assignment was supported by the reactivity of Pel to antibodies raised against poly-β-1,6-N-acetylglucosamine and chitosan (poly-β-1,4-N-acetylglucosamine) as well as binding to N-acetylgalactosamine-specific lectins.

Protein quantification assays also have limitations in their application to biofilm and ECM samples. The overall protein concentration of *in vitro A. fumigatus* ECM was found to be 2% (w/w) by the bicinchoninic acid (BCA) assay. However, this assay can incorrectly estimate protein concentration, as the complex molecular environment of the ECM can contain biomolecules such as glucose and lipids that can cause interfering absorbance in the presence of the BCA reagent or may limit accessibility of the protein peptide bonds to the BCA reagent (specifically Cu^{2+} ions) [72–74]. The major antigens, DppV, catalase B and Asp f1, were identified by immunoblot, and the hydrophobic nature of the colony suggested the presence of hydrophobic proteins [75]. However, the contributions and identities of other potential proteins were not determined.

Despite the technical limitations of the above methods and inability to generate a complete accounting of the *A. fumigatus* ECM, the assays allowed an informative comparison between *A. fumigatus* ECM samples formed during host invasion, which was observed to depend upon whether the aspergillosis was localized (aspergilloma) or invasive (IPA) [64]. ECM was present in both pathological settings. However, hyphae in the IPA were separated and surrounded by a thinner layer of ECM relative to that of an aspergilloma. Aspergilloma appeared as a ball of strongly agglutinated hyphae, which was devoid of host cells and surrounded by a dense ECM network. Whole-biofilm immunoassays revealed that galactosaminogalactan (GAG) and galactomannan were major polysaccharides observed in the ECM, and α-1,3-glucan was only detected in aspergilloma at the periphery of the ECM. *In vivo* labeling with anti-GAG antibody was more intense as compared with *in vitro* labeling, suggesting that GAG was a major component of ECM produced during the development of *A. fumigatus* in tissues of patients with either aspergilloma or invasive aspergillosis. Later, it was observed that GAG mediates adherence to host cells, serves to control the host immune response by shielding β-glucans, and is a virulence factor that is required for biofilm formation [76–78]. The prevalence of GAG was later supported by studies using atomic force microscopy showing that GAG was highly exposed and able to serve as a fungal adhesin [79]. The biosynthesis of GAG has also been under study with efforts to understand the mechanism of deacetylation of residues in GAG, for example [80,81]. Immunocytochemical assays performed with antibodies raised against the three major secreted antigens mentioned above (DppV, catalase B, and Asp f1), showed that these antigens were not embedded in the *in vivo* ECM [64]. Thus, the immunoassays of putative polysaccharide and protein components permitted comparative analysis of the ECM from *in vitro* and *in vivo A. fumigatus* biofilms and demonstrated that each system included many similar components such as GAG, galactomannan, α-1,3-glucans, and melanin. In addition, some components, such as the antigenic proteins, were enriched in the *in vitro* ECM but not detected in the *in vivo* ECM [63,64]. These studies highlight the strengths of using immunoassays to detect particular predicted ECM components. However, this approach is limited to studying only predicted components as well as those for which specific antibodies are available. Furthermore, using this approach, it is not possible to quantify the contributions of each component in the total ECM.

Macromolecular screening for *C. albicans* ECM composition

Many of the biochemical methods available to identify ECM components were collectively implemented in a recent single study of *C. albicans* biofilm by Zarnowski *et al.* (Figure 2) [65]. In this study, the researchers first profiled the contributions to the crude ECM of each of the macromolecular classes: protein, polysaccharide, lipid, and nucleic acid. This initial profiling was performed using a combination of spectrophotometric and colorimetric assays. Specifically, the protein enrichment was determined to be 55% (w/w) using a BCA assay; the carbohydrate content was determined to be 25% (w/w) using the phenol-

sulfuric acid method; the nucleic acid concentration was determined to be 5% (w/w) using the absorbance of the crude ECM at 260 nm; and, finally, the remaining 15% (w/w) was assigned to lipid. The crude ECM was then processed in order to further characterize specific *C. albicans* ECM constituents using additional biochemical techniques. Specific proteins were identified using proteomic mass spectrometry following enzymatic digestion of crude matrix material. Most of the 565 identified proteins play a role in metabolism or in the production and modification of polysaccharides. Interestingly, when the proteomic analysis was repeated using *in vivo* biofilms formed in a rat catheter model, the majority of identified ECM proteins were mammalian, suggesting that host factors may greatly impact biofilm composition, a topic that warrants further study. Sequence analysis found that much of the nucleic acid was noncoding. Lipids were profiled using gas chromatography, and nearly all of the lipids were found to be glycerolipids.

Further analysis of the carbohydrate contributions to the ECM primarily relied upon monosaccharide analysis and solution NMR following purification and fractionation steps. Initially, size-exclusion chromatography identified both high-molecular weight and low-molecular-weight fractions that constituted 38.3 and 61.7% of the total carbohydrate, respectively. Monosaccharide analysis concluded that the high-molecular weight fraction was primarily mannose, while the low-molecular-weight fraction was mostly arabinose. 1D and 2D solution NMR analysis of the abundant neutral carbohydrate fractions supported the presence of both mannan and glucan residues, and the most-abundant mannan polysaccharide was investigated using small-angle x-ray scattering. While 2D correlative NMR methods were not able to identify a linkage between the mannan and glucan residues, a novel mannan–glucan complex was predicted based upon the constant ratio of each in chromatography fractions as well as colocalization of anti-mannan and anti-glucan antibodies observed in confocal imaging of *in vitro* biofilms. This colocalization also was observed in biofilms formed *in vivo*. As previously mentioned, β-1,3-glucan can bind to antifungals. However, only small amounts of matrix β-1,3-glucan were observed via immuno-TEM, suggesting that additional ECM components are likely able to sequester antifungals. This concept was explored using a solution-state ^{1}H NMR binding assay, in which line-broadening of matrix peaks suggested an interaction of the matrix with fluconazole. It was also determined that multiple ECM components were necessary for drug binding, which was further supported in a later study [82].

This meticulous study provided new information about the relative abundance of the different macromolecules in *C. albicans* ECM that could be digested and accessed, and used this information to predict the presence of a novel exopolysaccharide complex [65]. However, many biofilms are resistant to the solubilization that is required for such solution-based analyses, and harsh degradative conditions are often used to attempt to overcome this challenge and generate some material for analysis [83,84]. Furthermore, in addition to the previously stated limitations of the BCA assay to determine ECM protein concentration, the phenol-sulfuric acid method can incorrectly estimate the enrichment of carbohydrate. For the phenol–sulfuric acid method to be quantitative, the standard samples must contain representative portions of the types of monosaccharides found in the experimental sample, which is difficult to predict for complex carbohydrate mixtures such as ECM [85]. As a consequence of the limitations of these solution-based assays, estimates of contributions of proteins, polysaccharides, and other biomolecules can be dramatically misrepresented depending on the efficiency of the solubilization and the extent of material loss during sample processing, and extreme care should be taken when interpreting results from such analyses. These limitations, coupled with the importance of defining biofilm matrix composition as well as comparing biofilms and the influence of potential inhibitors, have encouraged the development of new approaches to help transform more qualitative biofilm descriptors into quantitative parameters of molecular composition [58,59].

Solid-state NMR to quantify *A. fumigatus* ECM composition

We recently developed an approach that utilizes solid-state NMR to quantitatively characterize and define biofilm and ECM composition [61,86]. Solid-state NMR, in general, permits analysis of the entire, intact ECM without preparatory chemical or enzymatic processing. There is no intrinsic size or mass limit as in solution NMR, which requires high-molecular tumbling rates to achieve high-resolution NMR spectra [61]. More specifically, and as reviewed in more detail in the direct context of biofilm and ECM characterization, solid-state NMR employs magic-angle spinning to mechanically spin samples of multicellular biofilm communities or isolated ECM to help achieve the necessary resolution to obtain quantitative spectra of these heterogeneous and insoluble systems [61,62]. Solid-state NMR has been applied to study other similarly complex and insoluble systems such as bacterial whole cells and cell walls [87–91] and intact plant leaves [92]. In 2013, we reported the first quantitative determination of the chemical composition of intact ECM of a microbial biofilm by using solid-

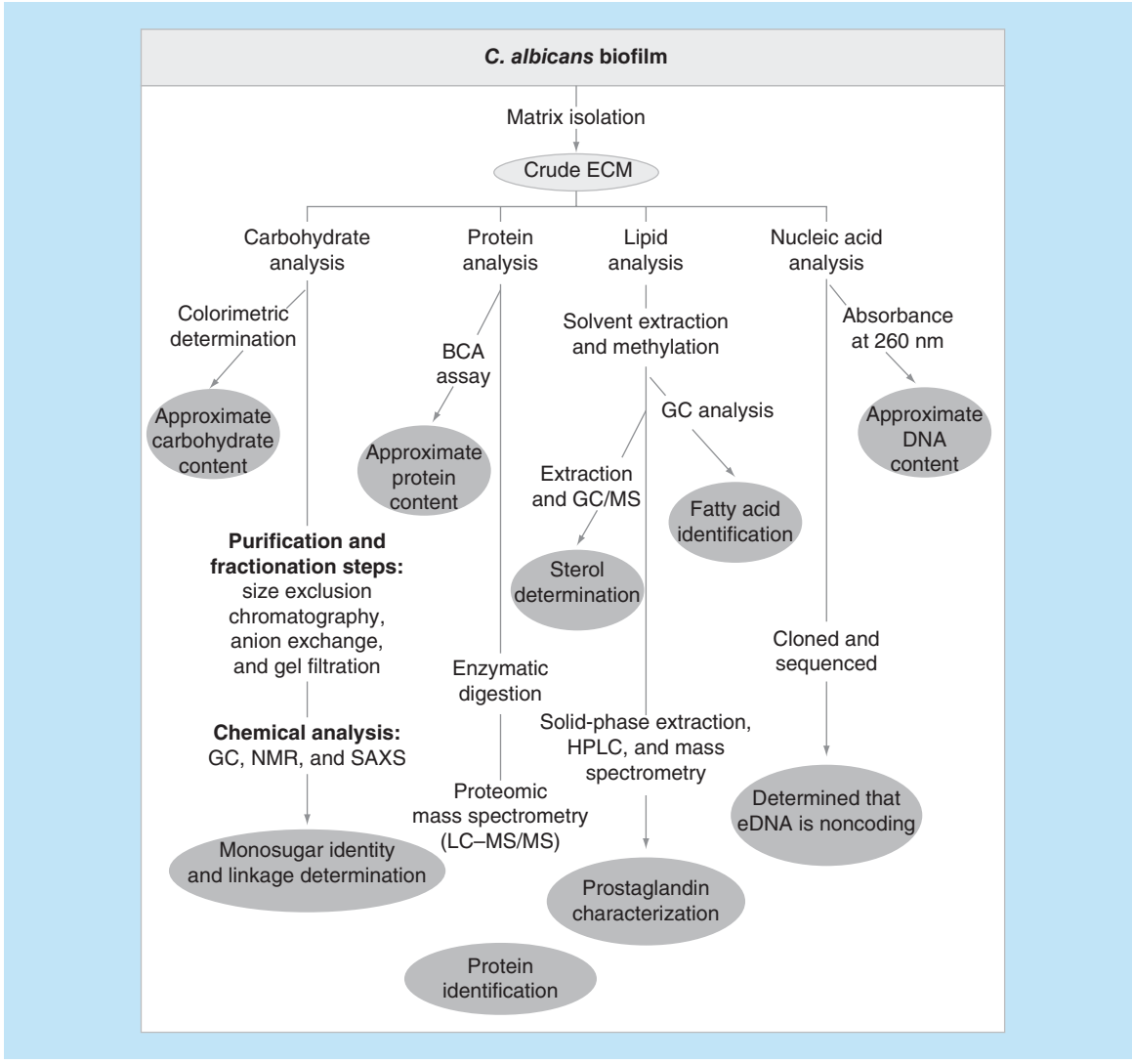

Figure 2. Schematic representation of a protocol employed to characterize the composition of *Candida albicans* biofilm. This methodology relies upon extensive processing of the biofilm and extracted matrix. Differences in solubilization efficiency of ECM components can result in compositional estimates that may vary widely from prep-to-prep [65]. An advantage of this approach, which uses an integrated effort, is the identification of specific matrix parts in the ECM, although there are caveats with quantification of these parts.
BCA: Bicinchoninic acid; ECM: Extracellular matrix; GC: Gas chromatography; HPLC: High-performance liquid-chromatography; LC–MS/MS: Liquid chromatography–mass spectrometry; NMR: Nuclear magnetic resonance; SAXS: Small-angle x-ray scattering.

state NMR, biochemical analysis, and electron microscopy [86]. These initial experiments were performed on an important human pathogenic bacterium, the uropathogenic *E. coli* (UPEC) strain UTI89. We discovered that the spectral additional of just two components, the functional amyloid protein curli and a modified form of cellulose, completely recapitulated the ^{13}C NMR spectrum of intact UTI89 ECM. Thus, we determined that UTI89 ECM is 85% curli and 15% modified cellulose by mass. However, this bottom-up approach used for analysis of UTI89 required separate samples of putative ECM components, which are not readily known or available for many biofilm systems [61].

To overcome this limitation, we developed an alternative top-down solid-state NMR approach that permits spectroscopic dissection and annotation of complex material and can be applied to biofilms or other multicomponent systems for which there is less known (or even nothing known) about potential constituents [61]. We first developed this method to examine *Vibrio cholerae* ECM composition, and as discussed below, subsequently implemented the top-down approach in *Aspergillus fumigatus* [61,83]. This approach uses two types of 1D solid-state NMR experiments: cross-polarization magic-angle spinning (CPMAS) [93,94] and rotational-echo double-resonance

(REDOR) [95]. The ^{13}C CPMAS spectrum contains information about the quantities of carbon types, including carbonyls, aromatic carbons, polysaccharide carbons (anomeric and nonanomerics) and aliphatics, present in the ECM. By ensuring full incorporation of ^{15}N into the matrix via defined growth medium, it is possible to further annotate the carbon pools according to C–N and C–P couplings using both $^{13}C\{^{15}N\}$ and $^{13}C\{^{31}P\}$REDOR NMR experiments [61,83].

We applied this top-down solid-state NMR methodology to quantify the ECM composition of the fungal *A. fumigatus* biofilm grown in RPMI 1640 nutrient medium (selected because of its optimum for growing mammalian cells) [47]. The ^{13}C CPMAS spectrum of *A. fumigatus* ECM showed contributions from a range of biomolecules (Figure 3A). To use CPMAS to quantify the number of nuclei at corresponding chemical shifts relative to others in a spectrum, differences in cross-polarization (CP) efficiency and relaxation must be taken into account. Such differences can arise due to variations in local dynamics and in the spatial distributions of ^{1}H nuclei in the sample that are coupled to the carbons. By way of experimental detail, we monitored the *A. fumigatus* ECM ^{13}C CPMAS intensities as a function of CP contact time, and observed a typical 'exponential rise–exponential decay' behavior that was extrapolated to zero contact time to quantify relative spin numbers. Together the ^{13}C CPMAS peaks due to polysaccharide anomeric and ring-sugar carbons, which are chemical shift resolved and thus were able to be uniquely assigned, accounted for approximately 43% of the total carbon mass.

To annotate the remaining 57% of the carbon mass, we capitalized on the unique C–N and C–P couplings that are present in different biomolecules (Figure 3B & C). For example, the ^{13}C spectrum of the ECM contained a peak near 173 ppm that was attributed to carbonyl carbons. Several types of biomolecules contain carbonyls, but all peptide carbonyl carbons are directly bonded to nitrogen (Figure 3C). Thus, by using $^{13}C\{^{15}N\}$REDOR as a spectroscopic filter to select only carbonyls that are directly bonded to nitrogen, we could determine an upper limit of the amount of protein carbonyls. Through determining the quantitative CPMAS peak intensities of different carbon types as described above, we discovered that the carbonyl peak accounted for 12% of the total ^{13}C spectrum of *A. fumigatus* ECM. Using $^{13}C\{^{15}N\}$REDOR, we found that only 76% of the carbonyl carbons were directly bonded to nitrogen. Thus, at most, only 9% (76 of 12%) of total ECM carbon could be attributed to peptide carbonyls of proteins. The percentage of possible α-carbons was similarly determined to be 7% (including α-carbons of both glycine and other amino acids). Amino acids contain an average of 5.4 carbons each, and so three additional carbons can be generally attributed to protein. These carbons mostly would be observed in the upfield aliphatic region of the ^{13}C CPMAS spectrum. Together, this analysis supports that approximately 40% of the total carbon mass can be attributed to protein. The aromatic region of the ^{13}C CPMAS spectrum accounts for an additional 3% of the total carbon mass, which was partially attributed to melanin.

The remaining 14% of the ^{13}C CPMAS spectral area contains contributions from carbonyls and aliphatics (excluding directly nitrogen-bonded carbons), including a sharp peak at 33 ppm that is characteristic of CH_2 groups typically found in lipids and accounts for 7% of the total spectral area. Spectral selection of portions of these regions by $^{13}C\{^{31}P\}$REDOR also suggested that the ECM contained some phospholipid. Thus, *A. fumigatus* ECM contained at least 7% lipid (due to the characteristic peak at 33 ppm) and up to 14% lipid by carbon mass. Taken together, the *A. fumigatus* ECM produced under these growth conditions was approximately 40% protein, 43% polysaccharide, 3% aromatic-containing components and up to 14% lipid.

Thus, atomic-level parameters of the intact isolated *A. fumigatus* ECM were measured and defined using the top-down solid-state NMR method. In addition, other valuable compositional parameters of the ECM constituents were obtained. The ^{15}N CPMAS spectrum showed that most of the ECM nitrogen is present in protein (appearing as an amide peak at 119 ppm). We were also able to detect low abundance nitrogen-containing motifs likely present as modifications to ECM constituents, for example, as amino or N-acetyl modifications. The $^{13}C\{^{15}N\}$REDOR results suggested that some of the exopolysaccharides were N-acetylated based upon the presence of peaks attributed to N-acetyl methyl groups and the observed proximity of the nitrogen to some of the sugar-ring carbons. This finding is consistent with some of the previously identified extracellular polysaccharides produced by *A. fumigatus* including GAG. N-acetyl groups also could occur as part of the N-acetylglucosamine groups present in N-linked glycosylation of ECM proteins. The possibility that some of the extracellular proteins were glycosylated was explored using traditional biochemical analysis. SDS-PAGE analysis of the ECM showed several bands corresponding to SDS-soluble proteins, and these bands were excised and subjected to proteomic mass spectrometry. The SDS-soluble proteins were identified as catalase B and Asp f2. Consistent with the NMR spectral detection of N-acetyl groups, these two proteins are reported to be N-linked glycosylated. Digestion with peptide-N-glycosidase F (PNGase) resulted in cleavage consistent with glycosylation of Asp f2 in this ECM sample.

Importantly, the overall solid-state NMR approach

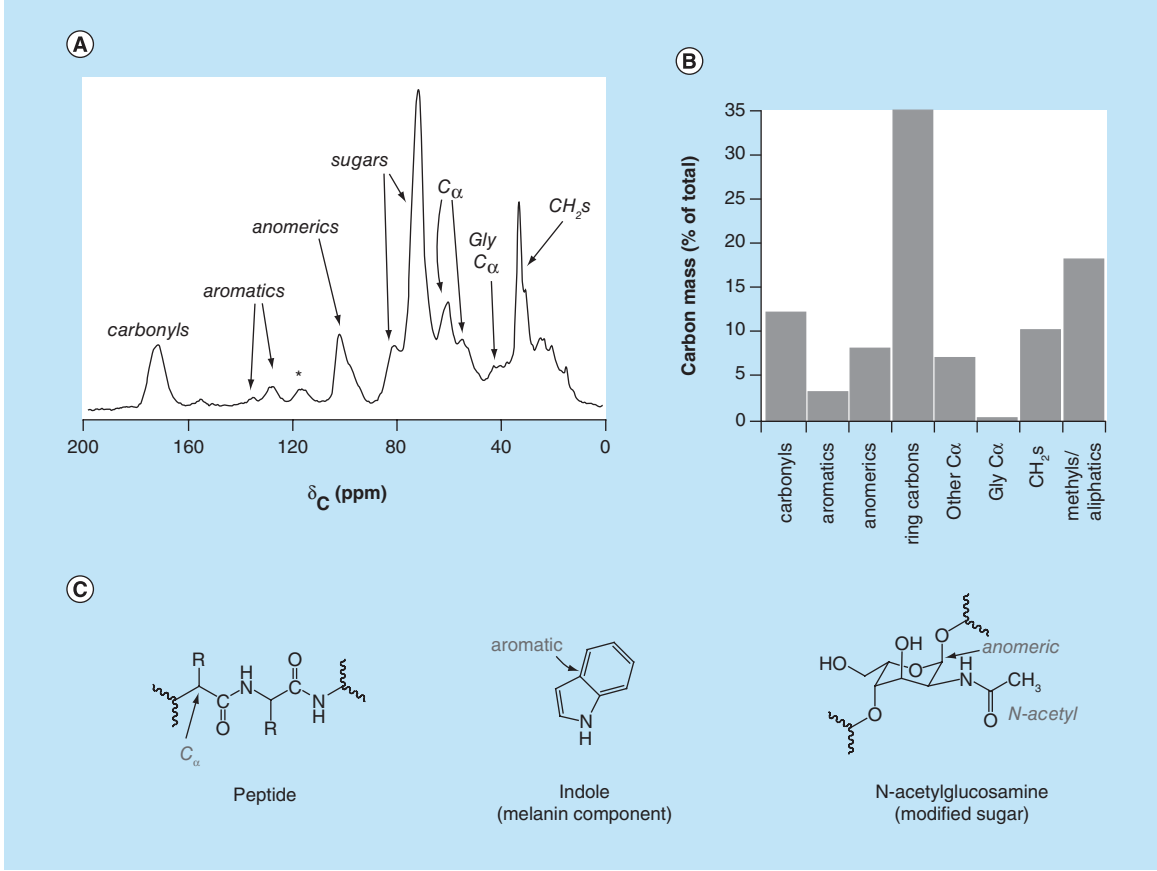

Figure 3. *Aspergillus fumigatus* extracellular matrix composition determined using solid-state nuclear magnetic resonance. (A) The ^{13}C cross-polarization magic-angle spinning spectrum of *A. fumigatus* extracellular matrix showed carbon contributions from a range of biomolecules including proteins, carbohydrates and lipids. **(B)** The contributions of specific carbon pools to the total carbon mass were annotated using cross-polarization magic-angle spinning and spectral editing via $^{13}C\{^{15}N\}$ and $^{13}C\{^{31}P\}$REDOR nuclear magnetic resonance experiments, and these contributions are summarized in the graph. **(C)** Chemical structures of representative biomolecules that were identified in the *A. fumigatus* extracellular matrix.
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provides absolute carbon intensities that can be compared across samples, whether due to different biofilm formers or the influence of external stimuli such as antibiofilm agents. We developed an approach to finely annotate the specific carbon types and to obtain a quantitative accounting of these pools in the biofilm ECM. Even quick inspection of the comparative 1D CPMAS spectra would permit facile identification of whether any significant differences were present among a sample set. Coupled with electron microscopy of intact biofilms and the isolated ECM material as well as biochemical analyses for the specific identification of some biofilm parts, we believe that this integrated approach with solid-state NMR analyses provides one of the most robust and powerful ECM characterization approaches available.

Conclusion

Connections between ECM composition and function are crucial to understanding the fundamental molecular basis for: fungal biofilm physiology, the recalcitrance of biofilm infections to antifungal treatment, and antifungal resistance. Robust and reliable methods to define and/or quantitatively compare ECM composition are crucial for driving these connections. The two major approaches to annotate ECM composition described in this review provide complementary details of ECM composition and connections with function. Solid-state NMR is uniquely suited to profile chemical composition of complex, insoluble systems like ECM [62]. The top-down solid-state NMR method described above can be applied to the study of any biofilm, including mixed species or *in vivo*-derived biofilms, and, importantly, does not require any prerequisite knowledge of biofilm composition [47,61–62,83]. The approach first involves the annotation of the fundamental carbon and nitrogen compositional pools, for example, quantifying the prevalence of carbonyls and methyls, etc. It also takes advantage of poten-

tially unique internuclear couplings to further dissect ECM composition and to specify the types of carbonyls present, for example. The analysis is performed on the intact ECM and provides parameters not accessible from solution-based biochemical methods that require solubilization of ECM parts, wherein much of the ECM is often not analyzed due to its recalcitrance to digestion and dissolution. Furthermore, solid-state NMR analysis permits one to simultaneously observe and quantify multiple macromolecular species and a complete analysis can be performed on the same sample. The advantages of the solid-state NMR approach thus include the nondestructive nature of the analysis and the ability to quantify the types of nuclei present in a sample. A disadvantage, is the limited sensitivity of NMR and the relatively large sample sizes and/or long spectral acquisition times required to achieve high signal-to-noise spectra using natural abundance ^{13}C samples. Isotopically labeled samples improve sensitivity, although it is attractive that ^{13}C profiling, for example, does not require labeling and can be performed using ^{13}C at natural abundance. NMR correlation experiments provide additional selectivity to specify the specific nature of carbon pools that are annotated through 1D spectra. These experiments require isotopic enrichment, for example, as we demonstrated with ^{15}N-incorporation in a customized RPMI 1640 medium for the analysis of *A. fumigatus* ECM composition described above [47]. Thus, it is important to choose the right tool for the experiment, taking into account the problem-solving advantages and the technical limitations that can include sample solubility, processing requirements, and sample size.

Future perspective

Ideally, the traditional biochemical methods applied to solubilized parts of the ECM should be integrated with quantitative compositional profiling by solid-state NMR. The NMR analysis provides a total snapshot of molecular contributions to the intact ECM and the prevalence of molecules in the ECM, such as the relative abundance of proteins versus polysaccharides, including an analysis of types of chemical modifications present in the samples. Yet, while each protein has a unique NMR signature, identification of individual proteins in the ECM is best performed using proteomic mass spectrometry and immunoassays. Similarly, the identities of lipids and small, soluble polysaccharides can be determined using solution-based biochemical methods on the individual purified components in isolation from one another. In addition to the study of complex, biomolecular mixtures, solid-state NMR can be applied to study in detail the isolated ECM components such as the high-molecular-weight polysaccharide fractions identified in the previously described *C. albicans* study [65], and does not require harsh, degradative conditions prior to analysis. In this way, solid-state NMR has been used to quantitatively determine structural information of similarly noncrystalline and insoluble bacterial [88,90,91,96] and plant cell walls [97–100].

Solid-state NMR also is well suited for the study of drug–matrix interactions, with inspiration from studies that mapped atomic-level interactions of the antibiotic [^{19}F]oritavancin with *S. aureus* cell walls (both in isolated cell walls and in the whole-cell context) by measuring several ^{19}F-^{13}C and ^{19}F-^{15}N distances between drug and specific cell-wall sites [87]. In the realm of antifungals, solid-state NMR approaches have been used to examine the possible mechanism(s) of action of amphotericin B in lipid vesicle systems used as surrogates for fungal membranes. Most structural studies have characterized the pore-forming assemblies of amphotericin [101], yet very recent solid-state NMR work examined samples prepared with phospholipids, ergosterol and amphotericin, and showed that amphotericin B could extract ergosterol out of phospholipids, and serve as a type of ergosterol sponge [102]. These types of structure-focused NMR approaches are ripe for identifying specific ECM components that bind to antifungals and for mapping the interactions between the ECM and ^{19}F-labeled drugs, for example. Together, solid-state NMR methods enable the quantification of the abundance of biomolecules in a biofilm and can be used to determine the ways in which the components could be interacting with antifungals to contribute to the antifungal resistance often exhibited by biofilms.

The ECM is able to inhibit the access of some antifungals to fungal cells in a biofilm and contributes to the challenge of treating biofilm-associated infections. The potential mechanisms of ECM-mediated antifungal recalcitrance include both matrix–matrix interactions and matrix–drug interactions. For example, the matrix could be self-associating to form an impenetrable barrier to drugs. Alternatively, the matrix could either bind to drugs or enzymatically inactivate the drugs, both of which prevent antifungals from reaching their intended target at the surface of or within the fungal cell. The ECM composition plays a key role in dictating the possible mechanisms that biofilms employ to decrease susceptibility to antifungals. In the past, knowledge of ECM composition has given rise to more effective strategies to treat biofilm-involved fungal infections such as the coadministration of amphotericin B with alginate lyase to degrade exopolysaccharides and enhance the antifungal activity of amphotericin B against *A. fumigatus* biofilms [46]. As knowledge of

Executive summary

Background

- Biofilm infections are recalcitrant to antimicrobial treatment and clearance by host immune responses.
- Many prescribed antifungal drugs are not effective against biofilm infections owing to several protective factors including poor diffusion of drugs through biofilms as well as specific drug–matrix interactions.
- Both *Candida albicans* and *Aspergillus fumigatus* form biofilms, and these biofilms are involved in a range of human infections, including lethal fungal infections among immunosuppressed individuals.
- A defining feature of biofilms is the extracellular matrix (ECM), which is a self-produced, noncrystalline material that encases microbial cells. ECM is typically rich in biopolymers and can contain proteins, polysaccharides, lipids, nucleic acids, and other molecules.
- The formidable survival traits of biofilms combined with our dwindling pipeline of effective antifungals make it imperative that we undertake in-depth analyses to improve upon our understanding of biofilms.
- The two approaches, traditional biochemical methods and a newly developed solid-state NMR method, can be used to annotate ECM composition and provide complementary perspectives on ECM composition and function.

Immunoassays to identify *A. fumigatus* ECM constituents

- Immunoassays were useful to validate the presence of specific ECM polysaccharides and proteins in *A. fumigatus* biofilm, including both *in vitro* and *in vivo* biofilms.
- Immunoassays and subsequent gas chromatography identified galactomannan and α-1,3-glucans as *A. fumigatus* ECM components.
- The major antigens, DppV, catalase B and Asp f1, were identified by immunblot in the *in vitro* ECM but were not detected in the *in vivo* ECM.

Macromolecular screening for *C. albicans* ECM composition

- The ECM of *Candida albicans* was examined and the contributions of each of the macromolecular classes were profiled.
- This initial profiling was performed using a combination of spectrophotometric and colorimetric assays, and the ECM was determined to be 55% (w/w) protein, 25% (w/w) carbohydrate, 5% (w/w) nucleic acid, and 15% (w/w) lipid.
- Carbohydrate characterization primarily relied upon monosaccharide analysis and solution nuclear magnetic resonance (NMR) following purification and fractionation steps, and both high-molecular-weight and low-molecular-weight fractions were identified.
- Many biofilms are resistant to the solubilization that is required for such solution-based analyses, and harsh degradative conditions are often used to overcome this challenge which can lead to misrepresentations of ECM composition.

Solid-state NMR to quantify *A. fumigatus* ECM composition

- A solid-state NMR approach was developed that permits analysis of the entire, intact ECM without preparatory chemical or enzymatic processing.
- Solid-state NMR does not require high tumbling rates in solution (solution NMR) or homogeneous samples, and provides quantitative information about composition.
- This solid-state NMR approach uses two types of 1D NMR experiments: cross-polarization magic-angle spinning (CPMAS) and rotational-echo double-resonance (REDOR).
- Spectral dissection using solid-state NMR determined that the *A. fumigatus* ECM was approximately 40% protein, 43% polysaccharide, 3% aromatic-containing components, and up to 14% lipid.
- In addition to the atomic-level parameters of *A. fumigatus* ECM gained using this top-down solid-state NMR method, general characteristics of the ECM constituents were obtained including glycosylation of extracellular proteins and types of modifications of exopolysaccharides.

Opportunities in drug discovery

- Measurements of ECM composition are crucial to understanding fungal biofilm physiology, the recalcitrance of biofilm infections to antifungal treatment, and antifungal resistance.
- Integrated approaches employing electron microscopy, biochemical methods including proteomics analyses, together with solid-state NMR compositional measurements would allow for comprehensive characterization and elucidation of the generation of quantitative parameters of ECM composition, enabling comparisons with samples from organisms treated with antifungal and antibiofilm agents.
- Solid-state NMR approaches can be used to map the ECM binding sites and bound conformations of candidate therapeutics in isolated ECM and in intact cells.

biofilm composition, including composition of mixed species and *in vivo* biofilms increases, we believe that effective strategies to treat biofilm-involved fungal infections will also improve. Knowledge of the ECM composition provides evidence of which mechanisms could be in play for a particular biofilm, and furthermore, provides routes to overcome the matrix-mediated antifungal resistance. These routes may involve combined antifungal and anti-virulence approaches. For example, the biophysical and biochemical ways in which the ECM is able to act as a drug barrier could be taken into account when designing an antifungal drug so that the drug meets size, charge, and reactivity requirements to be able to pass through the biofilm and impact the fungal cells. Complementary anti-virulence approaches could block matrix–matrix or matrix–drug interactions, either through co-administration of additional drugs or through mechanical disruption. Specific knowledge of those matrix–matrix and matrix–drug interactions is crucial to the design of such therapeutics. Thus, compositional and molecular-level descriptions of the ECM should help to drive the development of strategies to eradicate biofilm-associated infections and develop more effective antifungal treatments.

Financial & competing interests disclosure

We acknowledge support from the National Science Foundation CAREER Award, NSF Grant Number 1453247. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

References

Papers of special note have been highlighted as:
• of interest; •• of considerable interest.

1 Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. *Nat. Rev. Microbiol.* 2(2), 95–108 (2004).

2 Römling U, Balsalobre C. Biofilm infections, their resilience to therapy and innovative treatment strategies. *J. Intern. Med.* 272(6), 541–561 (2012).

3 Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science* 284, 1318–1322 (1999).

4 Bjarnsholt T, Alhede M, Alhede M *et al.* The *in vivo* biofilm. *Trends Microbiol.* 21(9), 466–474 (2013).

5 Mathe L, Van Dijck P. Recent insights into *Candida albicans* biofilm resistance mechanisms. *Curr. Genetics* 59(4), 251–264 (2013).

6 Douglas LJ. *Candida* biofilms and their role in infection. *Trends Microbiol.* 11(1), 30–36 (2003).

7 Mayer FL, Wilson D, Hube B. *Candida albicans* pathogenicity mechanisms. *Virulence* 4(2), 119–128 (2013).

8 Mowat E, Williams C, Jones B, Mcchlery S, Ramage G. The characteristics of *Aspergillus fumigatus* mycetoma development: is this a biofilm? *Med. Mycol.* 47(Suppl. 1), S120–S126 (2009).

9 Fanning S, Mitchell AP. Fungal biofilms. *PLoS Pathogens*. 8(4), e1002585 (2012).

10 Kuhn DM, Gannoum MA. *Candida* biofilms: antifungal resistance and emerging therapeutic options. *Curt. Opin. Investig. Drugs* 5(2), 186–197 (2004).

11 Balajee SA, Houbraken J, Verweij PE *et al. Aspergillus* species identification in the clinical setting. *Stud. Mycol.* 59, 39–46 (2007).

12 Kaur S, Singh S. Biofilm formation by *Aspergillus fumigatus*. *Med. Mycol.* 52(1), 2–9 (2014).

13 Bakare N, Rickerts V, Bargon J, Just-Nubling G. Prevalence of *Aspergillus fumigatus* and other fungal species in the sputum of adult patients with cystic fibrosis. *Mycoses* 46, 19–23 (2003).

14 Manavathu EK, Vager DL, Vazquez JA. Development and antimicrobial susceptibility studies of *in vitro* monomicrobial and polymicrobial biofilm models with *Aspergillus fumigatus* and *Pseudomonas aeruginosa*. *BMC Microbiol.* 14, 53–68 (2014).

15 Beauvais A, Loussert C, Prevost MC, Verstrepen K, Latge JP. Characterization of a biofilm-like extracellular matrix in FLO1-expressing *Saccharomyces cerevisiae* cells. *FEMS Yeast Res.* 9(3), 411–419 (2009).

16 Al-Fattani MA, Douglas LJ. Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. *J. Med. Microbiol.* 55(Pt 8), 999–1008 (2006).

17 Kumamoto CA. *Candida* biofilms. *Curr. Opin. Microbiol.* 5, 608–611 (2002).

18 Silva S, Henriques M, Martins A, Oliveira R, Williams D, Azeredo J. Biofilms of non-*Candida albicans Candida* species: quantification, structure and matrix composition. *Med. Mycol.* 47(7), 681–689 (2009).

19 Flemming HC, Wingender J. The biofilm matrix. *Nat. Rev. Microbiol.* 8(9), 623–633 (2010).

20 Flemming HC, Neu TR, Wozniak DJ. The EPS matrix: the "house of biofilm cells". *J. Bacteriol.* 189(22), 7945–7947 (2007).

21 Blankenship JR, Mitchell AP. How to build a biofilm: a fungal perspective. *Curr. Opin. Microbiol.* 9(6), 588–594 (2006).

• **Review of fungal biofilm composition.**

22 Colvin KM, Gordon VD, Murakami K *et al.* The Pel polysaccharide can serve a structural and protective role in the biofilm matrix of *Pseudomonas aeruginosa*. *PLoS Pathog.* 7(1), e1001264 (2011).

23 Jennings LK, Storek KM, Ledvina HE *et al.* Pel is a cationic exopolysaccharide that cross-links extracellular DNA in the

Pseudomonas aeruginosa biofilm matrix. *Proc. Natl Acad. Sci. USA* 112(36), 11353–11358 (2015).

24 Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* 15(2), 167–193 (2002).

25 Al-Fattani MA, Douglas LJ. Penetration of *Candida* biofilms by antifungal agents. *Antimicrob. Agents Chemother.* 48(9), 3291–3297 (2004).

26 Lewis K. Riddle of biofilm resistance. *Antimicrob. Agents Chemother.* 45(4), 999–1007 (2001).

• **Review of antimicrobial resistance associated with biofilms.**

27 Taff HT, Nett JE, Zarnowski R *et al.* A *Candida* biofilm-induced pathway for matrix glucan delivery: implications for drug resistance. *PLoS Pathog.* 8(8), e1002848 (2012).

28 Ramage G, Rajendran R, Sherry L, Williams C. Fungal biofilm resistance. *Int. J. Microbiol.* 2012, e528521 (2012).

29 Mukherjee PK, Chandra J. *Candida* biofilm resistance. *Drug Resist. Updat.* 7(4), 301–309 (2004).

30 Taff HT, Mitchell KF, Edward JA, Andes DR. Mechanisms of *Candida* biofilm drug resistance. *Future Microbiol.* 8(10), 1325–1337 (2013).

31 Bink A, Kucharikova S, Neirinck B *et al.* The nonsteroidal antiinflammatory drug diclofenac potentiates the *in vivo* activity of caspofungin against *Candida albicans* biofilms. *J. Infect. Dis.* 206(11), 1790–1797 (2012).

32 Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, Mccormick T, Ghannoum MA. Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. *J. Bacteriol.* 183(18), 5385–5394 (2001).

33 Nett JE, Sanchez H, Cain MT, Andes DR. Genetic basis of *Candida* biofilm resistance due to drug-sequestering matrix glucan. *J. Infect. Dis.* 202(1), 171–175 (2010).

34 Nett J, Lincoln L, Marchillo K *et al.* Putative role of beta-1,3 glucans in *Candida albicans* biofilm resistance. *Antimicrob. Agents Chemother.* 51(2), 510–520 (2007).

35 Nett JE, Sanchez H, Cain MT, Ross KM, Andes DR. Interface of *Candida albicans* biofilm matrix-associated drug resistance and cell wall integrity regulation. *Eukaryot. Cell* 10(12), 1660–1669 (2011).

36 Sadovskaya I, Vinogradov E, Li J, Hachani A, Kowalska K, Filloux A. High-level antibiotic resistance in *Pseudomonas aeruginosa* biofilm: the ndvB gene is involved in the production of highly glycerol-phosphorylated beta-(1,3)-glucans, which bind aminoglycosides. *Glycobiology* 20(7), 895–904 (2010).

37 Vediyappan G, Rossignol T, D'enfert C. Interaction of *Candida albicans* biofilms with antifungals: transcriptional response and binding of antifungals to beta-glucans. *Antimicrob. Agents Chemother.* 54(5), 2096–2111 (2010).

38 Martins M, Henriques M, Lopez-Ribot JL, Oliveira R. Addition of DNase improves the *in vitro* activity of antifungal drugs against *Candida albicans* biofilms. *Mycoses* 55(1), 80–85 (2012).

39 Martins M, Uppuluri P, Thomas DP *et al.* Presence of extracellular DNA in the *Candida albicans* biofilm matrix and its contribution to biofilms. *Mycopathologia* 169(5), 323–331 (2010).

40 Rajendran R, Williams C, Lappin DF, Millington O, Martins M, Ramage G. Extracellular DNA release acts as an antifungal resistance mechanism in mature *Aspergillus fumigatus* biofilms. *Eukaryot. Cell* 12(3), 420–429 (2013).

41 Shopova I, Bruns S, Thywissen A, Kniemeyer O, Brakhage AA, Hillmann F. Extrinsicextracellular DNA leads to biofilm formation and colocalizes with matrixpolysaccharides in the human pathogenic fungus *Aspergillus fumigatus*. *Front. Microbiol.* 4 (2013). http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3674311/

42 Krappmann S, Ramage G. Asticky situation: extracellular DNA shapes *Aspergillus fumigatus* biofilms. *Front. Microbiol.* 4 (2013). http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3695381/

43 Mowat E, Butcher J, Lang S, Williams C, Ramage G. Development of a simple model for studying the effects of antifungal agents on multicellular communities of *Aspergillus fumigatus*. *J. Med. Microbiol.* 56(Pt 9), 1205–1212 (2007).

44 Mowat E, Lang S, Williams C, McCulloch E, Jones B, Ramage G. Phase-dependent antifungal activity against *Aspergillus fumigatus* developing multicellular filamentous biofilms. *J. Antimicrob. Chemother.* 62(6), 1281–1284 (2008).

45 Seidler MJ, Salvenmoser S, Muller FM. *Aspergillus fumigatus* forms biofilms with reduced antifungal drug susceptibility on bronchial epithelial cells. *Antimicrob. Agents Chemother.* 52(11), 4130–4136 (2008).

46 Bugli F, Posteraro B, Papi M *et al. In vitro* interaction between alginate lyase and amphotericin B against *Aspergillus fumigatus* biofilm determined by different methods. *Antimicrob. Agents Chemother.* 57(3), 1275–1282 (2013).

47 Reichhardt C, Ferreira JA, Joubert LM, Clemons KV, Stevens DA, Cegelski L. Analysis of the *Aspergillus fumigatus* biofilm extracellular matrix by solid-state nuclear magnetic resonance spectroscopy. *Eukaryot. Cell* 14, 1064–1072 (2015).

• **Key paper that outlines the 'top-down' solid-state nuclear magnetic resonance (NMR) method to study extracellular matrix (ECM) composition. Describes the analysis of *A. fumigatus* ECM.**

48 Hawser SP, Baillie GS, Douglas LJ. Production of extracellular matrix by *Candida albicans* biofilms. *J. Med. Microbiol.* 47, 253–256 (1998).

49 Hoiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. Antibiotic resistance of bacterial biofilms. *Int. J. Antimicrob. Agents* 35(4), 322–332 (2010).

50 Robbins N, Collins C, Morhayim J, Cowen LE. Metabolic control of antifungal drug resistance. *Fungal Genet. Biol.* 47(2), 81–93 (2010).

51 Lewis K. Multidrug tolerance of biofilms and persister cells. *Curr. Top. Microbiol. Immun.* 322, 107–131 (2008).

52 Ramage G. Investigation of multidrug efflux pumps in relation to fluconazole resistance in *Candida albicans* biofilms. *J. Antimicrob. Chemother.* 49(6), 973–980 (2002).

53 Rajendra R, Mowat E, McCulloch E *et al.* Azole resistance of *Aspergillus fumigatus* biofilms is partly associated with

efflux pump activity. *Antimicrob. Agents Chemother.* 55(5), 2092–2097 (2011).

54 Mukerjee PK, Chandra J, Kuhn DM, Ghannoum MA. Mechanismof fluconazole resistance in *Candida albicans* biofilms: phase-specific role ofefflux pumps and membrane sterols. *Infect. Immun.* 71(8), 4333–4340 (2003).

55 Cegelski L, Marshall GR, Eldridge GR, Hultgren SJ. The biology and future prospects of antivirulence therapies. *Nat. Rev. Microbiol.* 6(1), 17–27 (2008).

56 Cegelski L, Pinkner JS, Hammer ND *et al.* Small-molecule inhibitors target *Escherichia coli* amyloid biogenesis and biofilm formation. *Nat. Chem. Biol.* 5(12), 913–919 (2009).

57 Cui J, Ren B, Tong Y, Dai H, Zhang L. Synergistic combinations of antifungals and anti-virulence agents to fight against *Candida albicans*. *Virulence* 6(4), 362–371 (2015).

58 Ramage G, Mowat E, Jones B, Williams C, Lopez-Ribot J. Our current understanding of fungal biofilms. *Crit. Rev. Microbiol.* 35(4), 340–355 (2009).

59 Sutherland I. Biofilm exopolysaccharides: a strong and sticky framework. *Microbiology* 147, 3–9 (2001).

60 Sutherland IW. The biofilm matrix – an immobilized but dynamic microbial environment. *Trends Microbiol.* 9, 222–227 (2001).

61 Cegelski L. Bottom-up and top-down solid-state NMR approaches for bacterial biofilm matrix composition. *J. Magn. Reson.* 253, 91–97 (2015).

• **Review that compares two solid-state NMR methods for analysis of biofilm composition.**

62 Reichhardt C, Cegelski L. Solid-state NMR for bacterial biofilms. *Mol. Phys.* 112(7), 887–894 (2014).

• **Review that details the use of the 'bottom-up' solid-state NMR method to quantify ECM composition.**

63 Beauvais A, Schmidt C, Guadagnini S *et al.* An extracellular matrix glues together the aerial-grown hyphae of *Aspergillus fumigatus*. *Cell Microbiol.* 9(6), 1588–1600 (2007).

• **Key paper to describe *A. fumigatus* ECM composition.**

64 Loussert C, Schmitt C, Prevost MC *et al. In vivo* biofilm composition of *Aspergillus fumigatus*. *Cell Microbiol.* 12(3), 405–410 (2010).

• **Key paper to describe *in vivo A. fumigatus* ECM composition.**

65 Zarnowski R, Westler WM, Lacmbouh GA *et al.* Novel entries in a fungal biofilm matrix encyclopedia. *mBio* 5(4), e01333–e01314 (2014).

• **Key paper that outlines many of the biochemical methods available to study ECM composition. Describes the analysis of *C. albicans* ECM.**

66 Muller FMC, Seidler M, Beauvais A *et al. Aspergillus fumigatus* biofilms in the clinical setting. *Med. Mycol.* 49(Suppl. 1), S96–S100 (2011).

67 Mukherjee PK, Zhou G, Munyon R, Ghannoum MA. *Candida* biofilm: a well-designed protected environment. *Med. Mycol.* 43(3), 191–208 (2005).

68 Serra DO, Richter AM, Klauck G, Mika F, Hengge R. Microanatomy at cellular resolution and spatial order of physiological differentiation in a bacterial biofilm. *mBio* 4(2), e00103–e00113 (2013).

69 Serra DO, Hengge R. Stress responses go three dimensional – the spatial order of physiological differentiation in bacterial macrocolony biofilms. *Environ. Microbiol.* 16(6), 1455–1471 (2014).

70 Martinez LR, Mihu MR, Tar M *et al.* Demonstration of antibiofilm and antifungal efficacy of chitosan against candidal biofilms, using an *in vivo* central venous catheter model. *J. Infect. Dis.* 201(9), 1436–1440 (2010).

71 Berk V, Fong JC, Dempsey GT *et al.* Molecular architecture and assembly principles of *Vibrio cholerae* biofilms. *Science* 337(6091), 236–239 (2012).

72 Kessler RJ, Fanestil DD. Interference by lipids in the determination of protein using bicinchoninic acid. *Anal. Biochem.* 159, 138–142 (1986).

73 Brown RE, Jarvis KL, Hyland KJ. Protein measurement using bicinchoninic acid: elimination of interfering substances. *Anal. Biochem.* 180, 136–139 (1989).

74 Schoel B, Welzel M, Kaufmann SHE. Quantification of protein in dilute and complex samples: modification of the bicinchoninic acid assay. *J. Biochem. Biophys. Meth.* 30, 199–206 (1995).

75 Bruns S, Seidler M, Albrecht D *et al.* Functional genomic profiling of *Aspergillus fumigatus* biofilm reveals enhanced production of the mycotoxin gliotoxin. *Proteomics* 10(17), 3097–3107 (2010).

76 Gravelat FN, Beauvais A, Liu H *et al. Aspergillus* galactosaminogalactan mediates adherence to host constituents and conceals hyphal beta-glucan from the immune system. *PLoS Pathog.* 9(8), e1003575 (2013).

77 Beauvais A, Fontaine T, Aimanianda V, Latge JP. *Aspergillus* cell wall and biofilm. *Mycopathologia* 178(5), 371–377 (2014).

78 Lee MJ, Liu H, Barker BM *et al.* The fungal exopolysaccharide galactosaminogalactan mediates virulence by enhancing resistance to neutrophil extracellular traps. *PLoS Pathog.* 11(10), e1005187 (2015).

79 Beaussart A, El-Kirat-Chatel S, Fontaine T, Latge JP, Dufrene YF. Nanoscale biophysical properties of the cell surface galactosaminogalactan from the fungal pathogen *Aspergillus fumigatus*. *Nanoscale* 7(36), 14996–15004 (2015).

80 Bamford NC, Snarr BD, Gravelat FN *et al.* Sph3 is a glycoside hydrolase required for the biosynthesis of galactosaminogalactan in *Aspergillus fumigatus*. *J. Biol. Chem.* 290(46), 27438–27450 (2015).

81 Lee MJ, Geller AM, Bamford NC *et al.* Deacetylation of fungal exopolysaccharide mediates adhesion and biofilm formation. *mBio* 7(2), pii: e00252–16 (2016).

82 Mitchell KF, Zarnowski R, Sanchez H *et al.* Community participation in biofilm matrix assembly and function. *Proc. Natl Acad. Sci. USA* 112(13), 4092–4097 (2015).

83 Reichhardt C, Fong JC, Yildiz F, Cegelski L. Characterization of the *Vibrio cholerae* extracellular matrix: a top-down solid-state NMR approach. *BBA Biomembr.* 1848(1 Pt B), 378–383 (2015).

84 Yildiz F, Fong J, Sadovskaya I, Grard T, Vinogradov E. Structural characterization of the extracellular polysaccharide

from *Vibrio cholerae* O1 El-Torr. *PLoS ONE* 9(1), e86751 (2014).

85 Hall MB. Efficacy of reducing sugar and phenol–sulfuric acid assays for analysis of soluble carbohydrates in feedstuffs. *Anim. Feed Sci. Technol.* 185(1), 94–100 (2013).

86 Mccrate OA, Zhou X, Reichhardt C, Cegelski L. Sum of the parts: composition and architecture of the bacterial extracellular matrix. *J. Mol. Biol.* 425(22), 4286–4294 (2013).

87 Cegelski L, Steuber D, Mehta AK, Kulp DW, Axelsen PH, Schaefer J. Conformational and quantitative characterization of oritavancin–peptidoglycan complexes in whole cells of *Staphylococcus aureus* by *in vivo* ^{13}C and ^{15}N labeling. *J. Mol. Biol.* 357(4), 1253–1262 (2006).

88 Zhou X, Cegelski L. Nutrient-dependent structural changes in *S. aureus* peptidoglycan revealed by solid-state NMR spectroscopy. *Biochemistry* 51(41), 8143–8153 (2012).

89 Toke O, Cegelski L. REDOR applications in biology: an overview. In: Solid state NMR of biopolymers. McDermott AE, Polenova T (Ed.). John Wiley and Sons, West Sussex, UK, 473–490 (2010).

90 Kim SJ, Cegelski L, Preobrazhenskaya M, Schaefer J. Structures of *Staphylococcus* cell-wall complexes with vancomycin, eremomycin, and chloroeremomcyin derivatives by ^{13}C{^{19}F} and ^{15}N{^{19}F} rotational-echo double resonance. *Biochemistry* 45, 5235–5250 (2006).

91 Nygaard R, Romaniuk JA, Rice DM, Cegelski L. Spectral snapshots of bacterial cell-wall composition and the influence of antibiotics by whole-cell NMR. *Biophys. J.* 108(6), 1380–1389 (2015).

92 Cegelski L, Schaefer J. Glycine metabolism in intact leaves by *in vivo*^{13}C and ^{15}N labeling. *J. Biol. Chem.* 280(47), 39238–39245 (2005).

93 Schaefer J, Stejskal EO. Carbon-13 nuclear magnetic resonance of polymers at the magic-angle. *J. Am. Chem. Soc.* 98(4), 1031–1032 (1976).

94 Schaefer J, Stejskal EO, Buchdahl R. High-resolution carbon-13 nuclear magnetic resonance study of some solid, glassy polymers. *Macromolecules* 8(3), 291–296 (1975).

95 Gullion T, Schaefer J. Rotational-echo double-resonance NMR. *J. Magn. Reson.* 81(1), 196–200 (1989).

96 Cegelski L, Kim SJ, Hing AW *et al.* Rotational-echo double resonance characterization of the effects of vancomycin on cell wall synthesis in *Staphylococcus auerus*. *Biochemistry* 41, 13053–13058 (2002).

97 Wang T, Park YB, Cosgrove DJ, Hong M. Cellulose-pectin spatial contacts are inherent to never-dried Arabidopsis primary cell walls: evidence from solid-state nuclear magnetic resonance. *Plant Physiol.* 168(3), 871–884 (2015).

98 Dick-Perez M, Wang T, Salazar A, Zabotina OA, Hong M. Multidimensional solid-state NMR studies of the structure and dynamics of pectic polysaccharides in uniformly ^{13}C-labeled Arabidopsis primary cell walls. *Magn. Reson. Chem.* 50(8), 539–550 (2012).

99 Wang T, Zabotina O, Hong M. Pectin–cellulose interactions in the Arabidopsis primary cell wall from two-dimensional magic-angle-spinning solid-state nuclear magnetic resonance. *Biochemistry* 51(49), 9846–9856 (2012).

100 Dick-Perez M, Zhang Y, Hayes J, Salazar A, Zabotina OA, Hong M. Structure and interactions of plant cell-wall polysaccharides by two- and three-dimensional magic-angle-spinning solid-state NMR. *Biochemistry* 50(6), 989–1000 (2011).

101 Matsuoka S, Ikeuchi H, Matsumori N, Murata M. Dominant formation of a single-length channel by amphotericin B in dimyristoylphosphatidylcholine membrane evidenced by 13C-31P rotational echo double resonance. *Biochemistry* 44(2), 704–710 (2005).

102 Anderson TM, Clay MC, Cioffi AG *et al.* Amphotericin forms an extramembranous and fungicidal sterol sponge. *Nat. Chem. Biol.* 10(5), 400–406 (2014)