The Importance of Fungal Pathogens and Antifungal Coatings in Medical Device Infections

*Carla Giles[a], Stephanie J. Lamont-Friedrich[a], Thomas D. Michl[a], Hans J. Griesser[a], Bryan R. Coad[a,b] **

a. *Future Industries Institute, University of South Australia, Adelaide SA 5000, Australia*

b. *School of Agriculture Food & Wine, The University of Adelaide, Adelaide SA 5000, Australia*

Mailing addresses:

Future Industries Institute, University of South Australia, Mawson Lakes Blvd, Mawson Lakes, SA 5095, Australia

School of Agriculture, Food and Wine. University of Adelaide Waite Campus. Urrbrae SA 5064, Australia

* Corresponding Author
Dr Bryan R. Coad, PhD
bryan.coad@adelaide.edu.au
Twitter: @DrBryanCoad
ORCID ID: 0000-0003-3297-3977
Telephone: +61 8 8313 7260

Abstract

In recent years, increasing evidence has been collated on the contributions of fungal species, particularly *Candida*, to medical device infections. Fungal species can form biofilms by themselves or by participating in polymicrobial biofilms with bacteria. Thus, there is a clear need for effective preventative measures, such as thin coatings that can be applied onto medical devices to stop the attachment, proliferation, and formation of device-associated biofilms.

However, fungi being eukaryotes, the challenge is greater than for bacterial infections because antifungal agents are often toxic towards eukaryotic host cells. Whilst there is extensive literature on antibacterial coatings, a far lesser body of literature exists on surfaces or coatings that prevent attachment and biofilm formation on medical devices by fungal pathogens. Here we review strategies for the design and fabrication of medical devices with antifungal surfaces. We also survey the microbiology literature on fundamental mechanisms by which fungi attach and spread on natural and synthetic surfaces. Research in this field requires close collaboration between biomaterials scientists, microbiologists and clinicians; we consider progress in the molecular understanding of fungal recognition of, and attachment to, suitable surfaces, and of ensuing metabolic changes, to be essential for designing rational approaches towards effective antifungal coatings, rather than empirical trial of coatings.

Keywords

antimicrobial surface, *Candida*, fungal disease, medical devices, mold, mycoses, surface modification, yeast

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1.0 Introduction

The usage of medical devices continues to increase rapidly and they have become essential components of modern health-care. Artificial hip and knee implants, stents, heart valves, vascular grafts, and other implanted devices are widely used to save lives and to restore function and quality of life for large numbers of patients. There is also a need for non-implanted, shorter-term- usage medical devices, such as various catheters, orthopedic fixation screws, and contact lenses. Clinical needs have given rise to a substantial biomedical devices industry and to an extensive body of research in biomaterials science, device design, and clinical studies of devices.

While usage has increased, there are continuing problems and shortfalls with current devices. Improved materials, device designs, and surgical practice have overcome some of the challenges, but others remain, including that of device-associated infections. Microbial organisms are capable of colonizing the surfaces of a wide range of synthetic materials used to fabricate medical devices. In fact, microbial organisms seem to be able to grow and form infectious biofilms on just about any material, from metals to ceramics to polymers, even including materials and devices as biologically foreign as Teflon™ and silicone hydrogel contact lenses. Microbial biofilm colonies produce an exocellular polysaccharide matrix that can protect them against antibiotics and the host body's innate defense system. Biofilms on biomedical device surfaces are, therefore, much more difficult to eradicate by antibiotics than circulating microbes, often resulting in the need for surgical revision of a substantial proportion of infected implants.

The bacterial colonization of medical devices is usually pictured as comprising several stages (Stoodley et al., 2002) and has been studied extensively. More recently it has become evident that fungal species can also form biofilms on biomedical devices, either by themselves or in conjunction with bacteria. The formation of fungal biofilms is conceptually similar to that of bacteria; however, some important differences are apparent (Soll and Daniels, 2016). An illustration is given in Figure 1. Initially, microbial (fungal) cells arrive at the device surface and attach, driven by their propensity to settle onto a solid substrate rather than remaining in the planktonic state. Once a sufficient number of cells is attached, intercellular communication causes a switch in phenotype resulting in the production of extracellular matrix and biofilm formation (Mitchell et al., 2016). With biofilms of polymorphic yeasts such as Candida, yeast cells develop pseudohyphae and hyphae creating a filamentous layer that extends away from the basal layer of cells (Gulati and Nobile, 2016). As the biofilm grows and matures, it eventually starts to release planktonic cells. The formation of an

infectious biofilm on the medical device interferes with healing, for example tissue integration of hip and knee implants. The release of microbial cells from the biofilm leads to bloodstream infections, which can be lethal for patients severely weakened by surgery or trauma, or that are immunocompromised. The situation is further complicated by the fact that with implantable devices, detection usually only occurs when a full-blown infectious biofilm has formed on the medical device; often the only viable remedy is revision surgery and replacement of the device.

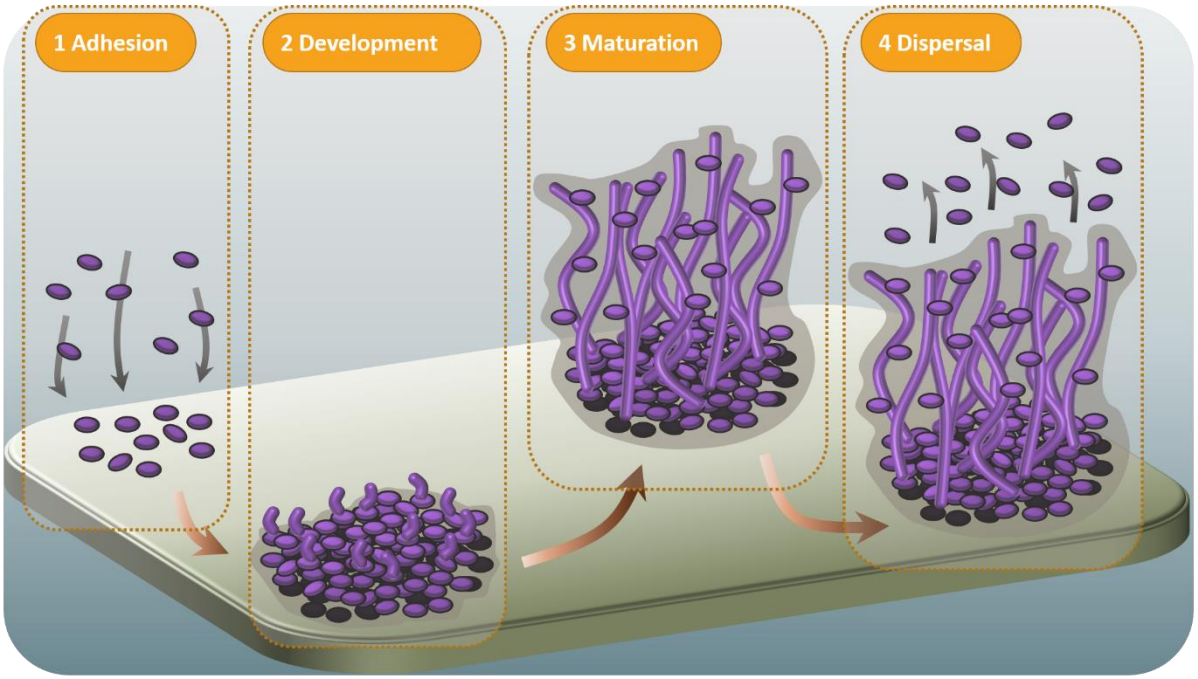

Figure 1. Schematic of fungal biofilm formation typical of *Candida albicans*. 1) In the adhesion phase, cells adhere firmly to the substrate. 2) Cells replicate, begin to secrete extracellular matrix and develop pseudohyphae. 3) The mature biofilm develops with hyphal filaments extending away from a basal layer of yeast-forms. 4) Yeast cells disperse from the biofilm and spread, disseminating the infection and beginning the cycle again.

Accordingly, research has focused on preventing the initial attachment of microbial cells onto the surfaces of materials used for medical devices. In particular, the development of thin coatings that can be applied to medical devices to confer resistance to microbial colonization has been of much interest. Such coatings are designed to prevent the establishment of infectious biofilms while not affecting material properties such as the visual clarity of contact lenses or the flexibility of vascular grafts. Nor should, of course, such coatings have adverse effects on cells or fluids (e.g., blood, tear fluid) of the human host.

For many years, device-associated infections have been discussed in terms of bacterial biofilm formation. In recent years, however, evidence has accumulated that fungal species often play a major role (Coad et al., 2016; Coad et al., 2014; Imbert, 2016; Kojic and Darouiche, 2004; Luis et al., 1989; Muakkassa and Ghannoum, 2016; Peleg et al., 2010). Pathogenic fungi, particularly various *Candida* species, can form biofilms of their own or associate with bacteria in polymicrobial, poly-Kingdom biofilms. Detection and identification of fungal species are more difficult than for human pathogenic bacteria, which have been well-known for a long time to microbiologists. This may be why fungal contributions to device infections were overlooked for quite some time, and why infections at times did not respond well to the administration of antibacterial drugs – after the

bacteria were killed, fungal organisms were unimpeded by competition and continued to flourish. It is therefore clear that there is a need to address the prevention of fungal colonization in the design of infection-resistant coatings for medical devices.

Here we review the literature on strategies on the design of antifungal coatings and the resulting outcomes. Strategies for combatting biofilms on surfaces (i.e. antimicrobial materials, surfaces, and coatings) are a hot topic in materials-led research, but the large majority focuses on antibacterial strategies. In order to be clinically useful, they must be effective in assisting the body to clear infection, and must be non-harmful to the patient. With fungal biofilms, or poly-Kingdom biofilms, the challenge is also considerable, but not insurmountable, we posit. What is required are approaches that are specifically directed to combatting fungi without damaging mammalian cells and tissue; this is a considerably more difficult challenge than preventing bacterial biofilm formation since fungi are eukaryotic organisms and thus much closer biologically to human cells than bacteria are, being prokaryotic. Ultimately one may need to implement dual-action coatings that can combat both fungi and bacteria, but for the present review we focus on antifungal strategies. We also emphasize strategies that aim to inhibit fungal attachment instead of eradication, as it appears challenging to kill fungi without affecting human cells. After an overview of fungal infections on medical devices, we will review materials science strategies for preventing colonization. Our aim is to stimulate critical thinking and rational design of strategies towards advances in antifungal coatings that will ameliorate the problem of fungal infections on medical devices.

2.0 Fungal infections on medical devices

2.1 Fungal pathogens contribute to poly-Kingdom biofilm infections, and scope for resistance

Biofilms on medical device surfaces have a recognized importance in clinical infections. It is estimated that 65 % of all microbial infections in humans involve biofilms (Ramage et al., 2006). The physical nature of the biofilm, which is a structured microbial community encapsulated within a protective extracellular matrix, means that cells within a biofilm display phenotypic traits that are vastly different to those found when the cells are in a planktonic state (Costerton et al., 1995). Bacterial species are a primary cause of device related infections; however, biofilms are communities of organisms and the contributions of many different bacterial species in biofilm infections have been recognized (Peters et al., 2012). Moreover, the important role that fungi play in biofilms either alone or participating in poly-Kingdom infections is much less well-understood. On the specific topic of medical devices with infections derived from, or contributed by fungi, there have been a few reviews (Kojic and Darouiche, 2004; Pierce et al., 2015; Ramage et al., 2006; Williams et al., 2016).

Polymicrobial biofilms on indwelling medical devices are multifaceted and difficult to treat (Qu et al., 2015). This problem is partly due to the complex interplay between surfaces, bacterial and fungal colonizers, and their biological and physical interactions. For example, C. albicans hyphae can form a scaffold, which supports and protects S. aureus (Qu et al., 2015). This behaviour is particularly a problem due to the fact that these microbial species are frequently encountered in the clinical setting, and associated with high mortality rates such as that in catheter-related line bloodstream infections (CLBSI) (Sievert et al., 2013). Treatment of polymicrobial biofilms is challenging due to the difference in cellular make-up, metabolism, and biochemical pathways in prokaryotic and eukaryotic organisms. Therefore, there is a lack of clinically relevant therapies which are capable of killing both (Qu et al., 2015). Thus, common antimicrobial therapies used to combat these infections often use multiple classes of drugs, but fail to be effective, having a 70 % failure rate (Kim et al., 2013).

Biofilms are associated with contributing to antibacterial resistance (Costerton et al., 1999). The same is true for fungal biofilms and their resistance to antifungal agents (Borghi et al., 2016). For mixed (fungal/bacterial) polymicrobial biofilms there is evidence showing that the scope for resistance development is potentially more threatening than monomicrobial biofilms. For example, biofilms containing *C. albicans* and *S. aureus* are associated with increased bacterial resistance to vancomycin and daptomycin, more so than single species biofilms (Harriott and Noverr, 2009). This observation is also mirrored for resistance to antifungals where it has been shown that *Staphylococcus epidermidis* (*S. epidermidis)* can protect *C. albicans* against fluconazole and amphotericin B in a co-cultured biofilm (Adam et al., 2002). Clearly, the role that fungi play in biofilms, the treatment difficulties, and their increased potential to develop resistance are key issues.

2.2 Causative fungal species and review of occurrence

The most frequently used devices that can develop *Candida spp.* infections are urinary catheters, central and peripheral venous catheters, endotracheal tubes, and dentures, which are used in the millions of units per year (Kojic and Darouiche, 2004; Ramage et al., 2006). The infection risk on these devices is variable, but the pervasiveness of the problem can be seen in urinary catheters, which had a usage rate of tens of millions of units used in 2006 and an associated infection risk between 10 and 30 %. Other biomaterials that are prone to fungal infections include prosthetic joints, intracardiac prosthetic devices, haemodialysis and peritoneal dialysis catheters, breast implants, neurosurgical shunts, voice prostheses, and penile implants. Other yeast species, such as *Cryptococcus*, *Trichosporon*, and *Saccharomyces*, have also been described in case reports to infect biomaterial implants (Ramage et al., 2009).

Risk of infection, site of implantation and life expectancy of the medical device directly impacts the opportunity of combatting fungal biofilms on these surfaces. Prosthetic implants have around a 1 % chance of developing a *Candida* spp. infection, despite being deeply implanted and designed to last the life span of the patient (Kojic and Darouiche, 2004). Therefore, strategies that reduce the risk of infection are attractive, as correction of infected deep implant medical devices is directly associated with intensive surgery, high healthcare costs and poor patient outcomes. Conversely, usage of voice prostheses is relatively low (thousands of units per year) yet the infection risk approaches certainty (50 – 100 %) (Ramage et al., 2006). This prevalence demonstrates a great need for anti-infective strategies to suit many devices and usages.

Yeasts belonging to genus *Candida* are the primary causative agents for fungal biomaterials infections and can disseminate as fungal blood sepsis (candidemia). In 2006, a three-year surveillance study explored candidemia-related mortality rates across all Australian intensive care units. It was found that greater than 50 % of the patients with candidemia had died as a direct result from their *Candida spp*. systemic infection. Of the 179 patients, *C. albicans* was detected in 62 % of cases (111 people), and *C. glabrata* in 18 % of cases (32 people) (Playford et al., 2008). Besides yeasts, filamentous fungi belonging primarily to *Aspergillus spp*. have also been known to contribute to infections on medical devices and form biofilms. Williams and Ramage have reviewed *Aspergillus* biofilms and describe serious infections that have developed on catheters, prosthetic devices, pace makers, heart valves, and breast implants (Williams et al., 2016). The therapeutic strategies to address biofilms of *Aspergillus fumigatus* have also recently been reviewed (Muakkassa and Ghannoum, 2016). Finally, *Fusarium spp.* is associated with the wearing of contact lenses, which can lead to fungal keratitis. Strategies that reduce fungal proliferation on lenses and in cleaning solutions would help to combat eye diseases and blindness (Imamura et al., 2008).

2.3 Medical device materials

Fungal biofilms can develop on a wide variety of implantable devices and, by implication, biomaterials, including contact lenses, prosthetic implants, dentures and cochlear implants. A recent in vitro study explored biofilm formation of five different Candida spp. (*C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei*) on three clinically significant materials, PVC, Teflon™ and polyurethane. It was found that all *Candida spp*. readily formed biofilms on Teflon™, except *C. glabrata*, which exhibited greater biofilm formation on PVC (Estivill et al., 2011). Hence, one has to consider the context of both the colonising species and the material properties. An *in vitro* study conducted in 2005 investigated altering the surface chemistries of substrates through the addition of surface-modifying end groups (SMEs), in order to prevent or reduce *C. albicans* biofilm growth (Chandra et al., 2005). The results showed that various SMEs affected *C. albicans* biofilm formation differently. For example, Elasthane 80A surfaces modified with 6 % polyethylene oxide reduced metabolic activity of *C. albicans* biofilm by 78 % (Chandra et al., 2005). These different rates of colonization, depending on the material, add an additional dimension to this already complex problem.

2.4 Clinical manifestations

A list of all clinical case studies involving infected implants is beyond the scope of this review. Instead, we provide four examples illustrating the clinical significance of fungi-related device infections. We contrast strategies for treating devices that are easily removed (such as contact lenses or dentures) versus implanted devices such as prosthetics and cochlear implants which are designed for long term implantation within the body. While biofilms on short-term devices can be treated by cleaning or removal of established biofilms, there is a greater emphasis on prevention in the case of implanted device.

A review by Ramage et al. provides an excellent summary table of medical implant devices that develop *Candida*-derived infections, their usage and infection risk (Ramage et al., 2006). In Table 1 we present a summary of information for the four examples shown below. This is meant to provide insight into common and less-common fungal infections on various biomaterial implant devices and their clinical significance related to this context.

Table 1. The clinical significance and causative agents of fungal infections pertaining to a selection of biomaterial implant devices

Biomaterial device	**Fungal infection**	**Causative fungal species**	***Clinical significance***
Dentures	Candida-associated denture stomatitis	*C. albicans* *C. glabrata* *C. parapsilosis* *C. tropicalis*	Dentures are one of the most common medical implant devices associated with fungal infections.
Contact lenses	Fungal keratitis	*Fusarium spp.* *Candida spp.*	Contact lens use is an increasing risk factor for fungal keratitis.
Prosthetic joints	Fungal prosthetic joint infection	*C. albicans* *C. parapsilosis* *C. glabrata* *C. tropicalis*	Although rare, fungal prosthetic infections are difficult to combat with surgical techniques, and expected to rise in the coming years.
Cochlear implants	Fungal biofilm	*C. albicans*	When the cause of implant infections is unknown, the use of antibacterial prophylaxis can eliminate bacterial pathogens but is ineffectual against fungal pathogens.

2.4.1 Dentures

Accounting for device usage and infection risk, dentures are one of the most common biomaterial devices causing fungal infections (Ramage et al., 2006). Dental plaque frequently forms on these devices and the importance of fungi in these polymicrobial biofilms is recognized. For example, Peleg et al., explored the symbiotic relationship between *Streptococcus* spp. and *C. albicans* in the oral cavity. The results suggest that the presence of the bacteria promotes adhesion of fungal cells, as the bacteria are capable of adsorbing protein components from the saliva, which encourage *C. albicans* adhesion (Peleg et al., 2010). The onset of denture-induced stomatitis from polymicrobial biofilms can result in oral candidiasis where *C. albicans* is the primary organism responsible (Chandra et al., 2005; Estivill et al., 2011), while *C. glabrata, C. parapsilosis* and *C. tropicalis* infections occur at a lower incidence (Nett et al., 2010). Fungal biofilms formed on denture surfaces are common, however, effective methods of control are currently not understood. Preventing these will require a better understanding of how *Candida* spp. in particular adhere onto the various polymeric substances that comprise denture materials. These polymers are mostly acrylic hybrids consisting of a mixture of polymethyl methacrylate (PMMA), hydroxyapatite or polyamide resin, polyurethane, polycarbonateurethane and poly(ethyleneterephthalate) (PET) (de Freitas Fernandes et al., 2011; Pereira-Cenci et al., 2008; Von Fraunhofer et al., 1988). Additionally, the supportive framework of dentures is composed of hybrid acrylic resins (PMMA, hydroxyapatite, polyamide resin) or a flexible polymer, such as nylon (de Freitas Fernandes et al., 2011).

A study conducted in 2007 described the physical properties of denture materials that affect *Candida* spp. adhesion to materials surfaces. Such properties include surface free energy, surface roughness and presence of saliva. This *in vitro* study concluded that overall adhesion and colonization of *C. albicans* and *C. glabrata* on materials surfaces was reduced by saliva, surface roughness, and bacteria, but not by surface free energy (Pereira et al., 2007). Another study conducted by the same group in 2008 explored the interactions between *C. albicans, C. glabrata* and *Streptococcus mutans* in biofilms, when in the presence of saliva. The materials used for test substrates were hydroxyapatite, PMMA and soft denture liner. It was concluded that saliva, substrate material and the presence of other microorganisms, significantly affected *Candida* spp. biofilm growth (Pereira-Cenci et al., 2008).

Removal of established biofilms on dentures is possible by manual cleaning or soaking in cleansers. In 2011, a study aimed to evaluate the efficacy of current denture cleansers against fungal and polymicrobial biofilms grown on polyamide resin with different surface roughness and surface free energy. *Candida* spp. biofilms were grown on PMMA resin and polyamide resin before being treated with either enzymatic cleaner solution, a cleanser solution, 0.5% sodium hypochlorite solution or water. Ultrasonic waves were used to remove any remaining adhered fungal cells, and these were plated up and colony-forming units were counted. Polyamide resins were shown to support higher *Candida* spp. biofilm growth than PMMA materials, with all cleanser solutions removing *Candida* spp. biofilms on both PMMA and polyamide resin materials. The cleanser solution composed of 0.5 % sodium hypochlorite was concluded to be the most effective cleanser (de Freitas Fernandes et al., 2011).

2.4.2 Contact lenses

Fungal keratitis refers to opportunistic fungal infection and inflammation of the cornea and was first described in 1879 (Cheng et al., 2015; Hassan et al., 2010; Leber, 1879). Fungal keratitis is caused by *Candida* spp., *Aspergillus* spp. and *Fusarium* spp., with the latter being the most common. If left untreated, fungal keratitis can lead to endophthalmitis and blindness (Cheng et al., 2015).

Currently, a limited range of topical antifungal agents exists for treatment. The most common treatments include topical amphotericin B, topical natamycin, topical fluconazole, oral ketoconazole, and recently added, topical and systemic voriconazole (Cheng et al., 2015). A 2015 review of fungal keratitis highlighted the major challenge is finding a most suitable drug or combination of drugs for effective treatment (Cheng et al., 2015). Fungal keratitis is often incorrectly diagnosed and treated, at times resulting in corneal grafting to restore vision. Occasionally, antifungal drug treatment and corneal grafting are undertaken prior to establishing a correct diagnosis (Hassan et al., 2010).

Although fungal infections related to contact-lens use are rare, a marked increase in risk associated with contact lens use was evident from 2005, increasing from 40% to 52% (Iyer et al., 2006). Because loss of eyesight is a devastating outcome of fungal keratitis, it is important to investigate disease causes and possible preventative measures associated with contact lenses (Imamura et al., 2008).

Outbreaks associated with increased contact lens use are an interesting case study. The US Centers for Disease Control and Prevention received 164 confirmed reports of *Fusarium keratitis* beginning June 2005 until June 2006, spanning 33 states of the USA. It was found that the outbreak was directly associated with the use of a particular contact lens solution, and consequently contact lens users were urged to stop using that solution (Alfonso et al., 2006; Chang et al., 2006).

It is important to develop appropriate assays to understand the role of materials and their properties in contact lens associated keratitis. Imamura et al. have explored models for contact-lens associated fungal biofilms and characterized biofilm formation on soft contact lenses, focussing on *Fusarium* spp. and *C. albicans*. That study provided important insights into future prevention of contact-lens related infections by providing insights into the influence of lens type, and susceptibility of lens care solutions to contribute to infections (Imamura et al., 2008).

2.4.3 Total joint arthroplasty

A case report in 1998 examined prevalence of fungal infections in total joint arthroplasties. It concluded that fungal infections in total joint arthroplasties were rare, with only 21 cases previously reported in the literature, most of these caused by *Candida* spp. However, the limited success rate in combatting these infections was evident with all of the cases having to be removed in order to eliminate the source of fungal infection (Brooks and Pupparo, 1998). Furthermore, re-implantation was reported to be an unsuccessful option. An important virulence factor in periprosthetic infection is the ability for *C. albicans* to form biofilm (Azzam et al., 2009). Due to its persistence, surgical treatments involving irrigation and debridement are sometimes unsuccessful, which shows that surface attachment to the device and in tissues surrounding the device needs to be considered. The incidence of all infections for implanted prosthetic devices is expected to rise with the ageing population. Based on data from the United States Census Bureau, it is estimated that the demand for primary total hip arthroplasties will grow by 174 % (to 572,000 procedures), by the year 2030. It was also predicted that the demand for total knee arthroplasties would grow by 673 % to 3.48 million procedures by the year 2030, indicating an increasing demand for improved diagnostics methods and treatments (Kurtz et al., 2007).

2.4.4 Cochlear implant

The application and administration of antimicrobials are frequently used pre-operatively as a prophylactic measure that can prevent the first stage in biofilm formation, that of initial adhesion. However, it is clear that there is potential for fungal infections to develop in certain cases, probably from adventitious fungal cells in the wound environment. Cristobal et al. presented a case study of

cochlear hardware implantation in a young girl where oral and topical antibiotics were administered one week pre- and post-implantation (Cristobal et al., 2004). Oral cefdinir is a broad-spectrum cephalosporin antibacterial agent and topical ofloxacin is a broad-spectrum fluoroquinolone both of which are effective against Gram negative and positive bacteria. Four weeks post-implantation, the patient developed an infection and fluid sampling showed no bacterial cultures, only cultures of *Candida* spp. These results were dismissed as laboratory contamination and the patient was dismissed with a short course of oral amoxicillin. After a further two weeks, the patient presented with further signs of infection which was confirmed to be *C. albicans,* and the cochlear implant had to be removed. Analysis of the explanted device showed a mature biofilm of *C. albicans* covering the device. This case shows that antibacterial therapies were effective in clearing bacterial infections. However, it could be speculated that this may have provided increased opportunity for *C. albicans* (not affected by these antibacterial agents) to colonize the medical device surface and proliferate. This line of thought underscores the importance of anti-infective coating strategies that are targeted towards eliminating both bacterial and fungal pathogens.

3.0 Materials science approaches

Clinical cases for long-dwelling implantable materials, as well as the difficulties of treating established biofilms, show the advantage (or necessity) for medical devices to possess an ability to prevent colonization by microbial pathogens. Broadly speaking, materials science strategies can be categorized into releasing and non-releasing types. A critical discussion of these approaches and their potential advantages and disadvantages have been recently given (Coad et al., 2016). With antibacterial surfaces, several reviews have broadly captured the state-of-the-art (Campoccia et al., 2013; Tiller, 2011; Vasilev et al., 2009). However, in these reviews, antifungal surface coatings have been either a minor point or not considered at all. We summarized strategies for antifungal surface coatings of the non-release type previously (Coad et al., 2014). The sections below provide an opportunity to update this review in the specific context of medical devices and to further expand it by also reviewing releasing strategies.

3.1 Drug releasing strategies

3.1.1 Release from bulk

Placing antifungal drugs directly into or onto the material of the medical device is a straightforward method of modifying how the surface of the material will interact with microbes (Figure 2). However, the material and drugs themselves must be suitable for this approach, ultimately limiting the number of drug-material combinations possible.

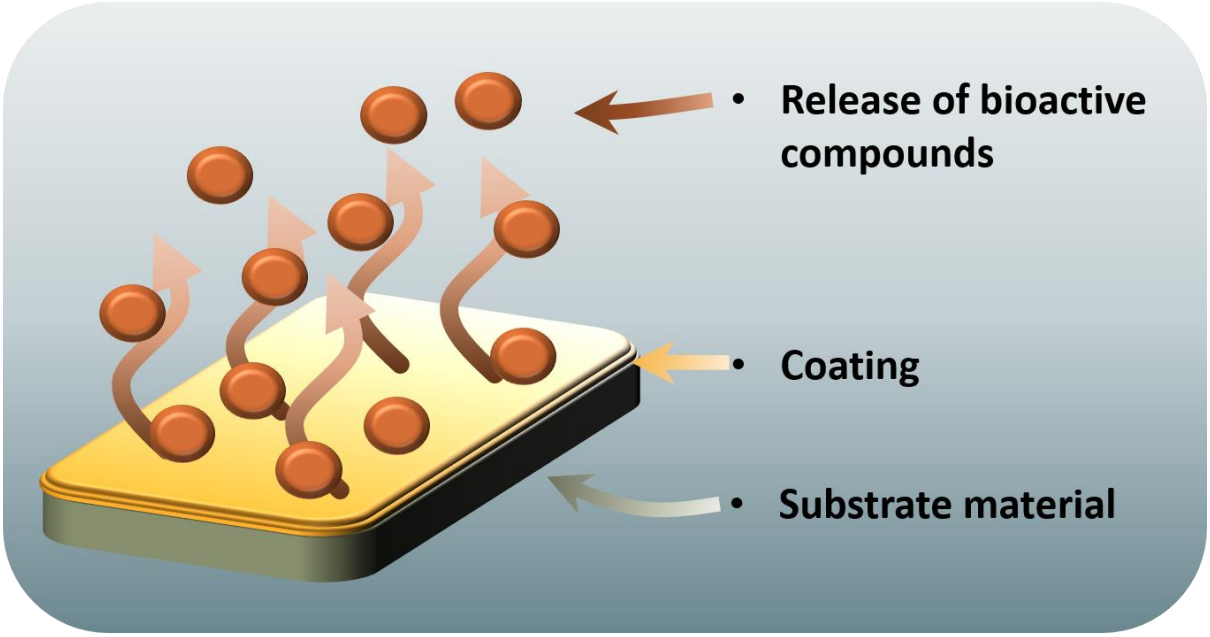

Figure 2. Materials that release bioactive compounds. Medical device materials with imbibed antifungal agents, either in the bulk or in a surface coating, are released by diffusion. The kinetics of release and diffusion of the compounds affect their antimicrobial performance.

Using a proprietary aromatic polyurethane (Pellethane) for medical catheters, Schierholz and co-workers loaded a combination of the antibiotic rifampicin and antifungal miconazole into the bulk of the material via a diffusion-controlled mechanism (Schierholz et al., 2000). Out-diffusion then released these compounds into biological solutions; kinetic measurements were made up to 21 days. The modified polyurethane surfaces were challenged with clinical isolates, among them three strains of *C. albicans*, although the focus of that work was on bacteria. The zone of inhibition was larger for all bacterial and fungal pathogens than for the "gold-standard" catheters coated with chlorhexidine and silver sulphadiazine. More importantly, the activity did not diminish after sterilization with ethylene oxide and materials retained 90 % of the drug loading after 12 months of storage under ambient air. The last point raises an aspect which is often not taken into account: sterilization and storage conditions of medical devices can potentially negate the viability of many approaches documented in the literature. This highlights an essential point to address if coatings are to proceed to human application.

Braem et al. designed a mesoporous diffusion barrier in the form of silicon oxide for controlled drug release from a porous titanium implant (Braem et al., 2015). The porous titanium membranes were fouled with foetal bovine serum and the broad-spectrum anti-biofilm molecule toremifene was successfully released with a delay of two days without an initial burst in concentration. This approach resulted in a 70 % metabolic reduction of *C. albicans* in RPMI media. The study demonstrates that engineering approaches, under-represented in the literature, can also lead to more controlled and sustained drug release.

Apart from pharmaceutical antifungal drugs that can be released from a material, halogenated materials have also been shown to be highly efficient at eliminating pathogens. Sun and co-workers attached the N-halamine precursor 5,5-dimethylhydantoin (DMH) via an isocyanate coupling agent to a polyurethane, followed by halogenating the DMH to the corresponding N-halamine using a 10 % aqueous sodium hypochlorite solution (bleach) (Sun et al., 2012). The

resulting material proved to be highly biocidal and fast-acting (log 4 reduction of *C. albicans* within 60 minutes of contact time). Additionally, this modified polyurethane could be quenched using aqueous thiosulfate solution, recharged after use again with bleach, and retained more than 91 % of the initial chlorine concentration after six months of storage. Analogously, Jie and co-workers used aqueous potassium iodide to prepare polyurethane-iodine complexes, which caused complete eradication of *C. albicans* and *Aspergillus. niger* after two or twenty minutes, respectively (Jie Luo et al., 2010). Further testing using animal models to date have not been conducted, as free halogens, hypochlorites and N-halamines are known to be indiscriminately cytotoxic (Gernhardt et al., 2004). However, there are applications, such as urinary catheters, that have minimal contact with human cells and thus such biocidal coatings could potentially have merit.

3.1.2 Release from thin film coatings

Thin film coatings provide an opportunity to store or associate antifungal compounds in a material extraneous to the medical device surface itself. Thus, they provide the ability to modify a wider range of devices. Furthermore, this technique allows for the use of more "exotic" combinations of polymers for building the coating, and antifungal molecules for in-diffusion (such as peptides, polysaccharides or synthetic polymers and analogues). Some compounds cannot be delivered orally or systemically and may be less likely to induce resistance in the microorganism when administered via local release from a coating on a biomedical device.

A system of physisorbed thin film coatings (alternating polyelectrolyte layers) was used to load a synthetic antifungal beta-peptide, as documented in three publications by Raman, Karlsson and co-workers. For their purpose, an initial layer of polyethyleneimine, polystryenesulfonate and polyallylamine was deposited onto either glass or silicon substrates (Karlsson et al., 2010). This step was followed by alternating layer deposition of poly-L-lysine (PLL) and poly-L-glutamic acid (PGA); resulting in layers of up to 800 nm in thickness, which were then in-diffused with a synthetic antifungal beta-peptide that was fluorescently tagged with a coumarin unit. This coumarin unit enabled quantification of the release kinetics of the beta-peptide via fluorescence over a time frame of 400 h. In the next step, the coatings were challenged with *C. albicans* in RPMI media and found to deliver a reduction in the viability of the yeast after two hours, with further reduction in metabolic activity observed after seven hours.

Similarly, other substrates were explored, such as polyethylene catheters which were coated with the layer-by-layer technique using PLL and PGA (Raman et al., 2014). Using the same antifungal beta-peptide, activity was observed against *C. albicans* in RPMI media spiked with foetal bovine serum, which is a model for biofouling by blood proteins that can negate activity.

Subsequently, polyethylene catheter tubes were explored using a different layer-by-layer coating method using either poly-L-lysine and poly-L-glutamic acid or chitosan (CH) and hyaluronic acid (HA); followed by beta-peptide loading (Raman et al., 2016a). The CH/HA layers had almost double the loading capacity of the PLL/PGA films and exhibited weak antifungal activity against *C. albicans* without the drug. Finally, the coatings were challenged in a 48 hour *in vivo* infectious central venous catheter rat model, using *C. albicans* where the coatings showed less robust biofilm formation (Raman et al., 2016b).

A different, "release upon stimuli", layer-by-layer approach was used by Cado and co-workers to immobilize the modified antimicrobial peptide cateslytin via a cysteine residue into a matrix of HA and CH (Cado et al., 2013). The covalently-bound antimicrobial peptide was intended to be released only if pathogen-secreted enzymes (hyaluronidases) degraded the linkage and thus

released the active drug. *In vitro* results demonstrated that *C. albicans* growth was effectively inhibited within one hour and the organism completely killed within 24 hours.

The PLL/PGA multilayer approach was used by Etienne et al. to embed the antifungal peptide CGA47-66. This coating exhibited moderate efficacy *in vitro* against *C. albicans,* but gave more encouraging results in an *in vivo* oral cavity rat model, showing almost no trace of *C. albicans* under microscopic exanimation (Etienne et al., 2005). Enumeration of viable organisms would have been a useful study to quantify the efficacy of the antifungal treatment.

A radically different approach was used by Manna and co-workers by combining the non-adherent qualities of slippery liquid-infused porous surfaces (SLIPS) with the biocidal action of a fungicide (Manna et al., 2016). For their purpose, silicone oil, which was used to generate the slippery liquid interface, was infused with triclosan. Due to the matching hydrophobic character between the silicone oil and triclosan, out-diffusion of the fungicide was considerably slowed. Three weeks were required to release one-third of the antifungal agent, and a total of 120 days to release a cumulative 45 % of the total triclosan loading. This duration of release is much longer than the few days obtained with most systems and could assist in avoiding infections during slow wound healing, such as in elderly patients. Antifungal testing against *C. albicans* showed negligible fungal adhesion and growth on the surface, and release of triclosan killed planktonic cells. Thus, “dual-action” surface coatings not only prevented cellular adhesion and biofilm formation on the surface but also reduced pathogens in the surrounding fluids. Reduced cellular adhesion was, however, also observed for mammalian cells, which might limit the application to medical devices where tissue integration is not important.

Drug-releasing biomaterials enable delivery of drugs that are otherwise inefficient or even impossible to deliver systemically. Anidulafungin, an approved antifungal drug, is one of those drugs, but its hydrophobic nature requires intravenous administration. For this reason, Gharbi et al. used the hydrophobic inner cavity of cyclodextrins (CDs) to generate stable complexes with anidulafungin or the terpene thymol (Gharbi et al., 2012). This approach improved the water solubility of anidulafungin by a factor of almost 3500 compared to the bare drug. Loaded CDs were then immobilized onto gold surfaces via amide coupling to thiol immobilized tethers, yielding antifungal surfaces toward *C. albicans* that were rechargeable after use. Additionally, the authors speculated that the anidulafungin-loaded CDs prevented fungal adhesion whereas thymol-loaded CDs were antifungal by killing. However, additional microbiological testing, besides fluorescent microscopy, was not undertaken.

A simpler, yet remarkably efficient, approach was taken by Darouiche and co-workers in three separate publications where titanium cylinders were coated by dipping them into a mixture of chlorhexidine and chloroxylenol via an undisclosed proprietary technique (Darouiche et al., 1998). This step was followed by a zone-of-inhibition test against *C. albicans* after two, four, six and eight weeks of incubation in human serum. Surprisingly, neither the size of the zone of inhibition, nor the quantity of the antiseptics appeared to change dramatically over the surveyed time course.

In a follow-up study, silicone catheters were impregnated via the same method with the same antiseptics, sterilized using ethylene oxide, infected with either *C. albicans* or *C. krusei*, and placed percutaneously into rabbits (Darouiche et al., 2006). This method resulted in a lower number of devices becoming infected (50 % instead of 100 % in case of the untreated control) for both *Candida* spp. and a reduction of retrieved CFU from the implant by a factor of 20 or more.

The final paper in that series involved the dip coating of urinary catheters with chloroxylenol and thymol, gas sterilization, and incubation in synthetic urine *in vitro*. Zone of inhibition tests against various pathogens including *C. albicans* were performed at different intervals for up to two weeks (Mansouri and Darouiche, 2008). Again, clinical relevance was evident in the demonstration of efficacy after sterilization.

Finally, drug delivery of antifungals was facilitated by using a biodegradable polymer, polyglycolic acid, formed as injection-moulded microneedles. The microneedles were modified by ink-jet coating with a water-soluble drug release layer, into which either itraconazole or voriconazole was deposited. The focus was primarily on the fabrication of the microneedles, their coating and their ability to penetrate porcine skin *in vitro*. Yet, a simple diffusion inhibition assay revealed activity against *C. albicans*; without studying the release kinetics in detail (Boehm et al., 2015; Boehm et al., 2016).

3.1.3 Release from hydrogels

In the wider drug release literature, release from hydrogel polymers is a popular approach. Here we discuss an illustrative example using an antifungal drug. Amphotericin B is a highly potent antifungal compound but it has human cell toxicity and poor water solubility (Scheld et al., 2014). To deliver amphotericin B from a surface coating matrix, dextran-based hydrogels (labelled Amphogel) were prepared by Zumbuehl et al. via a simple swelling and loading procedure (Zumbuehl et al., 2007). Releasing hydrogels proved to be highly antifungal against *C. albicans* (100 % kill rate within two hours) and persistent (up to 53 days of repetition). Interestingly, the fungal growth in the supernatant was not affected, suggesting that the hydrophobic drug does not diffuse out into the media, at least not to a concentration exceeding the minimum inhibitory concentration (MIC). This probably results from its low water solubility, with the killing occurring upon contact with drug molecules localized on the hydrogel surface. This may be advantageous for cytotoxic drugs such as amphotericin B, but does not address the problem of pathogenic spread into the surrounding tissue, which needs to be combatted via diffusing drug molecules. *In vivo* testing in mice over a period of three days yielded a log 6 reduction of *C. albicans* and no presence of host cells in the Amphogel. Similar to other approaches previously discussed, this technology offers a promising route for applications where host infiltration is not desired. Unfortunately, the fabrication method used in that study does not address the issue as to how the Amphogel could be coated readily onto a medical device surface.

3.1.4 Release from grafted polymers

Compared to thin-film layered coatings and hydrogels, approaches that use surface-grafted polymer layers as the storage matrix for antifungal drugs offer some advantages (schematically illustrated in Figure 3). They are typically thicker than thin film coatings, which increases the potential antifungal reservoir, and they are covalently attached to the substrate. Advances in polymer grafting technologies now seek to release drugs from materials in a more controlled manner, and also incorporating means for obtaining release triggered by external stimuli.

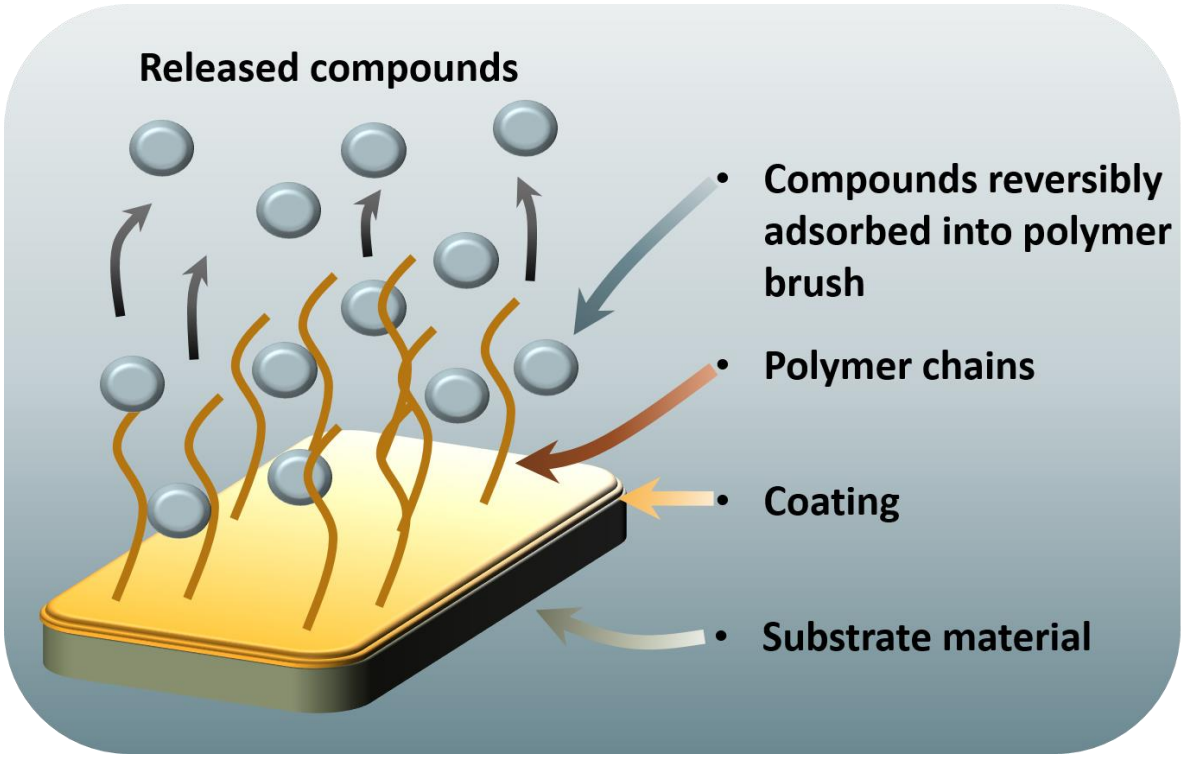

Figure 3. Schematic of grafted polymer chains (or polymer brushes) that serve as a storage matrix for the loading of antifungal compounds into the surface layer. Once implanted, desorption of the compounds results in diffusion away from the matrix into the fluids surrounding the device.

A 'release on demand' coating was developed by Segura and co-workers by gamma irradiation of silicone rubber, followed by the exposure to glycidyl methacrylate to form a grafted polymer layer up to 20.9 % by weight (Segura et al., 2014). The reactive epoxy groups of the grafted polymer were then used to covalently bind ergosterol, a sterol essential in fungal cell biology. The grafted ergosterol was then used to reversibly associate with the antifungal drugs natamycin and nystatin, which have an affinity for ergosterol, as well as the azole-class drug miconazole. As expected, there was selective uptake, into the grafted layer, of natamycin and nystatin, whereas nonselective in-diffusion was observed with miconazole, which does not specifically associate with ergosterol. Liposomes mimicking mammalian and fungal cell membranes (containing cholesterol or ergosterol, respectively) caused different rates of release and endpoint concentrations, with faster release upon contact with ergosterol-containing liposomes. Unfortunately, these encouraging results only translated partially in fungal testing against *C. albicans* and *A. fumigatus*, as natamycin-loaded samples did not cause any pathogen inhibition in a contact assay. In contrast, nystatin-loaded surfaces did inhibit *A. fumigatus* and caused *C. albicans* to remain in its less virulent yeast morphology. However, less than a log 2 reduction in CFU/cm^2 of *C. albicans* was achieved. These results demonstrate that even stimulated drug release does not necessarily translate into effective inhibition of fungal pathogens when required.

A similar, but non-selective, approach was undertaken by Sun et al. A layer of the biocompatible polymer poly(N-vinyl-2-pyrrolidinone) (PNVP) was grafted onto plasma-activated polymethylmethacrylate (PMMA) denture resins, reaching grafting densities close to 8 % by weight (Sun et al., 2013). The grafted PNVP was then infused with either miconazole or the disinfectant chlorhexidine, releasing the former for up to 60 days or the latter for 14 days to reach solution concentrations above the MIC. Samples challenged with *C. albicans* caused a marked reduction in metabolic activity over the same period of time and no fungal adhesion was observed by

microscopy. Furthermore, the PNVP coatings could be either “quenched” by immersion into PNVP aqueous solution or “recharged” with the antifungal drug. Lastly, lack of cytotoxicity against 3T3 mouse fibroblasts was confirmed; however, it should be considered that the antifungal tests were done in PBS media, whereas the cytotoxicity tests were conducted in DMEM media with added serum. This difference raises the question as to what role the media and sera, into which the antifungal drug is being released, play, and how they affect the release kinetics and activity. Ideally, antifungal and cytotoxicity tests should be done in the same medium, to avoid artefacts due to association between drug molecules and components of one or the other medium. Unfortunately, to date, there are no publications comparing the effect of media on the release kinetics and potency of antifungal drugs.

3.1.5 Silver

Silver has been of considerable interest for its ability to ward off diseases even prior to the emergence of germ theory and modern medicine. Hence, it is not surprising that its qualities have also been used in the modern setting of antimicrobial coatings (Marambio-Jones and Hoek, 2010). Silver has a recognized antibacterial and antifungal effect and has therefore received attention for incorporation into commercial medical devices. However, in clinical studies, the overall benefit has not been clearly established and considerable discrepancies exist in findings reported in the literature (Brennan et al., 2015; Schierholz et al., 1998). More recently, evidence has emerged indicating cytotoxicity of silver (ions), as well as inactivation of silver ions in protein-containing media (AshaRani et al., 2009; Marambio-Jones and Hoek, 2010; Xiu et al., 2012). However, research into the use of silver as a surface-active antimicrobial remains a highly active research area, though antifungal applications have received far less attention than antibacterial usage. In this context, we now highlight a few examples of antifungal surface coatings based on silver.

A straightforward coating approach, by Roe and co-workers, involved the reduction of silver nitrate by tetramethylenediamine in the presence of Tween 20 as a surfactant and deposition onto catheters (Roe et al., 2008). As expected, the growth of *C. albicans* and other pathogens on silver-coated catheters was inhibited *in vitro*. The same coatings were also produced using the radioactive isotope 110 of silver and implanted subcutaneously into the dorsum of mice for ten days. Results revealed that almost no silver was excreted by urine, yet up to 8 % of the total implanted silver was lost through faeces, mostly within the first four days. A further 3 % was recovered from the tissue surrounding the implant site and almost no silver was detected in the ribcage or other organs. Hence, the authors suggested that the silver would be unlikely to cause systemic toxicity; though there was no mention of effects on adjacent, exposed tissue. Additionally, the authors speculated that there could be the possibility for the emergence of pathogens with a higher resistance to silver due to increased effectiveness of efflux pumps.

A more targeted approach was investigated by Prucek et al., who synthesized magnetic binary nanocomposites of silver and iron oxide (Prucek et al., 2011). The aim of these magnetic nanocomposites was to direct the antimicrobial particles via a magnetic field to the target area, and to allow for the facile collection of the silver particles at the end of the treatment. The nanocomposites proved to be highly antifungal against *C. albicans*, *C. tropicalis* and *C. parapsilosis* in solution. However, the authors noted that the nanocomposites were cytotoxic towards NIH3T3 cells; this is consistent with the many reports about silver’s cytotoxicity.

The antimicrobial activity of silver was also investigated in combination with nitric oxide (NO) in the work of Privett and co-workers. NO has a demonstrated antimicrobial effect, also capable of mitigating the proliferation and adhesion of various microbes including fungi (Brunelli et

al., 1995; Neufeld and Reynolds, 2016; Ullmann et al., 2004). For this purpose, amine containing xerogels were produced on glass slides via siloxane chemistry and then charged with pressurized NO to generate diazeniumdiolate groups that are known to release again the short-lived NO radical molecule (Privett et al., 2010). The xerogels were challenged with *C. albicans* under flow conditions, which revealed a decrease (20-35 %) in fungal adhesion that was more pronounced at lower flowrates. This result led the authors to suggest that NO alone, while reducing adhesion, may not be sufficient for preventing fungal colonization. For this purpose, silver suldaziazine was added in sub-fungicidal concentration to the fungal solution and the drug appeared to work synergistically against fungal adhesion with the NO releasing xerogels.

3.2 Non-release coatings

In this section, two strategies are summarized that could prevent colonization of medical devices by pathogenic fungi. First, we discuss barrier-type coatings that physically disrupt the adhesion process. Second are surfaces or coatings that contain antifungal molecules.

3.2.1 Anti-fouling/anti-adhesive coatings

Anti-fouling/anti-adhesive coatings work by reducing the surface affinity of biomolecules to preferentially adsorb to surfaces (Figure 4). These can be structurally quite simple coatings, such as hydrogels, or involve more complex architectures, such as polymer brushes. In the biomaterials literature the ability of hydrogel coatings, of various chemical compositions, to reduce or altogether prevent attachment of mammalian cells is well known. A number of studies have also investigated the use of such “antifouling” or “anti-adhesive” coatings to prevent attachment and biofilm formation by fungal organisms. For example, Roosjen et al. covalently-attached poly(ethylene oxide) polymers to glass surfaces, resulting in a polymer brush layer. This low-fouling polymer layer was effective in causing a 70 % reduction of growth of *C. albicans* and *C. tropicalis* after four hours compared with the uncoated glass surface. Interestingly, the observed reduction for Gram-positive and Gram-negative bacteria was generally higher, and it was reasoned that the smaller size of the bacteria was the cause (Roosjen et al., 2003). In subsequent work, polyacrylamide was densely grafted from a model substrate using SI-ATRP. On these brushes, the adhesion of *C. albicans* was decreased by 70 % (compared to the uncoated surface). However, the strength of adhesion was found to be very weak, not only on the brush surface but also on the control (silicone) surface where more than 99 % of the pathogen cells could be removed by passing an air bubble over the surface (Roosjen et al., 2006).

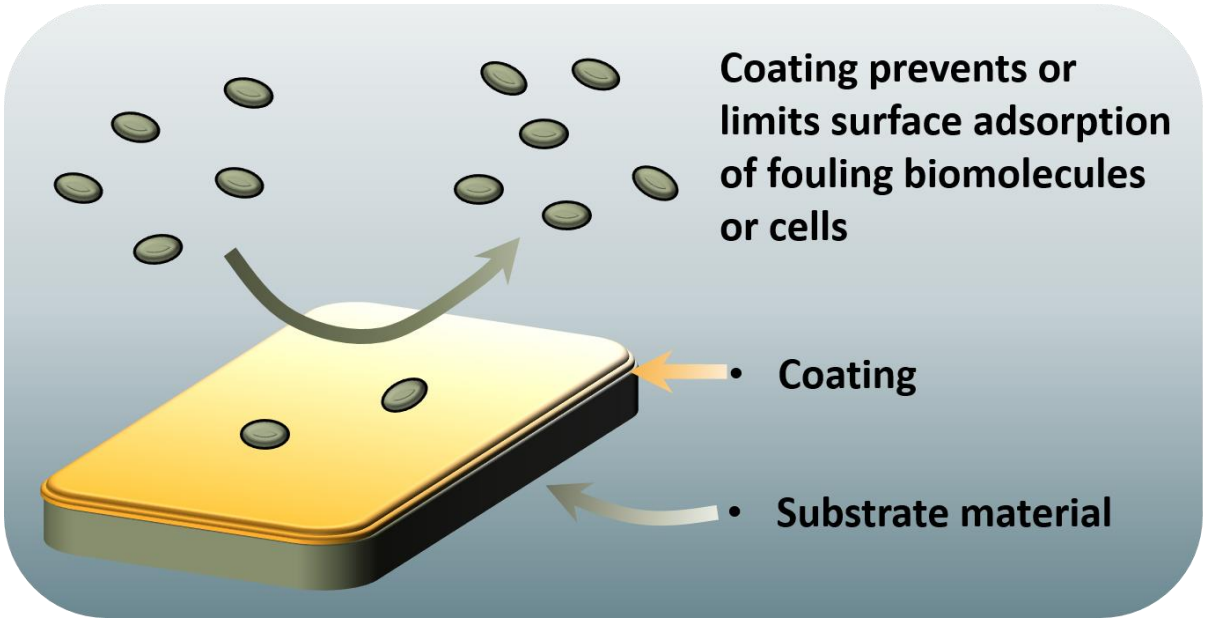

Figure 4. Anti-fouling (anti-adhesive) coatings repel adsorbing biomolecules. This strategy aims to prevent the first stage in biofilm formation on the device (adhesion) without necessarily combatting the pathogens themselves. Such coatings on medical devices, however, do not address how to encourage integration with mammalian tissues.

These two studies, and others, show that barrier coatings of polymer brushes are effective at reducing yeast adherence by more than two-thirds. This reduction is, however, less than observed for mammalian cells. Clinical utility of such coatings would depend upon the implantation site of the device, and whether flow forces surrounding the surface coating could provide forces sufficient to remove weakly adhered organisms. The lumen of urinary catheters would seem a possibility. Furthermore, a question remains about viable, yet non-device adhered organisms and whether these could then cause infection in surrounding tissues. Moreover, given the fast replication rates of micro-organisms, a reduction of 70 % amounts to a rather short-lived reduction; three orders of magnitude or more are considered desirable.

A study conducted in 2010 explored how hydrophobic interactions impact adherence of *Candida* spp. to acrylic surfaces that compose denture materials, aiming to reduce microbial biofilm formation and denture-induced stomatitis (Yoshijima et al., 2010). For this purpose, hydrophilic substrates were created by coating acrylic plates with carrageenan and hydrocolloid, and challenged with 27 isolates from *Candida* spp. (including *C. albicans*, *C. parapsilosis*, and *C. glabrata*). Additionally, a hydrocarbon adherence assay was used to measure cell surface hydrophobicity, which involved measuring the turbidity of the aqueous fungal cell suspension. The authors concluded that coating acrylic plates with hydrophilic materials decreased the adherence of hydrophobic hyphal *Candida* spp., without affecting the adherence of non-hydrophobic *Candida* spp. cells.

3.2.2 Contact killing

Using well-known chemical reactions, many antifungal drugs can, in principle, be covalently attached onto suitable complementary chemical groups on medical device surfaces. Such covalent bioconjugation immobilizes the drug molecules permanently on the device surface (Figure 5). The antifungal agents are thus incapable of leaching into solution (and adjacent tissues) which offers both advantages and disadvantages. It is important to consider interfacial phenomena between the

coating and cells. On the one hand, this approach presents attaching pathogens with a locally high concentration of the antifungal agent on the surface; on the other hand, the immobilized drug molecules do not act upon fungal colonization of the surrounding tissue that is not in immediate contact with the coating. Furthermore, the antifungal agents of choice must target an extracellular component of the pathogen; intracellular targets are obviously not amenable to surface-bound drug molecules. In the case of fungi, this strategy often targets either the β-D-glucan synthase enzyme (using echinocandin-class drugs), ergosterol (using polyenes), or the lipid bilayer itself (using cationic polymers or antimicrobial peptides). Furthermore, immobilization of a compound on a surface inherently leads to a restriction of its rotational freedom.

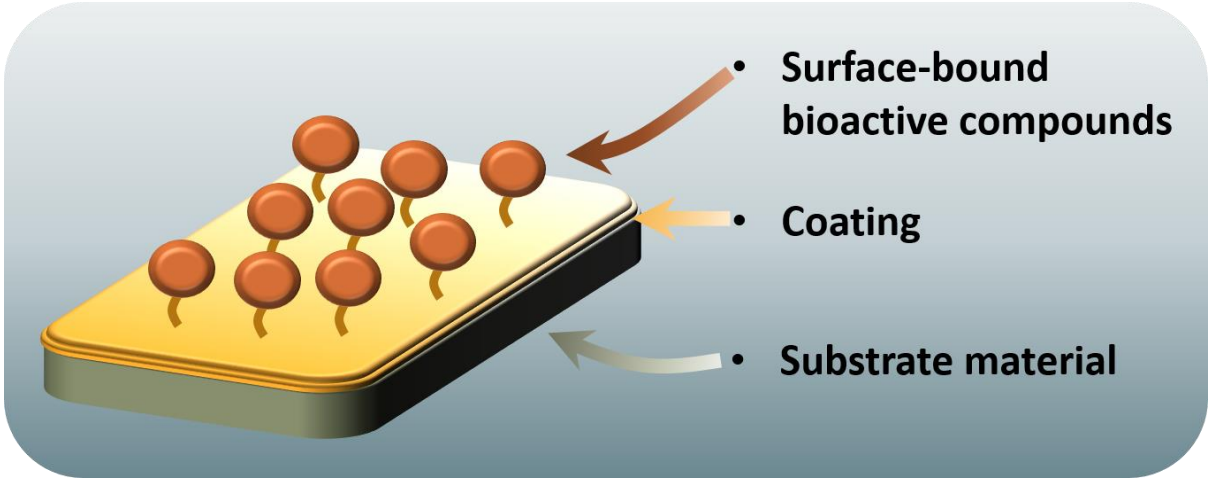

Figure 5. Schematic of materials with surface-bound bioactive compounds. Medical device materials with covalently attached biomolecules could act as "contact-killing" surfaces upon exposure to fungal pathogens. For this to be true, it is important to analytically verify that prepared surfaces do not leach bioactives, otherwise, the mechanism of action could be unclear and confused with releasing materials.

In the interest of both scientific rigour and quality control in manufacture, sensitive surface analysis methods must be employed to determine if the compound indeed remains covalently attached to the medical device surface; otherwise, an antifungal effect may be observed, but could be due to possible release or desorption of adsorbed compounds (Coad et al., 2016); such unintended release would require additional *in vivo* testing of toxicity to adjacent tissue and possible effects on organs.

3.2.2.1 Grafted antifungals

Regulated antifungal drugs that are approved for clinical use are a promising class of antimicrobial compounds because they have a well-known mechanism of action and known toxicity limits. Compared with antibacterial drugs, there have been very few reports of surface-grafted antifungal drugs since the time of our review on the topic of surface-immobilized antifungals (Coad et al., 2014).

Coad et al. demonstrated irreversible attachment of the antifungal drug caspofungin using a substrate-independent coating technique which could be applicable to different bulk biomaterials as an essentially two-dimensional surface coating (Coad et al., 2015; Griesser et al., 2015; Michl et al., 2017a). It was shown that using only water or buffer washes was not sufficient in removing the reversibly-bound caspofungin. In fact, after such washes, the majority of the caspofungin (two-

thirds) was available to reversibly desorb. However, SDS washing at 70 ^{o}C was shown to be necessary for preparing a surface with only covalently attached caspofungin. Covalently-bound caspofungin was effective in eliminating *C. albicans, C. tropicalis, C. parapsilosis* and *C. glabrata* using a contact-killing assay. The surface coating was also found to have no adverse effects on attachment and growth of mammalian primary fibroblasts (Griesser et al., 2015). Finally, the killing ability of surfaces with attached caspofungin was not diminished even after pre-conditioning the surface with adsorbed layers of protein (Michl et al., 2017a).

Kucharikova et al. examined caspofungin binding to titanium surfaces (Kucharíková et al., 2015). Biological testing revealed that the caspofungin-treated materials completely prevented biofilm formation *in vitro* and inhibited by 89 % *in vivo* (rat model). As a potential therapy for bone implantation, these surfaces showed very good potential for promoting osseointegration. However, the simple method of washing, the reported high surface density of caspofungin (much exceeding what one would calculate for a grafted layer), and the SEM images suggest the possibility that at least some of the caspofungin molecules present were in the form of physically adsorbed multilayers. This raises the question of whether exposure to the surface (contact killing) or exposure to released molecules (solution killing) is responsible for the antifungal effect. Such questions would be important to investigate for the longevity of implanted devices.

Another surface coating technique uses the concept of polymer brushes (as described above), not as a storage matrix, but as an attachment matrix to extend the reach of covalently-attached bioactives from the surface (Figure 6). This provides an opportunity to present antifungal drugs on polymers that have improved conformational flexibility that may help them interface with the fungal cell wall. Also, the supporting polymer brushes can themselves be low-fouling which reduces the potential for fungal attachment or surface fouling by biomolecules (see section 3.2.1). Michl et al. showed that polymer grafts from 2-hydroxyethylmethacrylate (HEMA) had a substantial effect on *C. albicans*, reducing their surface attachment and preventing formation of hyphae (Michl et al., 2017b). However, the yeast-form remained alive on the brush surface. When conjugated with caspofungin, greater that log 4 killing was observed. A slight biocidal effect was observed in the supernatants above the grafted surface, indicating a proportion of caspofungin had released into solution. However, this effect was small compared to cells exposed to the grafted surface suggesting the dominant effect of the contact killing mode of action. All of these studies reinforce the importance of considering the possible release of antibiotic agents and whether they can be classified as contact-killing or through a drug release mechanism (see section 3.1.2).

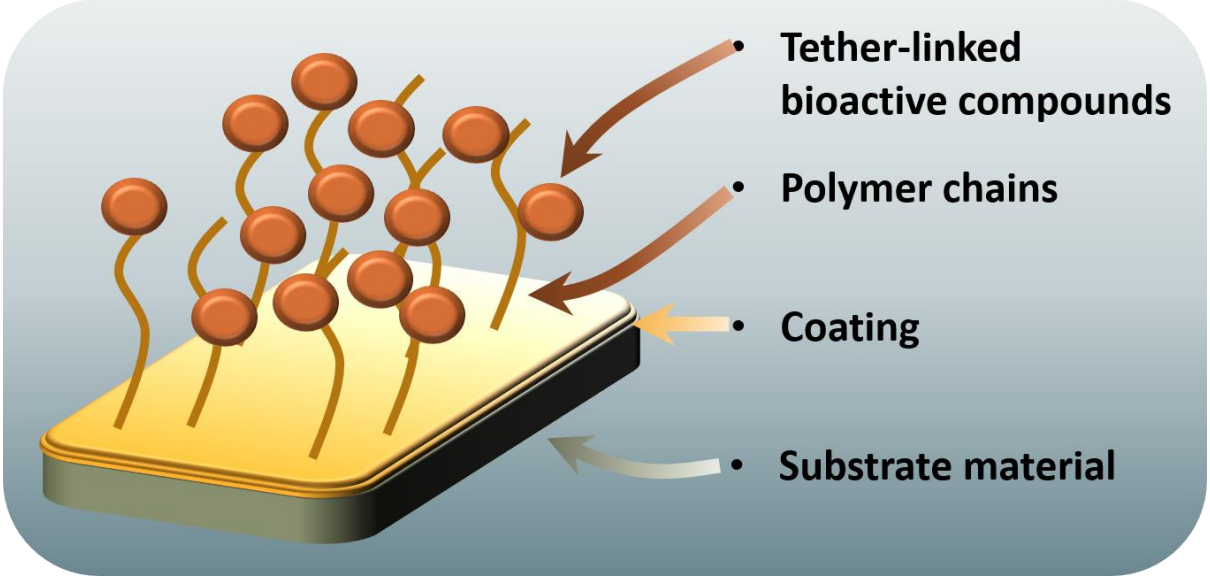

Figure 6. Schematic of materials with bioactive compounds tethered to polymeric linkers. In this strategy, bioactive compounds are covalently attached to polymer chains that extend away from the surface of the coating on the medical device. The polymer grafts present the compounds on flexible linkers providing a dynamic interface when exposed to fungal pathogens.

3.2.2.2 Peptides

Antifungal peptides are a novel class of antimicrobials that are being investigated as anti-infective surface coatings. Li et al. investigated surface tethered antimicrobial peptides to poly(allyl glycidyl ether) polymers grafted from polydimethylsiloxane (PDMS) or urinary catheter substrates (Li et al., 2014). These materials were antimicrobial against *C. albicans* as well as *E. coli* and *S. aureus*. To rule out non-covalent attachment to the substrate, the non-grafted PDMS material was shown not to adsorb the peptides. However, physisorption within the polymer brush was not tested (this mechanism could operate for the delivery of antifungals as shown in section 3.1.4). This possibility could have been investigated by first quenching the reactive epoxy groups with a small nucleophile and then adsorbing the peptides to see if they were subsequently released. Alternatively, biological evaluation of the supernatants could have been used to assess the release of any peptides. These constructs were found to be non-cytotoxic to smooth muscle cell lines.

3.2.2.3 Small MW organics

Lin and co-workers reported on the use of polystyrene which was modified with various chlorinated or iodinated amides for antifungal applications (Lin et al., 1996). Importantly, these modified polystyrenes were tested for their ability to leach halogens, after sufficient washing cycles. Testing against *C. albicans* and *Saccharomyces cerevisiae* (*S. cerevisiae*) revealed that there was no zone of inhibition around the modified polystyrene in diffusion tests, yet swift killing (in less than one hour) occurred upon contact in solution. This effect was more pronounced for shorter amide chains and more so for Iodinated- than for chlorinated-polymers. The biocompatibility and mode of action were not reported for these materials.

Lino and co-workers infused the hydrophobic antifungal amphotericin B into oxidized dextran, to improve its water-solubility, and then in turn conjugated to silica nanoparticles with a diameter of either 5 nm or 80 nm. Finally, these functionalized nanoparticles were then incorporated into a commercial dental resin at 2 % w/w (Lino et al., 2013). Both the particles as well as the particle-loaded resin were tested against *C. albicans, C. tropicalis, C. parapsilosis, C. glabrata*

and C. krusei. The functionalized 5 nm particles and resins loaded with nanopaticles showed a better performance compared to the 80 nm analogues which the authors explain with a higher surface to volume ratio. Furthermore, the authors claim that the particles and surfaces are contact-active only as decanted media lacked any antifungal activity; hence exploiting the poor water-solubility of amphotericin B to confine it locally.

Plasma polymerization, was used to deposit antifungal coatings in one step using a substrate-independent method (Lamont-Friedrich et al., 2016). For this, 1,1,2-trichloroethane was plasma deposited onto plastic substrates using previously detailed plasma polymerization methods (Griesser, 1989; Michl et al., 2014; Michl et al., 2015; Michl et al., 2016; Vasilev et al., 2011). Although non-releasing, low molecular weight chlorine species were present and were biocidal to fungal cells at the surface. The antifungal activity was demonstrated against the dimorphic *C. albicans* and non-dimorphic *C. glabrata*, by suppressing their surface colonization in excess of 24 hours. Finally, it was concluded that the mechanism of action and activity against other clinically significant fungi warrant future investigation.

3.3 Potential for generating microbial resistance from antifungal biomaterials

Is it possible that antifungal surfaces and coatings on medical devices that act prophylactically could contribute to the problem of antifungal resistance? For example, for releasing coatings, does a diminishing antifungal concentration away from the implant establish a sub-inhibitory concentration thereby inducing resistant populations? To our knowledge there are no studies that have looked at this question in the specific case of fungal pathogens. In bacterial applications, Campoccia et al. reviewed this scenario in the context of gentamicin releasing bone cements and the induction of gentamicin-resistant populations (Campoccia et al., 2010). *In vitro* and clinical data clearly show that the possibility exists. This possibility, however, is dependent on many factors, including the nature of the releasing matrix and the concentration of the inoculum, with these findings most relevant to orthopaedic implants and formulated as bone cement. Evidently, more studies need to be conducted in this area.

With contact-killing surfaces (non-release), again, no studies have investigated specifically the possible induction of antifungal resistance by contact with antimicrobials permanently tethered to materials surfaces. For bacteria, there have been arguments put forth based on limited studies. Intuitively, surfaces that are incapable of releasing antifungal agents (because they are covalently attached) would prevent diffuse concentration gradients and may be more effective in preventing acquired resistance. Antoci et al. used covalently attached vancomycin surface coatings for a study involving one-month continuous exposure to *S. aureus* and found no evidence of acquired resistance (Antoci et al., 2007). It can be argued that even if a very small proportion of an antifungal agent were to become desorbed, hydrolysed, or otherwise released from the surface, the resulting solution concentration would likely be several orders of magnitude below the minimum inhibitory concentration. In the worst-case scenario of the complete elution of all compound from a surface, there might be a total dose of several hundred ng per mL in the surrounding fluids, which could then be adsorbed as well as diluted in tissues or otherwise cleared through the circulatory system and the immune response. Such complete failures would be unlikely for a regulated implant. Due to the growing concern of antifungal drug resistance, however, the possibility of induced resistance needs to be assessed experimentally.

4.0 Challenges in the design of antifungal surface coatings

4.1 *in vitro* assays for evaluating antifungal surfaces

Currently, *in vitro* assays are limited in their ability to imitate *in vivo* conditions. Buhmann et al. discussed the significance of incorporating clinically relevant conditions when testing anti-infective surfaces *in vitro* to assist in predicting *in vivo* function. The article further suggested that the failure of many current anti-infective device coatings may be due to this limitation of *in vitro* tests not providing a realistic test environment. The importance of growth medium, fluid flow, intracellular interactions and the material properties including roughness, topography, chemical and mechanical properties were all emphasized as having a role. Consideration in designing *in vitro* assays that mimic *in vivo* conditions will aid in translating more anti-infective surfaces into the clinical setting (Buhmann et al., 2016).

In a letter replying to that paper, Qu and co-workers highlighted appropriate conditions for screening anti-infective surfaces in the microplate biofilm assay, and suggested suitable media, adhesion and biofilm formation conditions, and solutions to some of the problems associated with common device-associated biofilm producing microorganisms. Their letter also highlighted the need for co-culture of different bacterial and fungal species in consideration of polymicrobial *in vivo* conditions. The importance of screening anti-infective surfaces in the presence of proteins, using media such as foetal bovine serum, was also highlighted (Qu et al., 2017).

Finally, evaluation of contact-killing antimicrobial surfaces by the use of various standard methods can often yield conflicting results. In the case of evaluation of antibacterial surface coatings, it was recently shown that the evaluation of the antimicrobial efficacy of a surface coating was dependent on the choice of assay (van de Lagemaat et al., 2017). This example illustrates the importance of choosing an assay, how the results are interpreted, and the need to use complementary testing methods, as discussed below.

4.1.1 Assay Design

Multiple key outcomes in designing a microbiological assay to test a surface must be considered. The foremost concern should be the number of replicates. Due to the inherent variability of microbiological and cellular based assays, it is vital that a minimum of three biological replicates and three technical replicates are utilized with appropriate controls present. Replicates should be tested against different surface batches, allowing for the rigorous testing of the surface, ensuring reproducibility of the *in vitro* assay is present.

Outcomes of *in vitro* studies are dependent on the fungal strain used. Ideally the surfaces should be tested with both a typed strain (ATCC –American Typed Culture Collection) and a clinical strain. The use of a widely reported strain validates the work amongst peers, while the use of a wild-type clinical strain demonstrates potential effectiveness of the surface in clinical applications. Fungi are known to lose virulence after repetitive sub-cultures in laboratory conditions (Butt et al., 2006). Strains lacking particular virulence genes or gene-deleted strains are not suitable to use in initial surface testing and are more pertinent to the mechanism of action based experiments, where one requires a better understanding of the way the fungal cell and the surface interact. Care must also be taken when using drug resistant strains as these strains are phenotypically different compared to their wild type counterparts with reduced fitness that can produce a varied response (Ben-Ami et al., 2011). The other factor that must be considered in strain selection is whether or not the strain can produce a biofilm. The difference in results between planktonic and biofilm producing strains may significantly impact on the interpretation of a surface's function.

4.1.2 Assay selection

Selecting complementary assays can generate a more complete picture of how well the antifungal surface is performing. For a more complete understanding of the effects of an antifungal surface, multiple microscopy and quantitative methods are required (Martinez et al., 2010) where correlative data should be present.

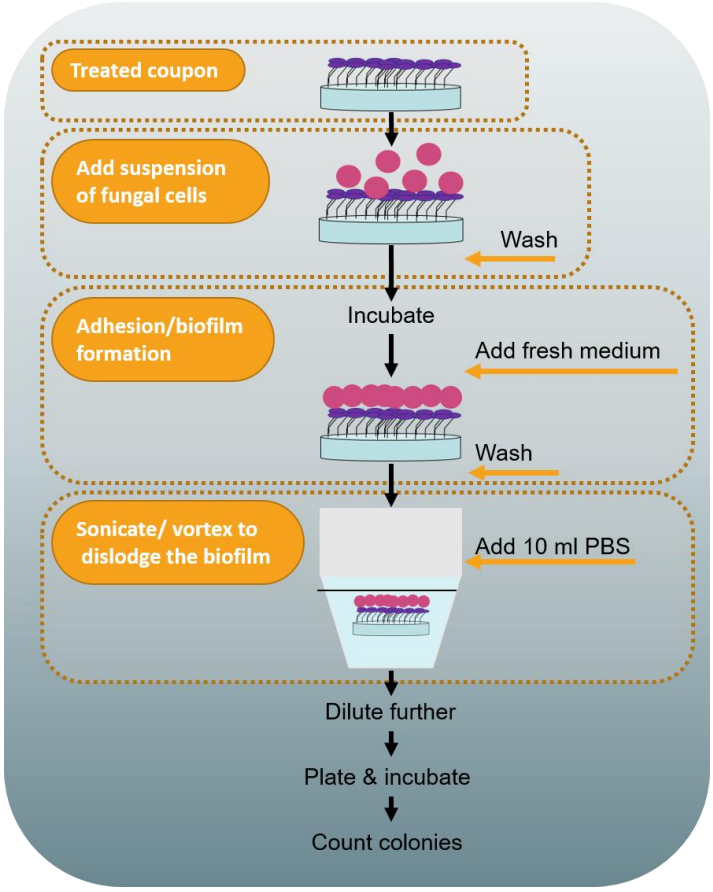

Figure 7. Schematic steps involved in the microtiter plate biofilm assay of antifungal surface coatings against biofilm-forming yeasts (Chandra et al., 2008). Treated coupons are materials with antifungal surface coatings applied. These are incubated with fungi where initial attachment to the surface is allowed to occur. After biofilm formation, test surfaces are washed and the biofilm is removed by sonication/vortexing. Viable colony forming units in solution are appropriately diluted, plated and counted.

Chandra et al. reviewed *in vitro* assay methods for *Candida* spp., summarising some precise methods for biofilm assays and including troubleshooting tips (Chandra et al., 2008). The general schematic for this assay is shown in Figure 7. This review further emphasized the need to use multiple microscopy methods including fluorescence microscopy, confocal scanning laser microscopy (CSLM) and scanning electron microscopy (SEM) to better determine the morphology and architecture of the fungal biofilm. Each method adds complementary information. Fluorescence microscopy provides whole biofilm images and is limited to a 2D plane of the biofilm; however, this method can be useful when investigating adhesion over time on antifungal surfaces. CSLM presents an insight into the 3D structure of the biofilm matrix in a low resolution, yet does allow for the observation of a hydrated biofilm matrix. While SEM can offer greater information on the surface topography, a major limitation is that the SEM preparation dehydration steps can particularly degrade the biofilm matrix (Chandra et al., 2008). Live-dead cell staining kits for fluorescent

microscopy are useful for discriminating yeast cells based on permeability to propidium iodide (Figure 8). Older staining methods including crystal violet are limited, due to the stains inability to differentiate between live and dead cells and the variation that is seen in the assay (Chandra et al., 2008).

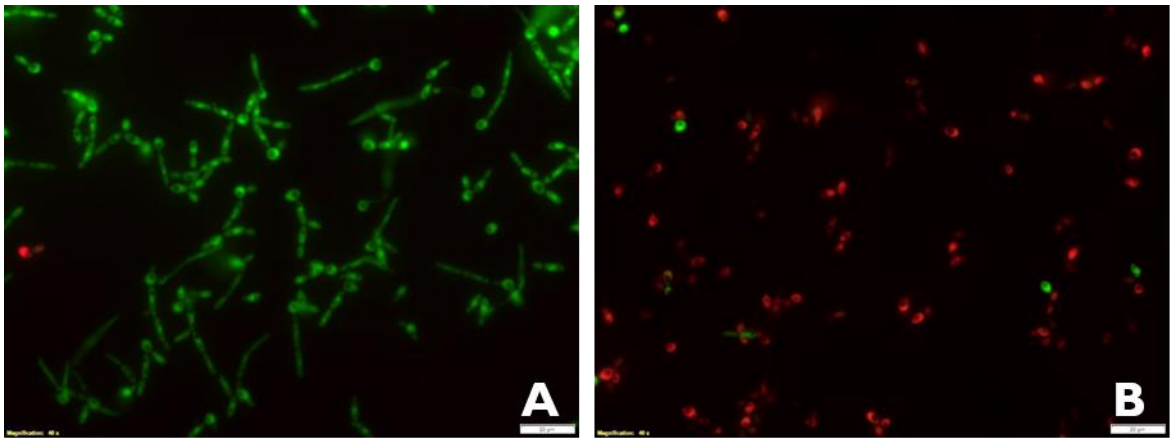

Figure 8. Live-dead cell assay for visualising cell viability on an antifungal surface coatings. *Candida albicans* was exposed to surfaces and imaged after 180 minutes using live-dead cell staining. A) On a control surface showing live cells (green) with normal pseudohyphal development. B) On a caspofungin-grafted surface coating, showing dead cells (red), cellular debris and no hyphal development. Magnification 40 X; scale bar 20 µm. Unpublished data from the Coad and Griesser research group. Methods for manufacture of caspofungin-grafted surface coatings were previously described (Coad et al., 2015).

The XTT assay is a commonly used colorimetric tetrazolium salt-based assay when used alone to determine surface activity and fungal metabolism has a number of limitations. Thus use of this assay must be considered carefully if it is to be deemed quantitative. Considerations include the lack of relationship linearity between organism number and colorimetric signal. Also, a variance in tetrazolium metabolism rates occurs in different fungal strains and species. Some strains retain a significant amount of the XTT product formazan intracellularly, and formazan is not water soluble as previously documented (Roehm et al., 1991). Furthermore, the differential release of formazan from biofilm and planktonic cells must also be considered. Finally, the XTT assay has not demonstrated a linear relationship between the concentration of XTT used and the colorimetric signal produced. Due to these assay limitations an appropriate standard must be present to validate the concentration of tetrazolium used and is not suitable for the comparison of different fungal strains (Kuhn et al., 2003). This assay is a good indicator of cellular activity; however, it must be conducted in conjunction with other assays to determine antifungal surface activity and care must be taken when the XTT assay is considered quantitatively.

An alternative quantitative method that can be utilized to examine antifungal surfaces is the microtiter plate biofilm assay (Coenye and Nelis, 2010) where the detailed methodology has been described previously (Chandra et al., 2008; Michl et al., 2017a; Michl et al., 2017b). This assay provides a quantitative measurement of the colony forming units and the biofilm formation on antifungal and control surfaces. Care must be taken in ensuring the adhesion and growth times are adjusted according to the species being tested. This method is limited to yeast-based fungi and bacteria but may possibly also be useful in quantitating polymicrobial biofilms with the use of appropriate controls and microorganism selection.

Nutrient flow based assays utilising a modified Robbins device have also been reported to test the activity of anti-Candida biofilms (De Prijck et al., 2010), with flow displacement and biofilm model systems reviewed previously for *Candida* species and bacteria (Coenye and Nelis, 2010).

Care must also be taken when selecting and designing an assay. Considerations include the clinical application for which the antifungal surface is aimed and the type of microbes which colonize this environment, while keeping in mind the type of surface and the appropriateness of the trial design (Buhmann et al., 2016). Fouling substances such as proteins or cellular debris must also be considered and an investigation into the effects of these factors and their activity need to be undertaken. The role of the immune system should also be considered in its interaction with the surface of the biomaterial or biomedical device. Awareness of the limitations of the *in vitro* assay is vital to the success of the investigations. The effects of nutrient availability, flow, pH, time, and shear forces have all been noted for their effects on cell adhesion and biofilm growth (Buhmann et al., 2016; Soll and Daniels, 2016). These variables should mimic conditions most similar to the biological/physiological environment the material surface will encounter in its intended application.

4.1.3 Cell culture assays

Care must be taken when making statements about the growth of mammalian cells on the surface of antimicrobial materials and the cytotoxicity of the surface to mammalian cells. Choice of cells is vital to soundly understand the toxicity of the surface. Selecting primary cells corresponding to cellular types that will likely be in contact with the device at its intended implantation site is best. Transformed or continuous cell lines are not considered 'normal' cells and often have differing phenotypes and physiology compared to primary cells and this must also be considered.

Hemocompatibility testing of a surface is also important for biomaterials intended to be exposed to blood to test for platelet activation, adhesion, aggregation or haemolysis of red blood cells (Adams et al., 2003; Hilpert et al., 2009; Kazemzadeh-Narbat et al., 2013). The use of erythrocytes to test cytotoxicity does not translate to other metabolically active cell types due to the haematopoietic origin of these cells, lack of a nucleus, no coding DNA, lack of redox energy, short lifespan and their inability to self-divide. For these reasons erythrocytes are not suitable for pre-implantation studies alone and a primary cell type from the local area of implantation should be considered.

4.2 *In vivo* evaluation: critical issues

A review by Soll and Daniels has extensively reviewed *in vivo* models that have been used to study the formation of *Candida* spp. biofilms on biomedical devices and model materials. That review describes, in detail, intravenous catheter models, urinary catheter models, denture models and indwelling subcutaneous pouch models (Soll and Daniels, 2016).

In vivo catheter models are common for testing antifungal strategies with biomedical devices (Martinez et al., 2010). Various small animal central venous catheter (CVC) animal models have been developed, including rat (Andes et al., 2004), rabbit (Schinabeck et al., 2004) and mouse (Lazzell et al., 2009), which have shown promise for understanding biofilm formation on CVC devices and possible treatments (Coenye and Nelis, 2010). The CVC model is limited by the ability to coat the internal surface of the catheter tubing where the *Candida* spp. is administered. However, studies have shown some success with pre-inoculation of a treated catheter in a rat *in vivo* model (Martinez et al., 2010). Tail vein administration of *Candida* spp. serves as a method to establish a disseminated *Candida* spp. infection and can be useful to monitor planktonic cell levels and systemic antifungal treatment (Nett et al., 2007; Nett et al., 2011).

An alternative *in vivo* model that may be more appropriate to testing surfaces is the rat indwelling urinary catheter model. This model successfully demonstrated the proliferation of *C. albicans* on the surface of the device, including the development of adherent yeast and filamentous cells in the extracellular matrix of the biofilm (Nett, 2014). This model utilized quantitative methods to monitor the infection within the bladder and the biofilm formation on the external surface of the catheter and SEM to study the biofilm matrix. The authors detail the problems associated with using silicone catheters for SEM assessment, where the biofilm can peel away from the surface during the dehydration step (Nett, 2014), such an artefact was not seen previously when polyethylene was used (Andes et al., 2004; Wang and Fries, 2011). A urinary catheter model would be suitable to test impregnated catheters or catheters with surface modification. The model also takes into account the effects of proteins, immune system factors, and cell adherence on the material surface. An ascending candiduria catheter mouse model has also been established (Wang and Fries, 2011).

Subcutaneous models have also been developed. Such models pre-inoculate the implant with microbes to mimic a typical surgical contamination load on the implant. If the surface is pre-inoculated using 60-90 minutes of adhesion time, this protocol is considered to mimic the conditions that the implant encounters when contaminated on insertion. This method models the ability of the surface to withstand microbial loads in an environment under physiological conditions (Coenye and Nelis, 2010). A subcutaneous pocket model in rats has also been developed using pre-inoculated catheter segments implanted in the subcutis of the rat (Řičicová et al., 2010).

Oral candidiasis is predominantly caused by denture-associated stomatitis, whereby *Candida* spp. biofilms form on the prosthetic denture surface. A rat denture stomatitis model was developed which imitates *Candida* spp. biofilm formation and the histology often seen in human cases. One limitation encountered in this model is the size of the device. A higher burden of enteric microbes is present in the rat compared to humans due to their natural cleaning behaviours and therefore appropriate antimicrobials should be administered prior to denture (Nett et al., 2011).

4.3 Surface analysis

Surface analysis is imperative for the characterization of biomaterials. Several prominent reviews describe the various analytical techniques available, in the context of medical implant materials (Born et al., 1998; Kingshott et al., 2011; Merrett et al., 2002). For antifungal biomaterials (or antimicrobial biomaterials in general) chemical and physical characterization of the surfaces of implantable materials provides the first understanding of the way in which these materials potentially act with biological systems. For example, for releasing coatings, at the very least, surface analysis can confirm the presence and provide an estimate of the active antimicrobial compound, and details of its release kinetics. These will be important for regulatory bodies in the approval process for novel medical devices.

Detailed surface analysis will also provide new insights into cell/surface interactions and could potentially reveal novel mechanisms of action. This approach is illustrated well by the case of contact killing surfaces. If it can be analytically verified that surface-attached antifungals are incapable of being released or desorbed from surfaces, then it can be proved that active antifungal compounds can act through novel surface interactions while conformationally constrained. This represents a different understanding of a drug's pharmacokinetic profile and such an arrangement can be thought of as new surface-active formulations of a drug. Perhaps immobilising "failed" drugs to surfaces (i.e. drugs not approved due to evidence for accumulation in kidneys and liver) might be one way of finding new uses for unused, yet highly effective antimicrobials, thus enlisting old

compounds for new purposes in the fight against device-related infections. Such devices, of course, would need testing to prove their efficacy and safety.

4.4 Selectivity

Selectivity, in the context of antimicrobials, refers to the efficacy of an antimicrobial agent to inhibit a pathogenic organism while providing minimal adverse effects on cells and tissue of the host. Perfectly selective antimicrobial agents would interact via a cellular pathway that is unique to the pathogen and thus absent in host cells. Of course, perfect selectivity is not achievable because human cells and microbes have shared evolutionary history and, therefore, possess a number of conserved biological structures, such as a cell membrane. However, subtleties are important here and phospholipid membranes provide a good example of the differences between fungi and bacteria. The cell membranes of bacteria are different from those of eukaryotic organisms. In bacteria, acidic phospholipids (bearing partial negative charge) tend to be presented on the outer and inner (cytosol) leaflets of the cell membrane. In contrast, eukaryotic organisms (including fungi and mammalian cells) have active metabolic processes that sequester these phospholipids on the inner leaflet of the cell membrane (Cerbón and Calderón, 1995; Devaux, 1991). The result is that some bacterial membranes tend to possess more negative character on their outer cell membrane. This difference explains the selectivity of cationic compounds such as quaternary ammonium ions, peptides, and polysaccharides towards selective killing bacterial cells (over mammalian cells). In contrast, because of their structural similarity, cell membrane arguments alone should not be used to explain how cationic agents might selectively kill fungal pathogens while not harming mammalian cells in so-called “broad spectrum” antimicrobials (Engler et al., 2012; Li et al., 2011). Instead, the particular structural biology of the fungal cell wall should not be overlooked (Gow et al., 2017). As mentioned above (section 3.2.2.1), contact-killing surfaces bearing antifungal agents that target the fungal cell wall, that also possess an acceptable low toxicity in human applications (as approved by regulatory bodies) could be promising in the development of antifungal medical devices.

4.5 Toxicity

The number of approved antifungal drugs and therapies has certainly been limited severely by their toxicity (Brown et al., 2012); the number of antifungal drugs is considerably less than that of antibacterial drugs. This is mainly a result of the narrow window of selectivity, as mentioned above. For disinfectants which have a non-selective mechanism of action (i.e. indiscriminate dissolution of the phospholipid membrane), poor selectivity is evident and they are likely too toxic for practical use with antifungal implantable materials. For non-clinically approved pharmaceuticals such as cationic small molecules (i.e. quaternary ammonium compounds, cationic peptides), it is unclear whether these would possess a sufficiently low degree of toxicity against human cells. In addition to the different cell membrane composition and metabolism mentioned above, fungi and bacteria operate by different metabolic processes. Finally, as we mentioned earlier, mammalian cell toxicity needs to be evaluated using more relevant cell types. Haemolysis is often used as a benchmark for mammalian cell toxicity; however, as a model system, red blood cells may not be a perfect model to test implantable devices that will contact tissue, as they lack mitochondria and nuclei. For example, with antifungal peptides, internalization and compartmentalization within vesicles have been shown to be an important factor beyond just how they bind to membranes (Muñoz et al., 2013; Muñoz et al., 2012). Furthermore, the solution (medium) that cells are cultured in (i.e. PBS versus cell culture solutions) have been shown to change the interpretation of toxicity of antifungal peptides (Helmerhorst et al., 1999). Lastly, modelling studies have revealed that the cell density is also an important factor when considering toxicity of cationic antimicrobials (Bagheri et al., 2015). Relevant

(metabolically active) cell types and *in vivo* testing are required for comprehensive evaluation of potential antifungal biomaterials.

4.6 Scale-up and manufacturing

Researchers in applied scientific research ought to keep in mind the steps and limitations to successfully translate an idea from a laboratory bench to a commercial product; scale-up potential and ease of manufacturing are key issues to be addressed. These issues are of importance as certain procedures are relatively difficult on a small laboratory scale but commonly used in industrial settings (e.g. high-pressure and -temperature procedures), with the opposite true as well: Reagents and reaction times which are feasible in a research setting might be too costly and/or wasteful in an industrial setting (Reisman, 1993). Hence, attention needs to be paid to the following aspects.

Process type: Whereas most processes in the laboratory are done in batches, industrial operations prefer continuous fabrications due to financial and safety concerns (Lee and Lee, 2014). Industrial-scale batch processes are only feasible for high-value products, which is the case for most medical devices. Hence, having to use a (semi-)batch process for medical devices is not inherently a deal breaker as demonstrated in the case of plasma treatment of contact lenses (Der Haegen Harro Mueller et al., 1995 ; Yasuda et al., 1975).

Chemicals used: The use of “exotic” reagents for research purposes presents little obstacle whereas it needs to be avoided on an industrial scale. Furthermore, reduced use of solvents, or their omission altogether, has clear economic and ecological benefits on a large production scale. Hence, solvent-free thin film deposition methods such as PVD, CVD and PECVD are widespread and well established in industry (Oehr et al., 2017).

Reproducibility / Homogeneity: One key aspect of concern is the human factor in the equation. Whereas most labour on a laboratory scale is conducted by human operators, mechanisation and automation are the norm on an industrial scale. Such automation by robots brings about better reproducibility and precision (e.g. dip coating at a well-defined speed) (Puetz and Aegerter, 2004) but reduces process flexibility (e.g. change of the substrate’s shape and size). Furthermore, for most coatings homogeneity is another key aspect. Methods that yield good homogeneity on a small laboratory scale, such as spin coating (Norrman et al., 2005), may be difficult if not impossible to translate onto larger substrates in an industrial setting. Furthermore, certain deposition methods, such as vacuum based depositions, are highly process dependent and require re-optimisation when translated from a small to a larger scale operation (Whittle et al., 2013).

In summary, almost no laboratory process can be scaled up to an industrial grade operation by simply “enlarging” all aspects involved. Many, if not most of these aspects have a finitely scalable region in which they operate and do so well. Hence, researchers who want to conduct translatable research need to keep the hurdles of scale-up and manufacturing in mind. Otherwise, their invention in the laboratory might never grow beyond that small scale.

5.0 Summary and future outlook

It is clear from the extensive literature on clinical studies of infections associated with biomedical devices and implants that the use of such devices carries a substantial risk of serious complications that arise from microbial biofilms that form on surfaces of devices and implants and once formed, are difficult to eradicate. Moreover, such device-associated infections serve as nuclei for systemic infections. It is also clear from the literature that fungal species can play a major role in

such surface-associated biofilm infections, either by themselves or in polymicrobial communities together with bacteria. While much research has focused on studying and trying to prevent *bacterial* biofilm formation on devices and implants, analogous work aimed at *fungal* biofilm infections is more recent and much more limited in volume. However, device-associated fungal biofilm infections are predominantly formed by *Candida* spp., which are causative of serious complications and a high mortality rate if they spawn a blood-borne infection (candidemia).

Prevention of formation of biofilms on medical devices and implants is an unmet clinical need that calls for materials that can resist colonization by fungal (and bacterial) biofilms. Unfortunately, materials currently available for the fabrication of biomedical devices and implants are readily colonized – in fact, microbes have an amazing ability to attach to and thrive on a wide range of synthetic materials, from metals and ceramics to polymers. This probably represents an evolutionary advantage. Thus, it is a considerable research challenge to design and fabricate materials and devices that can resist microbial colonization. At present there are no devices and implants that effectively deter biofilm infections.

Given the challenges in clinical treatment of biofilms once they have established on devices, the challenge is for biomaterials scientists and engineers to develop devices that prevent the initial attachment of microbes and thus eliminate the first step in infectious biofilm formation. Given that manufacturers have optimized the materials properties for devices and implants, for example the strength of hip implants and the flexibility of vascular grafts, rather than replacing entire materials it seems more promising to change the surface chemistry of existing materials, or apply thin coatings, to confer antimicrobial efficacy. A number of strategies have been explored to prevent fungal biofilm formation but to date, none has reached clinical usage.

Key limiting factors are that there is a rather limited number of antifungal drugs available that can be used to fabricate such coatings and that it is difficult to predict the effects of such coatings on human cells (and thus possible adverse effects on human tissue apposite to an implant). The toxicity of antifungal drugs is tested in systemic administration and this does not allow extrapolation to how a drug would affect human cells and tissue when drug molecules are bound to a device surface. This issue needs to be better understood to enable rational design of antifungal coatings.

But probably the biggest challenge is in translation from *in vitro* data to prediction of likely clinical performance. Novel antifungal materials, surfaces and coatings are tested using protocols that comprise the use of fungal cells in suspension and assessing their ability to attach onto surfaces and progress towards biofilm formation. Such testing is an enormous simplification of the *in vivo* situation where the formation of infectious biofilms proceeds within a complex biological environment, such as wound healing. It is no surprise then that for many antimicrobial materials, promising *in vitro* data were not matched by *in vivo* performance; failure of antimicrobial coatings to deliver adequate performance in animal models and clinical studies has been all too common and the reason why none is yet in commercial usage. Given the expense of *in vivo* testing, an urgent need is obvious for developing more realistic – and necessarily more complex - *in vitro* models for the formation of fungal biofilms on medical devices.

Yet, given the toll that fungal biofilm infections continue to take, research on novel strategies to prevent fungal biofilm formation on medical devices is an absolute necessity. Novel strategies must be based on understanding of specific fungal metabolic pathways that can be addressed without side effects on human cell metabolism, such as distinct pathways of formation of fungal cell wall components, and on effective collaboration between microbiologists, materials

scientists/engineers, and clinical researchers to ensure that molecular biology targets are translated to effective materials/coatings strategies and appropriate *in vitro* and clinical testing modalities.

5.1 Short-term outlook

The next five years will bring increasing awareness to the following important points.

- Fungal and polymicrobial infectious biofilms can form on a wide range of medical devices and implants and, once formed, are difficult to eradicate, often requiring replacement of the device.
- The main device-associated human biofilm pathogens are *Candida* species, particularly *Candida albicans*. If they spawn daughter cells from biofilms, serious blood infections can result.
- A number of strategies have been pursued to equip surfaces of materials/devices with the ability to reduce or prevent the attachment of fungal cells and ensuing biofilm formation, but none has yet found usage on medical devices.
- One class of strategies is based on releasing antifungal drugs from medical devices; this has short-lived efficacy and raises the question of the fate, distribution, and possible accumulation of released drug molecules.
- Another class of strategies is based on permanently binding drug molecules onto the surfaces of medical devices and relying on the ability of such surface-bound drug molecules to kill fungal cells before they can attach onto the device. This approach, while attractive from a regulatory point of view, requires in-depth materials science and also can be affected by drugs no longer being able to exert antifungal activity when bound to a device (as opposed to being freely diffusible into the cytosol).
- It might be worthwhile to pursue research into materials that use a combination of these strategies. Dual mechanism materials would compensate for detriments of each strategy alone. However, the regulatory framework for devices that have multiple functions is demanding.

5.2 Long term outlook

While a number of studies have reported substantial reductions in fungal biofilm formation in in vitro tests, analogous performance in animal and clinical studies has not been achieved. It is evident that the currently used in vitro models are far too simplistic and do not enable prediction of in vivo outcomes. This shortcoming is a big hurdle for rational progress, and more realistic test modalities should be a high priority in research on anti-infective devices.

A major issue also is selectivity: how can one ensure effective deterrence of fungal attachment while ensuring that the same device surface interacts benignly with human cells and tissue? The use of highly selective antifungal drugs that target a specific, cell wall-associated fungal metabolic process seems indicated.

It is essential to perform detailed analysis of antifungal surfaces and coatings to ensure that they are indeed what they were intended to be and hence observed biological responses can be interpreted reliably and with confidence in terms of chemical and physical properties of the surface of the device. A key question when binding antifungal drugs onto device surfaces is to ensure that all

molecules are indeed covalently anchored, lest some of them will drift away from the surface when placed in a biological test environment.

In conclusion, there is a clear need to address the clinical problem of the formation and maturation of fungal or mixed bacterial/fungal biofilms on biomedical devices. Such research is increasing rapidly. Based on molecular microbiological understanding of specific fungal metabolic pathways, collaboration between microbiologists, biomaterials scientists, and clinicians can be expected to lead to the development of coatings that will selectively deter biofilm formation while not causing adverse effects on human cells and tissue. While manufacturability, regulatory guidelines, and cost will all need to be considered in the scale-up of laboratory-scale coatings, we are hopeful that biofilm-resistant medical devices will reach the market in the not too distant future.

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