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Candida albicans is a common member of the human gut, skin and mucosal microbiomes. Systemic *C. albicans* infections are of special concern for immunocompromised patients such transplant recipients and AIDS patients. Drug resistant strains of *C. albicans* are an enormous health concern and like most antibiotic resistant microbes pose a greater risk due to the concomitant acquisition of traits associated with pathogenicity. Traditionally microbial control involves antibiotics that target specific essential enzymes; however, over the past fifty years there has been an exponential increase in resistance of pathogenic bacteria and fungi to known antibiotic compounds. Recent developments in electron microscopy have made the study of naturally occurring nano and micro-scale hierarchical structures specially on insect wings, lotus leaves, shark skin, gecko skin yielding to their super hydrophobic and antimicrobial responses. Nanostructured surface (NSS) are attractive alternatives to chemical antibiotics because they will not be susceptible to the same pathways towards resistance as chemical agonists.

Recent work has shown that microbes including gram negative bacteria and cellular yeast are vulnerable to mechanical assaults via surfaces that exhibit high-aspect ratio nanoscale topographies. Though extensive research has been done in mimicking naturally occurring NSS and designing new surfaces for antibacterial use, efficacy as antifungal agents specially against pathogenic fungi like *C. albicans* has been ignored. The long-term goals of my project are to define the properties of nano structured

materials and use these properties for the design and application of novel materials that control pathogenic fungal growth and biofilm formation. In this research I have characterized the nanoscale mechanical interactions between *C. albicans* cells and the cell-rupturing nanostructured surface from the wing of the Cicada *Tibicen ssp*; and have defined the timing and the conditions that control NSS-induced *C. albicans* cell rupture. I have found that the NSS changes the cell wall composition of this cellular yeast and that the NSS induced rupture of this microbe is significantly slower than NSS rupture of bacteria on similar surfaces. Different drug resistant strains of *C. albicans* displayed altered responses of rupture and morphogenesis which is attributed to the mechanism of drug resistance developed in them.

Biofilm production which is responsible for the virulence and development of resistance in *Candida* sps. is also significantly reduced when incubated on NSS. Finally, I demonstrate data showing Nano-cones on the cicada wing inhibit the transformation of yeast to hyphae thus reducing pathogenicity of *C. albicans*. Following this I used synthetic biocompatible NSS as coatings on catheters to determine their antifouling and antifungal efficacy in comparison to flat surfaces. This research is extremely necessary for a systematic study of microbial interaction with the Nanostructured surfaces and define properties to synthesize novel anti-microbial polymers which can be used as coating on indwelling medical devices to improve their lifespan and as coatings in the areas of sanitation.

TACKLING DRUG RESISTANT PATHOGENIC FUNGI THROUGH ANTIMICROBIAL NANOSTRUCTURED SURFACES

by

Naga Venkatesh Kollu

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Approved by

_____
Committee Chair

APPROVAL PAGE

This dissertation written by Naga Venkatesh Kollu has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

Committee Chair _____
Dennis R. LaJeunesse

Committee Members _____
Eric A. Josephs

_____
Christopher L. Kepley

_____
Tetyana Ignatova

_____

Date of Acceptance by Committee

_____

Date of Final Oral Examination

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

1.1 *Candida albicans*

Fungi makes up to 7% of the 8.7 million eukaryotic species on earth. Of the 611,000 species of fungi, only 600 species are reported as human pathogens[1]. The infections caused by these commensal pathogens range from the common superficial infections of the skin to life threatening systemic infections[2]. *Candida albicans* (C. albicans) is the most prevalent cause of fungal infections in people. Its species name, *albicans*, comes from the Latin word for "white"[3]. *C. albicans* is an opportunistic fungal pathogen commonly found in the mucosal membranes of all humans[4]. It is classified as an opportunistic fungus because it usually only causes diseases in those who are immunocompromised or whose natural flora have been altered. *C. albicans* are unicellular diploid and are present in the oral cavity of 75% of the population[5].

C. albicans is a common member of the human gut flora and does not proliferate outside the human body. It is detected in the gastrointestinal tract and mouth in 40–60% of healthy adults[6]. These opportunistic fungal pathogens cause recalcitrant infections of the oral cavity called oral candidiasis. The fungal infection Candidiasis causes conditions like Thrush, esophagitis, cutaneous skin candidiasis, vaginal yeast candidiasis, deep candidiasis[7]. *C. albicans* colonizes on skin and mucosal surfaces which can lead to acute infections[8, 9]. Though the superficial infections are very common they are often

non-lethal but in contrast systemic candidiasis is associated with a high crude mortality[10]. Indeed, *Candida* species are the fourth most common cause of hospital-acquired systemic infections in the United States with crude mortality rates of up to 50%[4].

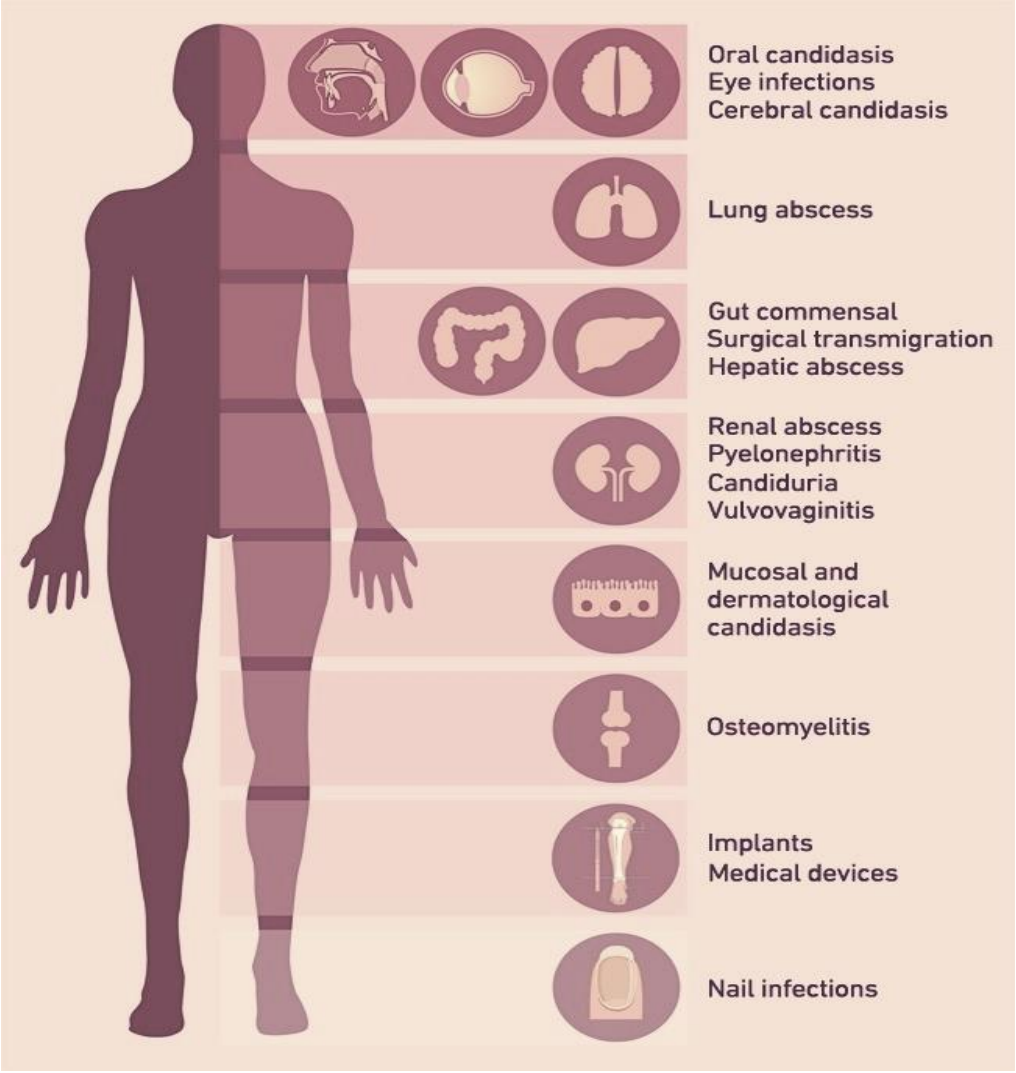


Figure 1.1. Organ/implant related infections caused by *C. albicans* in Humans

An estimate of 75% of all women suffer from vulvovaginitis at least once in their lifetime with 40–50% experiencing at least one additional episode of infection[11, 12]. This

problem is worse in third world countries where 5–8% suffer from at least four recurrent cases of vulvovaginal candidiasis per year mainly because of poor sanitation facilities[13]. As a major cause for hospital acquired infections *Candida* is the root cause for 15% of sepsis cases and 40% of bloodstream infections in clinical settings[14]. *Candida* often coexists with other dreadful microbes like *Staphylococcus epidermidis* and *Staphylococcus aureus* causing serious systemic infections. Candidemia is commonly polymicrobial and only a few rare cases of systemic infection of Staphylococcus spp. without *Candida* species were reported[15, 16], which may be due to the need of the fungal biofilm for the maintenance of the bacteria infection.

1.2 Morphogenesis and Pathogenicity of *Candida albicans*

C. albicans becomes pathogenic by its ability to transform into different morphological forms[17]. They exhibit two prominent proliferation modes the round cellular yeast where the cells elongate and bud off yielding daughter cells and the long tubular hyphal mode in which continuous lateral growth leads to elongated growth where in separate cells are delineated by septa. Most fungi exhibit polymorphism and the formation of hyphae and other cellular forms; in fact, the common mushroom fruiting body is constructed from collections of hyphae[18]. *C. albicans* has the ability to transform into different morphological forms as a response to the external signals, such as nutrient levels/sources, pH, and temperature[19]. Apart from cellular yeast and tubular hyphae, different morphologies of *C. albicans* include an opaque form which is a characteristic of mating-competent cells[20], a thick walled spore like chlamydospore formed under suboptimal conditions[21] and pseudo hyphae observed as elongated

ellipsoid cells with constrictions at the septa[22]. Various environmental conditions affect the morphology of *C. albicans* like low pH (< 6) facilitates the cellular yeast form while the higher alkaline conditions promotes hyphal growth[6], nutrient deficient conditions, stress induced by the presence of serum, higher incubation temperatures and CO_2 induce the formation of hyphal structures[23].

Microbial communication through quorum sensing, play a pivotal role in the morphogenesis of yeast[16]. Quorum sensing molecules like farnesol, dodecanol and tyrosol affect morphogenesis in *C. albicans*[24-26]. High cell densities (> 107 cells ml-1) have been reported to promote yeast growth, and low cell densities (< 107 cells ml-1) favor hyphal formation[27]. Morphogenesis can be induced through phenomena like Thigmotropism where cell contact with a surface or galvanotropism where morphogenesis is induced by exposure of cells to electric fields[28]. In this dissertation I describe a new thigmotropic response of *C. albicans.*

The pathogenic behavior of *C. albicans* is facilitated by two different mechanisms: 1) induced endocytosis by expressing specialized proteins called invasins on the cells surface and 2) active penetration by the hyphal structures mediated unclear molecular mechanisms[29]. Invasins mediate binding of fungi to host ligands such as E-cadherin on epithelial cells and N-cadherin on endothelial cells, ultimately resulting in engulfment of the fungi into the host cell[30, 31]. The role of hyphal morphogenesis for the virulence of *C. albicans* was established from the *in vivo* experiments in mice[32]. In these experiments, C. albicans cells carrying the mutations in two transcription factors, Cph1p and Efg1p, blocked the hyphal transition and also reduced the

virulence[33]. Many other genes have been shown to be involved with hyphal morphogenesis including genes that encode the hyphal wall protein Hwp1, the agglutinin-like sequence protein Als3, aspartic proteases Sap4, Sap5 and Sap6, and the hypha-associated proteins Ece1 and Hyr1[34].

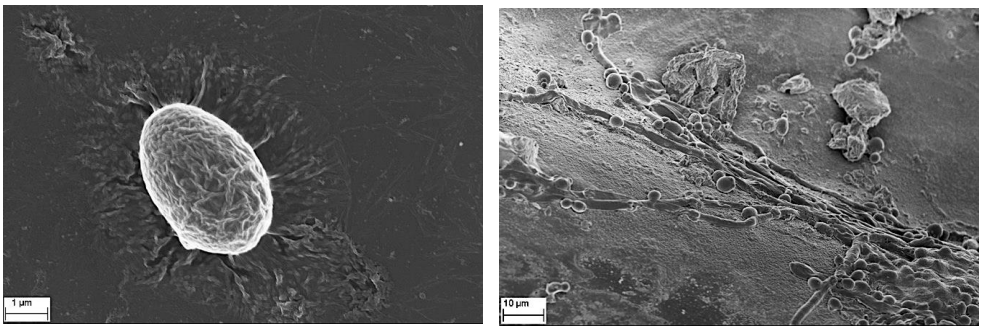


Figure 1.2. Common morphologies of pathogenic fungi *C. albicans* (a) Oval/spherical *C. albicans* yeast. (b) Tubular *C. albicans* hyphae

1.3 *Candida* Detection and Diagnosis

Poor diagnosis of fungal disease around the world resulting in over prescription of antibiotics and increasing harmful resistance to antimicrobial drugs. *C. albicans* can be identified by simple methods such as germ tube or colorimetric test or by using selective chromogenic agar tests[35]. Conventional *C. albican*s detection methods based on phenotype include microscopic examination, blood culture, and biochemical identification[36]. However, they are time-consuming, labor-intensive with low sensitivity. The long period of waiting time required to diagnose *C. albicans* infection often leads to a delay in the start of treatment with antifungal drugs. Additionally, several molecular biological methods have been applied to the detection of *C. albicans*, such as Polymerase Chain Reaction (PCR)[37], Real-Time PCR(RT-PCR), mass

spectrometry[38], and immunoassay[39]. Though there are no FDA approved PCR assays to detect *C. albicans* both commercial and inhouse PCR tests are widely used on serum or whole blood samples for detection. Definitive identification of the pathogenic fungi can be achieved by automated systems such as API *Candida* (bioMerieux), the MicroScan WalkAway System (Dade Behring), Vitek (bioMerieux), Auxacolor (Sanofi Diagnostic Pasteur), or Yeast Star (CLARC Laboratories) but these commercial techniques are often expensive and are limited by their prolonged time to obtain final results, averaging 24 to 48 hours[40, 41]. Despite considerable advances for candidiasis detection, the development of simple, compact and portable point-of-care diagnostics for rapid and precise testing that automatically performs cell lysis, nucleic acid extraction, purification and detection still remains a challenge.

1.4 *Candida* Biofilms

Most microorganisms in their natural habitats are attached to surfaces within a structured biofilm ecosystem rather than existing as free-floating planktonic organisms[42]. Biofilms are structured microbial communities attached to surfaces and encapsulated within a protective extracellular matrix. Biofilms are produced by both motile and non-motile organisms. Study of biofilms is gaining importance because a majority (65%) of all human microbial infections are biofilm based[43] and surprisingly more (80%) of the microbial infections in the United States are biofilm based[44].

Biofilms are complex communities of cells that are attached to a substrate and themselves by means of an organic glue-like substance known as the Extracellular Polymeric Substance (EPS)[45]. The EPS performs the important structural role of

stabilizing the cells and provides a protective environment against mechanical damage and shear caused by fluid flow at the interface[46-48]. The EPS is a complex matrix that is composed of polysaccharides, proteins, and extracellular DNA that is secreted by the members of the biofilm community by processing the nutrients available upon adhesion to the substrate[49]. The EPS allows its community members to share nutrients, communicate through chemical signaling molecules (i.e. quorum sensing), pass along genetic information through lateral gene transfer, and are even involved in promoting electron transfer[50]. All these functions are enhanced in biofilm communities compared to planktonic cells and increase antifungal drug resistance up to 1000-fold[51]. Indeed, the inefficacy of antifungal therapy against different fungal biofilms have been shown and stated by several studies[52-54].

A significant attribute in virulence of *C. albicans* is the formation of three-dimensional biofilm on biotic surfaces and abiotic surfaces like dental enamel, catheters and other biomaterial implants[55]. Formation of biofilms by *Candida* species occur in a systematic manner; cellular fungi attach to the substrates and grow horizontally to form a basal layer then hyphal cells are produced on the upper layers and with further secretion, biofilms are usually covered with extra cellular matrix [56, 57]. Adherence of fungal cells to biomaterial surfaces must first occur for colonization to take place[58]. The initial attachment of *Candida* cells to biomaterials is mediated by both nonspecific factors (cell surface hydrophobicity and electrostatic forces) and by specific adhesins on the fungal surface recognizing ligands in the conditioning films, such as serum proteins (fibrinogen and fibronectin) and salivary factors[59].

C. albicans biofilm formation has four developmental phases [Figure 1.3][60]:

i. Early phase - adherence of yeast cells to the device surface

ii. Intermediate phase - formation of a matrix with dimorphic switching from yeast to hyphal forms

iii. Maturation phase - increase in the matrix material taking on a three-dimensional architecture

iv. Dispersion – involves release or translocation of individual cells or clumps of cells from the biofilm or substrate

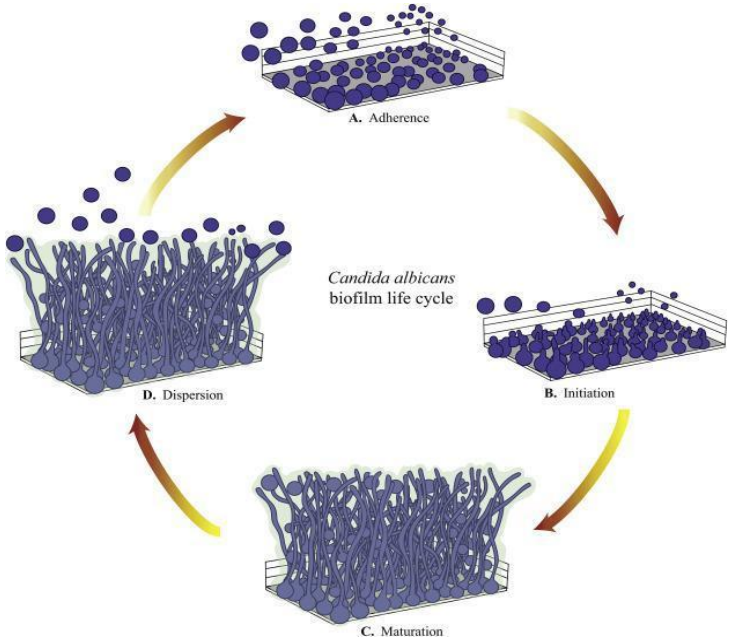


Figure 1.3. Stages of Biofilm formation[60]

Fully mature *Candida* biofilms have a mixture of morphological forms and consist of a dense network of yeasts, hyphae, and pseudo-hyphae in a matrix of polysaccharides, carbohydrate, protein, and unknown components[61]. The formation

and structure of *C. albicans* and *Candida* species biofilms is influenced by the nature of the contact surface, environmental factors, morphogenesis, and the species involved[60]. Though biofilms formation encourages the morphogenesis of yeast to hyphae all fungal morphologies indefinitely have the ability to form biofilms this was confirmed by testing the biofilm formation efficacy of hyphal gene knockouts of *C. albicans*[62]. Several transcription factors including Efg1p, Cph1p, Efh1p, Rap1p, Ino4p, and Tec1p have been shown to be essential for biofilm formation[57, 63]. Quorum sensing based cell-cell communication maintains biofilm by preventing unnecessary cell proliferation and controlling competition for nutrients[64]. This has important implications in the infectious process, particularly for dispersion of cells from the mature biofilm and the establishment of distal, secondary infections. It has been shown that farnesol acts as a quorum-sensing molecule that inhibits filamentation in *C. albicans*[24].

1.5 *Candida* Biofilms on Biomaterial Implants

Biofilms associated with *C. albicans* and other *Candida* species cause a variety of infections on implantable medical devices. Removal of these implants is required to eliminate the infection caused by irreversible adhesion of biofilms to these surfaces. Removal of implanted material is a painful process and might not even be often possible due to its anatomical location or state of infection. The implanted material might be attacked by the fungi either during contaminated administering conditions or from inside the body. As these surfaces are often surrounded by body fluids such as urine, blood, saliva and synovial fluid, their surfaces tend to acquire a glycol-proteinaceous conditioning film, which changes the original surface chemistry of the surface and

facilitates microbial adhesion[65]. The most important characteristic of biofilms produced by *C. albicans* and *Candida* species is resistance to conventional antifungal therapies[51, 62, 66, 67]. Intrinsic resistance of the biofilms can be caused by (a) biofilm matrix blocking the penetration of drugs (b) morphogenesis due to nutrient limitations (c) expression of efflux pump resistance genes and (d) presence of persister cells[68-70].

Implantable devices such as shunts, prostheses (voice, heart valve, knee, etc.), stents, implants (lens, breast, denture.), endotracheal tubes, pacemakers and various types of catheter support colonization and biofilm formation by *C. albicans* and *Candida* species (Table 1). Not only does *Candida* colonization of biomaterials precede infection, but it can also adversely affect the function of the implanted device. *C. albicans* and *Candida* species are the third leading cause of intra vascular catheter infections with a very high mortality rate[71]. These fungi are responsible for most catheter-related urinary tract infection, which remains a leading cause of nosocomial infections, with significant morbidity, and additional hospital costs[72, 73]. *C. albicans* and other *Candida* species are now the microbial pathogens that are most frequently isolated from the urine samples of patients in surgical intensive care units (ICUs), with about 10–15% of nosocomial urinary tract infections being caused by *Candida* species[74]. While most catheters are made of silicone research has shown that *C. albicans* and *Candida* species adhering to silicone displayed enhanced tolerance to fluconazole and is related to efflux pump[75]. Treating implant associated fungal infections typically requires surgical procedures to remove all infectious materials and necrotic tissue which may also involve the implant itself along with a long course of high dose antifungal therapy[76]. With the increase in

infections and resistance in *Candida* species research pertaining to the study of alternate approaches to existing fungal drugs is necessary and, in this research, I study the anti-fouling and antimicrobial efficacy of nanostructured surfaces.

Table 1.1. Risk of infections caused on medical implants in the human body by *Candida* species

Device	**Usage per year**	**Risk of infection (%)**	**Invasive *Candida* species**	**Ref**
Central and peripheral catheters	5 million	3-8%	*C. albicans* *C. glabrata* *C. parapsilosis*	*[77]*
Dialysis catheters	240,000	1-20%	*C. albicans* *C. parapsilosis*	*[78]*
Breast implants	130,000	2-10%	*C. albicans* *C. parapsilosis*	*[79]*
Urinary catheters	>10 million	10-30%	*C. albicans* *C. glabrata*	*[80, 81]*
Cardiac prosthetic devices	400,000	1-3%	*C. albicans* *C. glabrata* *C. parapsilosis*	*[82, 83]*
Neurosurgical shunts	40,000	6-15%	*C. albicans*	*[84]*
Dentures	>1 million	50-100%	*C. albicans* *C. glabrata*	*[85]*
Voice prosthesis	Thousands	5-10%	*C. albicans* *C. tropicalis*	*[86]*

1.6 Bacterial-Fungal Interactions in Infections

One of the most widely studied microbial interplays is the bacterial-fungal interaction[87, 88]. Many complicated infections are an interplay between two pathogens and often are found in patients with intravenous catheters, cystic fibrosis patients, the respiratory tract of ventilated patients, and burn wounds[89]. The current antibiotic drugs often target single species of microbes and harsh multi-microbial targeting drugs often have complications and side effects making these complicated infections challenging to treat. Most of these polymicrobial interactions are mediated by mechanisms that also serve as virulence factors, including quorum sensing, biofilm formation, production of secondary metabolites, and cellular signal transduction[90-92]. Bacterial-Fungal interactions are initiated by mutual support of their growth but potentially lead to suppression of one organism and dominant growth of another, based on the microenvironment of the host. Several studies have demonstrated that polymicrobial infections can be more severe and result in considerably higher mortality than infections with single pathogens[93, 94]. *P. aeruginosa* produces phenazines/pyocyanin , decanol, and 3-oxo-C12-homoserine lactone ($3OC_{12}HSL$), which inhibit *C. albicans* biofilm formation and hyphal development. These bacterial analytes induce the formation of reactive oxygen species (ROS) and increase the virulence of *C. albicans* by producing the proteolytic enzyme elastase (LasB)[95, 96]. The strong adherence and synergistic interaction between different *C. albicans* with pathogenic bacterial (e.g. *Actinomyces* spp, *Lactobacillus* spp, *Staphylococcus* spp) promotes stable, mature biofilms and enhanced colonization at multiple sites in the human body[91, 97, 98].

1.7 Antimicrobial Resistance

Traditionally the control of microbes has been done with chemical antibiotics, compounds that target specific essential enzymes; however, over the past fifty years there has been an exponential increase in resistance of pathogenic bacteria and fungi to known antibiotic compounds. Antibiotic resistance is a global threat to public health[99, 100]. Continued misuse of antibiotics runs parallel to the swift evolution of multi resistant microbial pathogens, which has led to an enormous rise of therapy resistant diseases. This behavior has resulted in the ineffectiveness of conventional therapies. At the cellular/molecular levels anti-drug resistance is manifested by the emergence of genetic alterations that either directly interfere with the binding of the drug to its cellular target, or by inducing gene expression that reduces drug susceptibility[101]. Antibiotic resistance issue is a serious concern in post-operative patients like implant recipients due to colonization of resistant pathogenic species at the transplant-tissue interface[102-104].

Inaccurate diagnosis of fungal sepsis in hospitals and intensive care units results in inappropriate use of broad-spectrum antibacterial drugs in patients with invasive candidiasis, fungal infections caused by yeasts. Different drugs and methods have been studied to control and kill the pathogenic fungi *C. albicans* and its biofilm. Despite the clinical and economic relevance of drug resistance in the context of yeast infections, this subject remains poorly studied, at least in comparison with the similar issue of antibiotic resistance in bacterial pathogens.

1.8 Anti-fungal Approaches and Resistance

In comparison to anti-bacterial research antifungal research has made very little progress. This is somewhat justified by the low occurrence of fungal infections, but recent increase in fungal infections by both planktonic and biofilm associated fungal infections has put us in jeopardy. Resistance to currently available fungal drugs and rising fatalities caused due to the coexisting bacterial and fungal species, aggressive research on new antifungal agents is becoming a priority[105]. Fungi are eukaryotic, with a closer evolutionary relationship with human hosts regarding basic cellular and molecular processes. These similarities lead to slow development/discovery of antifungal drugs, which complicates the process[106]. Nonetheless, detailed knowledge regarding the structure, composition and biochemistry of fungal cells has contributed to our understanding about the mechanism of action of many antifungal agents[107].

The fungal cell wall, which gives the cell a definitive structure and serves as a sensory interface with the external environment, is composed of chitin, glucans, mannans, and glycoproteins. The fungal cell wall is essential for adhesion and fungal pathogenesis and also acts as a protective barrier limiting the access of molecules to the plasma membrane[108]. The main antifungal drugs target the cell wall, specifically compounds that inhibit biosynthesis of chitin and β-glucan synthesis[109]. Chitin a β-1-4-linked N-acetylglucosamine polymer an essential component of fungal cell wall constitutes to a very small amount (1-2%) in cellular yeast but in considerable quantity in filamentous fungi (10-20%) and plays a key role in providing rigidity, structural support and maintains structural integrity of the thin fungal cells.

C. albicans during colonization or infection must adapt to environmental changes in the host. The *C. albicans* cell responds to these environmental challenges by altering the expression of specific genes, thereby altering specific biochemical synthetic pathways[110, 111]. These acute/ rapid responses will lead to more permanent alterations to the genome. Selection of adaptive mutations and the evolution of genetically altered variants of C. albicans that have adapted to the host's niche initiates conditions that lead to drug resistance[112-114]. Patients infected with C. albicans are treated with antifungal drugs, that are either systemic or topical based on their mode of introduction.

Table 1.2. Classification of commonly used antifungal drugs and their mode of action.

Antifungal Drug Class	Drug	Mode of action
Azoles	Fluconazole Voriconazole Posaconazole Itraconazole ketoconazole	Inhibitor of lanosterol 14α-demethylase
Echinocandins	Caspofungin Anidulafungin Micafungin	Inhibitor of 1,3-®-synthase
Polyenes	Amphotericin B Nystatin	Binding to ergosterol
Pyrimidine analogue	Flucytosine	Inhibitor of DNA/RNA/protein synthesis

Antifungal drugs are classified into three types (a) Azoles (b) Echinocandins and (c) Polyenes (table 1.2). Systemic drugs include flucytosine, Imidazoles, Triazoles, Haloprongin, Tolnaftate are examples of topical drugs. Mechanism of antifungal is mainly by inhibiting fungal cell wall and membrane biosynthesis and by alteration of microtubules or by inhibition of nucleic acid synthesis. Systemic antifungals like amphotericin B are toxic to mammalian cells. Most anti-fungal drugs currently available target highly divergent processes like Ergosterol genes synthesis pathways.

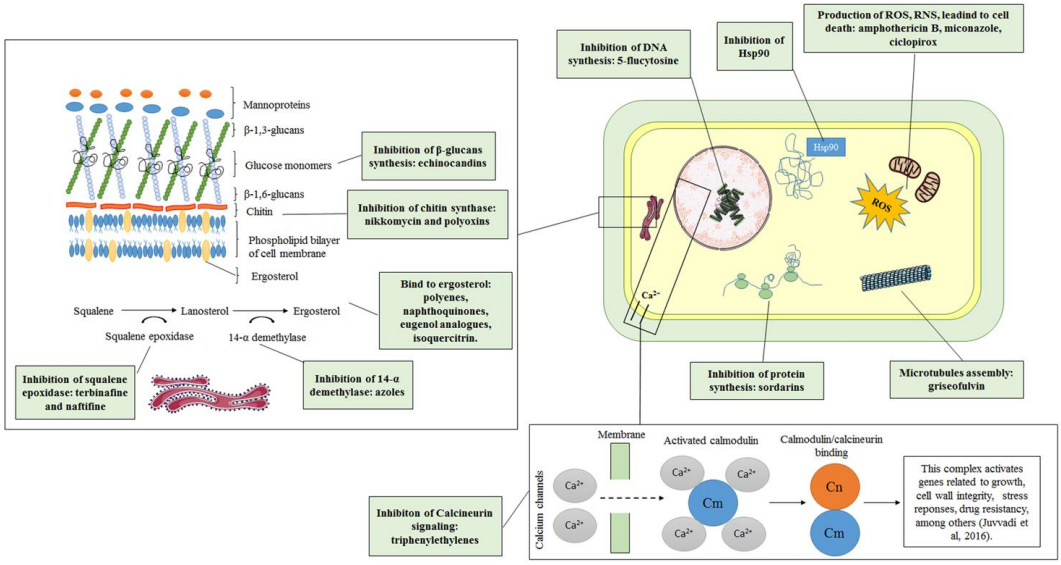


Figure 1.4. Targets of commonly used antifungal candidates [115]

Fluconazole is an example of commonly used anti-fungal drug and works by inhibiting biosynthesis of ergosterol, a key component of the fungal cell membrane. The drug targets 14α-demethylase encoded by ERG11 and mutation caused in it will result in amino acid exchanges that reduce affinity of enzyme for the drug[116]. Another important class of anti-fungal drugs echinocandins target the β-1,3-glucan synthase a key

component of fungal cell wall biosynthesis and mutations in FKS1 result in reduced drug binding[117]. In addition to anti-fungal drug used in therapeutic treatment, *C. albicans* encounters many innate antimicrobial agents taken up or generated by host. Saliva secreted in the oral cavity contains antimicrobial peptides including histatin 5 which is not membrane lytic but acts intracellularly to cause cell death[118]. Extracellular glycol-domain of the plasma membrane protein Msb2 of *C. albicans* is released into the environment which binds to Hst5 and other antimicrobial peptides evading the hosts antimicrobial response[119]. With increasing resistance in fungi towards the available antifungal drugs need for newer drugs and alternative methods for fungal infections needs to be investigated.

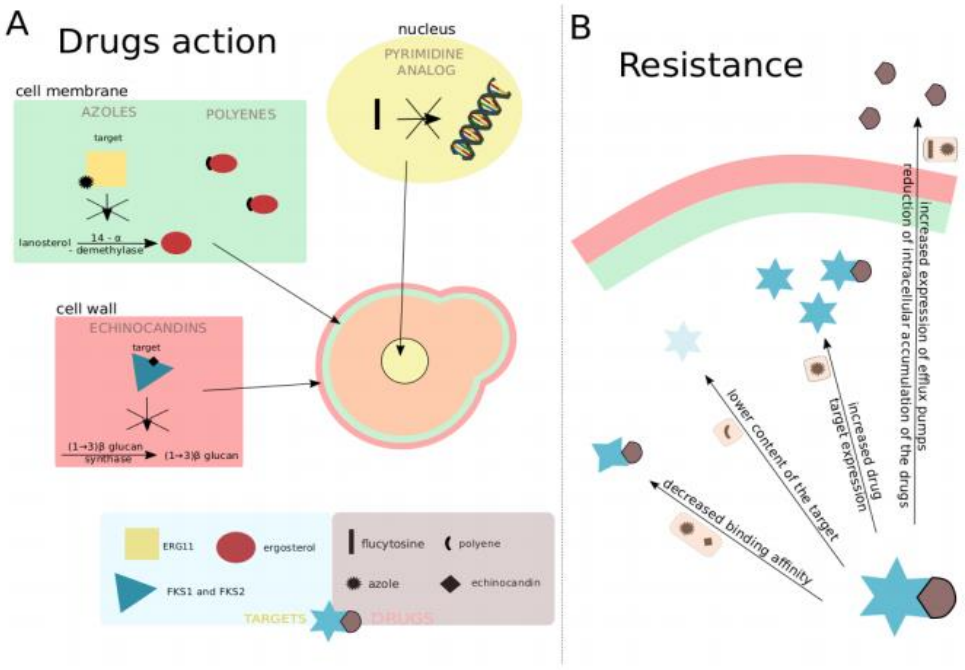


Figure 1.5. Antifungal drug actions and resistance mechanisms in *Candida* (A) Action mechanisms of drugs in different parts of the cells. (B) Common resistance mechanisms developed in *C. albicans* to antifungal drugs[120].

Commonly used drugs like caspofungin, micafungin, and anidulafungin of class Echinocandins act on mechanisms specific to fungal cell wall. Echinocandins target the protein complex responsible for the synthesis of β-1,3 glucans by blocking the enzyme glucan synthase[121]. This results in the decrease in the incorporation of glucose monomers linking β-1,3 and β-1,6 glucans, thereby weakening the cell wall and leading to fungal cell lysis[106, 122].

1.9 Antimicrobial Surfaces

In general, there are two ways to stop microbes from infecting humans or deteriorating materials: disinfection and antimicrobial surfaces. Many innovative technologies have been developed to impart antimicrobial function or activity to biomedical materials to prevent or treat infection and biofilm development[123], though most of these technologies are designed for controlling bacterial biofilm formation, Antimicrobial surfaces may be classified as anti-adhesive or bactericidal depending on the nature of the antimicrobial activity[124]. According to Munoz-Bonilla and Fernandez-Garcia (2012) the polymeric materials with antimicrobial activity can be classified as follows:

i. Polymers that exhibit inherent antimicrobial activity
ii. Polymers whose biocidal activity is conferred through chemical surface modification
iii. Those that incorporate antimicrobial organic compounds with either low or high molecular weight; and
iv. That involve the addition of active inorganic systems.

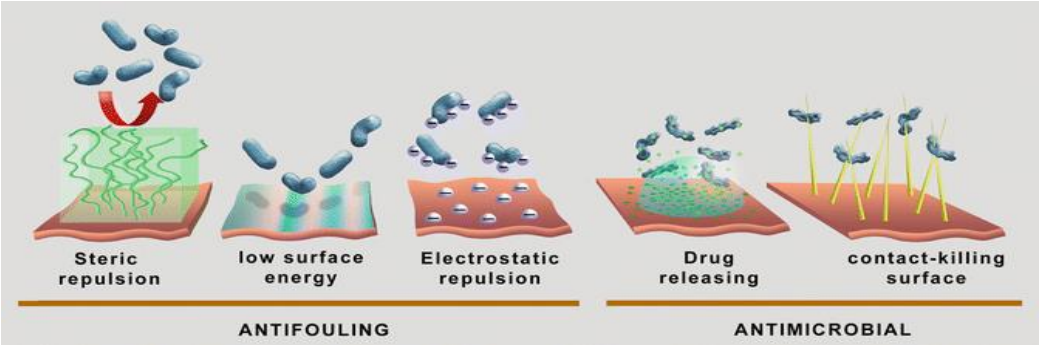


Figure 1.6. Antimicrobial Polymers for Anti-biofilm Medical Devices: State-of-Art and Perspectives[125]

1.9.1 Antifouling or Anti-Adhesive Surfaces

The first step for any microbial infection in adhesion and there is no possibility of microbial colonization if adhesion can be prevented. Microbes adhere to surfaces by physio-chemical surface interactions or species-specific active mechanisms mediated by adhesins[126, 127]. These can be structurally quite simple coatings, such as hydrogels, or involve more complex architectures, such as polymer brushes. Several studies have also investigated the use of such “antifouling” or “anti-adhesive” coatings to prevent attachment and biofilm formation by fungal organisms. A polymer brush layer formed by covalently attaching poly(ethylene oxide) polymers to glass surfaces decreased the growth of *C. albicans* by 70% compared to uncoated glass surfaces[128]. Acrylic plates coated with carrageenan and hydrocolloid were challenged with 27 isolates from *Candida spp*. and a decrease in adhesion of hydrophobic hyphal *Candida spp*., without affecting the adherence of non-hydrophobic *Candida sps*. cells were observed[129].

1.9.2 Intrinsically Anti-Microbial Bioactive Materials

Bulk materials that exert an antimicrobial action in the absence of modifications, such as loading with antibiotic substances or coating with active functional molecules, can generally be described as intrinsically antimicrobial. Silver has been described as one of the earliest materials used in surgery for its antimicrobial properties. Numerous substances are known to possess bactericidal and fungicidal properties, among them several metals, such as silver[130, 131], zinc[132] and copper, some polymeric materials, such as chitosan and its potentiated derivatives, and various bioactive glasses. *Candida sps* being complex eukaryotes sharing similar cellular ingredients as host, the cytotoxicity of the materials needs to be highly specific or can potentially affect the host cell/tissue. A detrimental effect can be determined even when the materials interact with host cells causing a loss of cell differentiation at the interface with the implant. Chitosan is a polycationic polymer derived from chitin and has been broadly used as a stable antimicrobial biomaterial platform with other biocidal organic and inorganic compounds[133-137].

1.9.3 Drug Releasing Surfaces

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. To address this, surfaces coated with cyclodextrins[138], chlorhexidine and chloroxylenol[139], chloroxylenol, thymol[140], along with commercial antifungal drugs have demonstrated a significant lower adhesion or reduced infections by *Candida sps*.

Drug release from bulk materials, thin film coatings, hydrogels and grafted polymers is being widely researched for use mainly as coatings on medical implants[141].

1.9.4 Contact Killing Surfaces

Antimicrobial releasing coatings deplete over a period of time, can produce non-specific response in the host and sustained low-level tail-release will contribute to the development of antibiotic-resistance[142]. Contact killing surfaces can solve the problem for long-term antimicrobial activity[143-146]. The combination of contact killing and non-adherent properties in surfaces is used to inhibit the deposition of biomolecules like proteins, serum and other body fluids[147]. Efficacy of contact-killing quaternary ammonium-coated surfaces has been demonstrated over a time period of several days by in-vivo experiments[148].

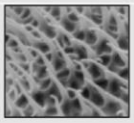
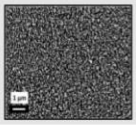
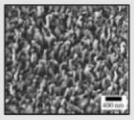
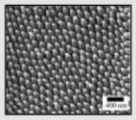
1.9.5 Nanostructured Antimicrobial Surfaces

Nanostructured surfaces occur abundantly in nature, from the superhydrophobic surfaces of lotus leaf which provide anti-adhesive quality to the various cuticular structures of different insects with anti-wetting, anti-drag and even optical properties[149]. These nano-architectures impart multi-functionality, affording a broad spectrum of favorable properties, often including the ability to self-clean[150], exhibit super hydrophobicity[151], and possess antibiofouling properties. Common examples of surfaces that exhibit combinations of these behaviors include: insect wings, such as those of cicadae[152-155], damselflies[156] and dragonflies[157, 158], shark skin[159-161], gecko feet[162-164] and plant leaves, most famously the lotus leaf[150, 165]. Naturally

occurring bactericidal surfaces are useful in providing a starting point in the design of antimicrobial structures[166].

Research on the impact of surface topography on microbial adhesion has been conducted since the last three decades but definite evidence on how these topographies modulate colonization is lacking[167-170]. Since most of these biofilms associated organisms exist on the micron scale, their attachment behavior on surfaces is also studied on micro and nanoscale topographies. Though the influence of surface topography and roughness of bacterial attachment has been recently studied, the interaction of a much complex fungal system has not been given great deal of attention. The conventional theories state that smooth surfaces render a repellent environment to the bacteria whereas rougher surfaces facilitate adhesion by providing more adhesion points[171, 172]. Contradictory to these, cells exhibited greater propensity for adhesion on the nano-smooth surfaces due to the production of extracellular polymeric substances (EPS)[173]. Periodic nanoscale arrays of high aspect ratio polymer posts have been reported to direct the attachment behavior of P. aeruginosa, B. subtilis and E. coli cells[174]. Cell adhesion on superhydrophobic surfaces are studied in an effort to create surfaces to reduce microbial colonization[175]. The rationale for this is that the air trapped between the dual-scale surface features present on such surfaces limits the available contact area for the bacteria[176]. Depressions with diameters of 2.5 μm etched into the surface of optical fibers tended to prevent bacterial attachment, relative to control optical fiber surfaces[177].

Table 1.3. Natural antimicrobial surfaces their nano structured topology and antimicrobial efficacy

Surface	Image	Features	Wettability	Antimicrobial efficacy
Cicada wing		Nanoneedles, height 200 nm, diameter 60 nm	Hydrophobic, water contact angle (CA) = 159	Lethal to P. aeruginosa,
Gecko skin		Hair (spinules) like curvature < 20 nm	Hydrophobic, CA = 151°–155°	Lethal to Porphyromonas gingivalis (g − ve)
Dragon fly wing		Nanograss	Hydrophobic, CA = 153°	Lethal to P. aeruginosa (−ve), S. aureus(+ve) and B. subtilis (+ve)
Sand dragon dragonfly		spherical capped nanocylinders	Hydrophobic, CA = 119°	Caused cell wall rupturing of S. cerevisiae
Annual DD cicada		Spherical nano cones	Hydrophobic, CA = 132°	Caused cell wall rupturing of S. cerevisiae

Preliminary research on *C. albicans* response to rough surfaces showed that the pathogenic yeast attached to scratch marks and pits were created by scratching with emery paper[178]. But *C. albicans* did not show any significant change in adhesion on surface pits in the size range 0.2–2 μm[179]. Recent research on *Candida* adhesion on patterned 120nm diameter pits showed decrease in adhesion compared to flat solid[180]. Adhesion of cellular *C. albicans* was demonstrated to be significantly higher on TiO_2 coatings when compared to TiO_2 nanoparticles[181, 182]. Understanding the mechanism and parameters for these antimicrobial surfaces will allow manufacturers to tailor-make antimicrobial surfaces that can minimize or prevent the formation of

biofilms, or even allow the synthesis of a surface that has the capacity to kill any microbes that come into direct contact.

1.10 Antimicrobial Efficacy and Nanostructured Topography of Cicada Wing

Natural superhydrophobic surfaces are often thought to have antibiofouling potential due to their self-cleaning properties, but the wings of Cicadas ruptured the Pseudomonas aeruginosa and have demonstrated to have anti-microbial properties as opposed to antibiofouling nature. Extensive research in electron microscopy techniques helped us analyze the structural topology of the natural available surfaces with innate anti-microbial super hydrophobic properties to create structured surfaces with similar properties by reverse engineering. Researchers have identified nano cone structures on the dorsal and ventral wing membrane sections of the Cicada wing and later confirmed their anti-bacterial efficacy[149, 151, 152]. Detailed AFM and SEM studies on the wings of Cicada sps (*Tibicen tibicen*) confirmed the presence of arrays consisting of hexagonally-packed spherically-capped conical protuberances with a spacing and height of 200 nm and radius of curvature of 35–55 nm at the apex. The nano structuring of surfaces with an ordered array of features increases the hydrophobicity as described by the Cassie and Wenzel models[175]. Sun et al., have systematically investigated both the wetting and optical properties of different cicada species as a function of their structure; also, the height, diameter and spacing of the nanopillars varies between cicada species[183, 184]. The natural surfaces of insect wings have been studied to exhibit complex chemical compositions of chitin, proteins, polyphenols and wax[185, 186]. The superhydrophobic nature of the Cicada wing is attributed to the sophisticated nano

structured topography and also the presence of wax yielding to a decreased wettability[187].

The first study on mechanical inhibition of bacteria by native nanostructure of cicada wing demonstrated a reduced cell numbers in a suspension of Pseudomonas aeruginosa by almost half within one hour, with a single cell being able to be ruptured in approximately 4 minutes. Cicada wings exhibited general bactericidal activity against Gram- negative cells, regardless of their morphology, while Gram-positive cells were found to be resistant to the antibacterial nature of the wing[152]. Mathematical models of bacterial interaction with cicada wings demonstrated the rupture of bacterial cells is due to the stretching of cell membranes suspended between the nano cones[188]. Gram-positive cells have a greater natural resistance to this effect than do Gram-negative cells, due to their greater rigidity. Nowlin et al experimentally demonstrates that there is a relationship between cell rupture/death of *Saccharomyces cerevisiae* and the nanostructure geometry of NSS[189].

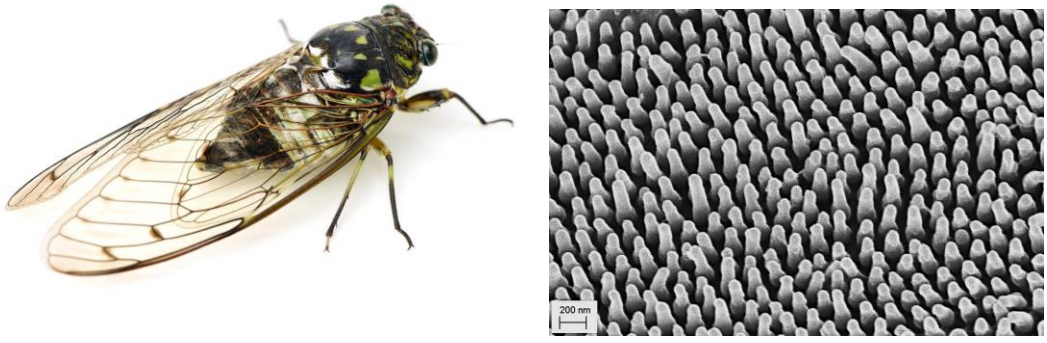


Figure 1.7. Scanning Electron Microscope image of Nanostructured surface of the Cicada wing (a)Cicada bug *Tibicen tibicen* (b)Scanning electron microscope image of Nanostructured surface of the Cicada win

CHAPTER II

SYSTEMATIC STUDY OF MECHANISM OF *CANDIDA ALBICANS* RUPTURE ON NANO STRUCTURED SURFACE OF CICADA WING

2.1 Introduction

Candida albicans is a unicellular, diploid fungi that is a common member of the human commensal microbiome of the gut, skin and mucosal membranes[190]. While *C. albicans* is found in all healthy humans, in certain circumstances this microbe is responsible for a variety of diseases from the common oral thrush to life threatening systemic infections[191]. *C. albicans,* unlike a few other *Candida* species, lack the ability to proliferate outside the human body and therefore pose a unique threat and problem to human health[192]. *C. albicans* infection is a serious problem mainly in immunodeficient patients particularly implant recipients, patients suffering from HIV where it causes conditions like Candidiasis and invasive Candidiasis which might lead to fungal sepsis[6]. *Candida* related medical issues are mainly because of its ability to form biofilms. Mature *Candida* biofilms host a mixture of morphological forms of the C. albicans including cellular, hyphal, and pseudo hyphal forms that are embedded in a complex polysaccharide matrix that attracts a community of pathogenic and non-pathogenic bacteria and other fungi[193]. The structure and composition of the *Candida* biofilm depends on the nature of contact surface, environmental factors, morphogenesis and the species involved[61]. C. albicans cells within the biofilm exhibit phenotypic

resistance to antimicrobials and host defense which might lead to reinfection[194]. *Candida* biofilms also host pathogenic microorganisms like Methicillin-resistant Staphylococcus aureus, which cause infection on indwelling medical devices like catheters, ocular lenses, cardiac valves and shunts[55, 195, 196]. The need for novel approaches for antifungal treatment and therapies is dire. Although antifungal drugs such as azoles (i.e. fluconazole and voriconazole), echinocandins (i.e. caspofungin and anidulafungin), and others (i.e. flucytosine and amphotericin B) have been critical for combating fungal infection, the prevention of these *Candida* infections is becoming problematic with the increase in antifungal drug resistance and the emergence of "*superbugs*" [197]. The increased misuse of antibiotics, pesticides and continued evolution of multi-drug resistant microbial pathogens cumulatively add to the growing resistance in microbes and the failure of existing antibiotics[198].

Candida associated biofilm infections have been reported to affect around 10% of the medical implant recipients in the US alone and lead to nearly 100,000 deaths per year[4]. Implant associated *C. albican*s infections after breast augmentation ranged from 1.1% to 2.5%[199] and *C. albicans* infections associated with breast reconstruction after mastectomy ranged from 1%-35%[200]. Unlike a few other *Candida* sps *C. albicans* lack the ability to proliferate outside the human body[192]. C. albicans and related yeast species are pathogenic mainly by their ability to exist in different morphological forms like the cellular yeast and tubular hyphal states. In particular, hyphal fungal cells have a special ability to produce proteins that protects and mask them from host immune oxidative stress responses and enable the fungus to evade phagocytosis, escape the

bloodstream and colonize on the organs/implants form biofilms and lead to failure[201].

Antimicrobial surfaces are a relatively new class of antimicrobial approaches which involve the application of inherently antimicrobial materials and surface coatings. Nanostructured antimicrobial surfaces have been proposed as a stand-alone treatment or as a potential addictive treatment with existing antimicrobial agents[202]. One strategy for the application of antimicrobial surfaces involves impregnating materials with soluble biocides that are released on contact there by rupturing and killing a biofilm forming microbe[203]. Another strategy involves the application of antimicrobial nanoscale surface morphologies. While nanomaterials derived from metal nanoparticles have been used extensively as coatings on medical devices, sanitation, wound dressings and also in food storage[204-207], antimicrobial nanostructured surfaces (NSS) are surfaces that inhibit microbial growth and viability using structural or mechanical cues. NSS were originally identified on the wings of certain insects such as cicadas, but more recently these have been fabricated using techniques from the semiconductor industry and materials like polymers or metal oxides[208-210]. Recent work has demonstrated that the nanoscale architecture on the cicadas not only inhibits biofilm formation but also controls microbial development[211]. Research has demonstrated that surfaces with a high aspect ratio nanoscale architecture are lethal to many gram-negative bacteria like *Pseudomonas aeruginosa*, *Escherichia coli*, *Pseudomonas fluorescens,* and *Branhamella catarrhalis[157, 212] a*s well as certain cellular yeasts such as *Saccharomyces cerevisiae[213]*. Cell physiology parameters like adhesion and cell wall rigidity play a role in cell-rupture, but there is a definite need to study underlying mechanisms involving

NSS-induced microbial rupture[214]. Biophysical models of bacterial cell interactions with nanopatterned cicada wing surfaces suggests that the bactericidal mechanism is biophysical and results due to a physical incompatibility between the cell and the surface without any specific biological interaction[153].

In this paper we determine and define the conditions and interactions of the fungi *C. albicans* with the nanostructured surfaces found on the wings of the dog day cicada, *Tibicen tibicen*. In our studies we identify critical timing events associated with specific physiological and morphological features of the *C albicans* cell. In our studies we demonstrate that the timing of *C. albicans* cell rupture depends on the timing of cell adhesion to the NSS and requires a cellular morphology rather than a hyphal form. We also demonstrate for the first time that fungal cell differentiation is controlled by physical signals from the NSS and may provide a novel means of controlling C. albicans virulence and pathogenicity at the interface between inorganic surfaces and human tissue, as in implants and other devices.

2.2 Materials and Methods

2.2.1 Yeast Strains and Cultures

A wild type strain of *Candida albicans* (ATCC 90028) was grown in Sabouraud dextrose broth (SDB) at 25^0C in 50 ml conical flasks[215]. Depending on the experimental needs, cultures were grown to either mid-log phase at O.D~0.6 or to stationary phase at O.D.~2.0. OD_{600} measurements were made using a Thermo Scientific Nanodrop 2000C spectrophotometer[216]. To induce hyphal growth, *C. albicans* hyphal forms are grown in a modified SDB media containing 10% bovine serum albumin (Sigma

F-3018) in 50ml conical flasks incubated overnight in a shaking incubator at 37^0C and 200 rpm[217]. Hyphal growth can be identified by the formation of granular sediments in the growth media.

2.2.2 Surface Preparation and Characterization

We investigated the interaction of *C. albicans* cells with three different surface types: flat surfaces, a nano structured surface (i.e. a natural Cicada wing) and etched cicada wings which had submicron depressions. We examined the interactions of the fungal cells on both native (i.e. uncoated surfaces) and Au-coated surfaces to control for differences in surface composition. In the process, each of the type of surfaces (e.g. flat, nanostructured wings and etched wings), were coated with a 7nm layer gold using a Leica EM ACE200 and monitored in real time with a quartz crystal microbalance (QCM). For the experiments we used a flat glass coverslip as control. The cicada (*Tibicen tibicen) wings* used in the experiments were purchased from BioQuip Products, Inc., California. Wings from whole cicadas were carefully dissected from the organism so as to not damage their surfaces. Isolated wings were sonicated in 70% ethanol for 10 minutes to remove any contaminants. Glass coverslips were cleaned using 70% ethanol and cleaned wings were then mounted on them using silicone glue (Silicone VC6-1/2). I used flat glass coverslip as a control. As an additional control we used an etched modified cicada wing that we processed using a PE-100-RIE Plasma Etch System etching the surfaces at 300FW power for 300 seconds. The plasma etched wing retained the general composition of the wing while removing all the nanocone architecture from the surface, revealing a surface dominated by submicron scale depressions. These etched wings serve

as negative control for all the experiments. The surface energy was measured via contact angle goniometry using a rame´-hart 260-F4 contact angle goniometer and DROPIMAGE advanced software. A 3µl drop of deionized water was dropped on all the 3 different native and Au-coated surfaces and averages of 4 different repetitions on similar surfaces were calculated.

2.2.3 Fluorescent Labelling and Exposure of *Candida albicans* Cells to Nanostructured Surfaces

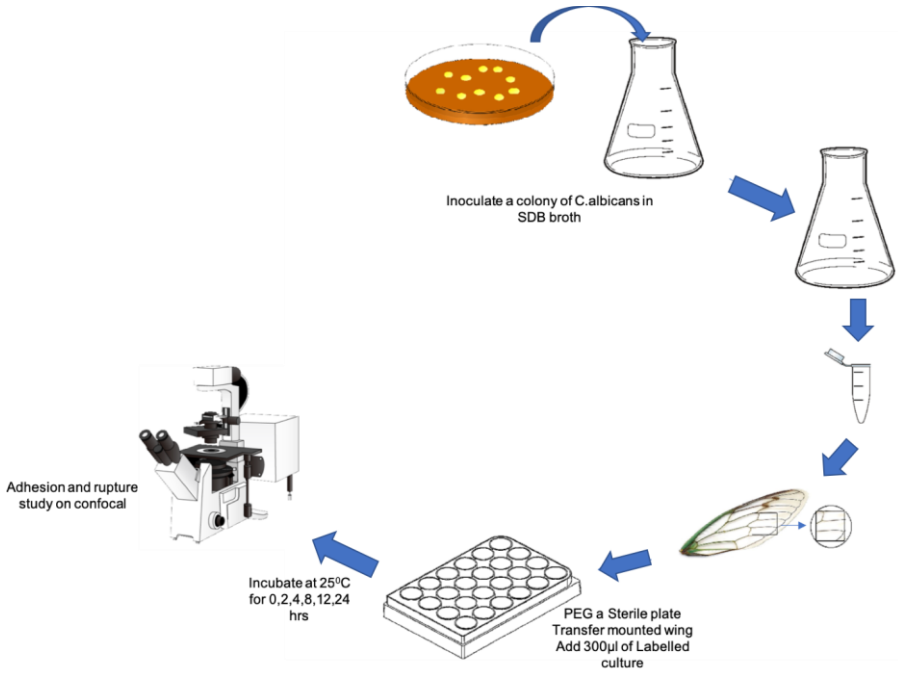


Figure 2.1. Schematic representation of experimental methodology of *C. albicans* interaction with Cicada wing

C. albicans cells were grown to desired O.D. and were labelled with calcofluor white (CFW) (Sigma-Aldrich, 18909) at 1:1000 concentration and Fun-1 cell stain obtained from (Molecular Probes F-7030) at 1:2000 concentration. The surfaces mounted

on the cover slip were placed in a PEGylated (Polyethylene glycol 8000) well of a 24 well plate. A PEG treatment was used to inhibit the non-specific interactions of yeast cells with the walls of the 24well plate. The fluorescent labelled *C. albicans* cells were diluted with fresh SDB media at 1:3 ratio and 300µl of the diluted yeast culture is pipetted into each well. The viability as per FUN1 labeling and the chitin composition of the cell wall of cellular and hyphal yeast formed were monitored on the surfaces at these time points O hrs ($T_{0)}$, 2hrs (T_2), 4 hrs (T_4), 8hrs (T_8) 16hrs (T_{16}), and 24hrs (T_{24}) (Figure 2.1).

2.2.4 Quantifying the Densitometric Change from CLSM Data

We monitored chitin levels in the C. albicans cell wall using the fluorescent dye Calcofluor white (CFW) and calculating the changes in its intensity in the confocal microscopy images using densitometry. I performed a densitometric analysis using Zen Blue software on the fluorescent microscopy images captured by measuring the grey values of the intensity of the fluorescent dye detected. Densitometry values were collected from 100 cells images collected over ten different experiments for each surface and at each time point.

2.2.5 Cell Viability

To measure cell viability of C. albicans cells on the different surfaces, we used an MTT assay *(*Molecular probes V13154) and followed the protocol as described by the manufacturer. For these experiments, we grew *C. albicans* yeast cultures to an OD_{600} of 0.6 which have approximately $1x10^6$ cells/ml and used 300µl of these cells that were cultured on the different surfaces for the MTT assay. Negative controls for these

experiments were replicates of C. *albicans* grown parallelly and treated with an antifungal drug (7 μg/ml) Voriconazole.

2.2.6 Biofilm Quantification

Biofilm formed on different nanostructured surfaces was quantified by a Crystal Violet 4Microtiter Dish Biofilm Formation Assay previously described[218]. The absorbance intensity of the crystal violet dye is measured using a BioTek Synergy Mx plate reader at 550 nm.

2.2.7 Scanning Electron Microscopy (SEM)

Candida albicans cells sampled were prepared on both the flat surfaces and the nanostructured cicada wings; cells were fixed overnight with 2.5% glutaraldehyde/2% formaldehyde solution in 0.1 M cacodylate buffer. The samples were them dehydration by an ethanol dehydration series of 30%, 50%, 70%, 95% and 100% for 10 min at each concentration. The samples are then dried and mounted on to aluminum SEM stubs and coated with a 5nm gold layer using Leica EM ACE200 equipped with real-time thickness monitoring using a QCM. The samples were then observed using a Zeiss Auriga scanning electron microscope.

2.2.8 Microcolony Assay

A microcolony assay was performed to test the effect of nanostructured surfaces on the morphogenesis of yeast to hyphae. A small volume 100μl of *C. albicans* yeast in a modified SBD media with the addition of 10% bovine serum albumin (Sigma F-3018) was inoculated on the various experimental and control surfaces and incubated at 37^0C overnight. *C. albicans* cellular forms that had been cultured in SBD media and *C.*

albicans hyphal forms that had been induced on glass coverslips served as negative and positive controls. To evaluate the microcolony assay, microcolony cultures on each surface were washed with PBS twice, labeled with CFW, and imaged using a confocal microscope as described. The results were confirmed using SEM imaging as described above.

2.3 Results

2.3.1 Surface Characterization

We examined the interactions of the yeast, *C. albicans* with three different surface nanoscale topologies: a flat topology (i.e. a 12mm round cover slip)(Fisherbrand 22-293232) a nanostructured topology that contained a ordered array of nanoscale cones that were 200nm tall, 200 nm wide with 30nm tips and spaced 200nm apart, (i.e. the wings from dog day *cicada,* (*Tibicen ssp*. [219].), and a nanoscale topology that consisted of an irregular arrangement of submicron depressions, (i.e. a cicada wing that had been plasma etched) (Figure 2.2). In addition to the 'native' uncoated surfaces we also examined surfaces that had been coated with a nanoscale layer of gold. Gold known for its biocompatible nature served as a control to maintain uniform surface chemistry in all of the test surfaces. Careful electron microscopy observations showed that gold nanoparticle coating on the cone surfaces retained the nanosurfaces aspect ratio coating the structures uniformly. As part of our characterization, we examined the surface energy of these materials using contact angle goniometry. We observed an increase in hydrophobicity with the deposition of 7nm gold later to the flat glass and the etched cicada wing, but a

decrease in the contact angle, which signifies a reduced surface hydrophobicity on the Au-coated Cicada wing when compared to the native wing (table 2.1).

Table 2.1. Contact angle of native and gold coated surfaces of different topologies

Surface	Contact angle
Control-Glass cover slip	$79.79^0 \pm 0.09^0$
Au coated Glass	$93.075^0 \pm 0.04^0$
Cicada	$114.6^0 \pm 0.07^0$
Au coated Cicada	$87.025^0 \pm 0.13^0$
Etched Cicada	$68.96^0 \pm 0.06^0$
Au coated Etched Cicada	$103.22^0 \pm 0.04^0$

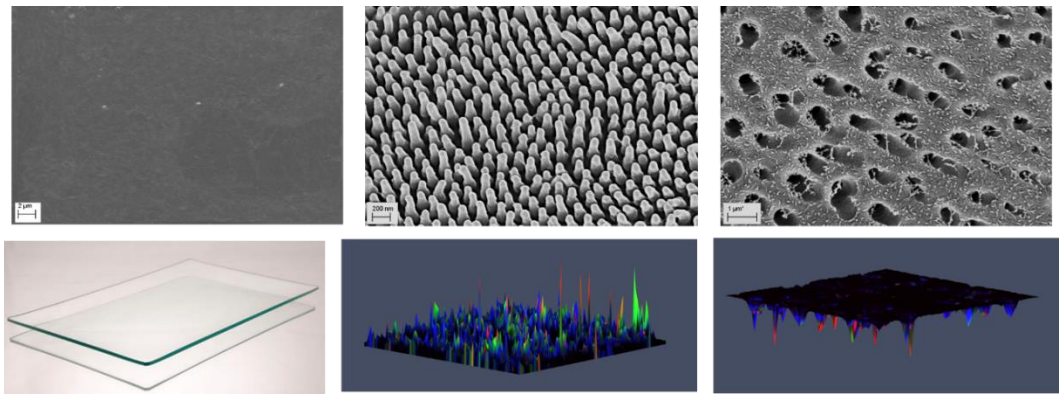


Figure 2.2. SEM images of experimental surfaces and visual representation of the topology of (1-A)(1-B) Flat surface, (2-A)(2-B)Nano-cone architecture on Cicada wing and (3-A)(3-B)Sub micron scale depressions on the Plasma Etched Cicada wing caused by the removal of nano-cones using Plasma etch process (4) Contact angles of native and 7nm gold coated substrates

2.3.2 Adhesion of *C. albicans* on Different Nanostructured Surfaces

Adhesion of microorganisms to substrates is the first step of pathogenesis. Microbial adhesion to inorganic materials depends on factors like surface roughness, hydrophilic/hydrophobicity of a surface, mechanical properties of the surface and microbes innate sensory mechanisms that control cell-surface interactions[220]. To examine C. albicans adhesion with different surfaces we cultured both cellular and hyphal forms of C. albicans with the six different surfaces and examined the number of cells bound to the surface using confocal microscopy (Figure 2.3).

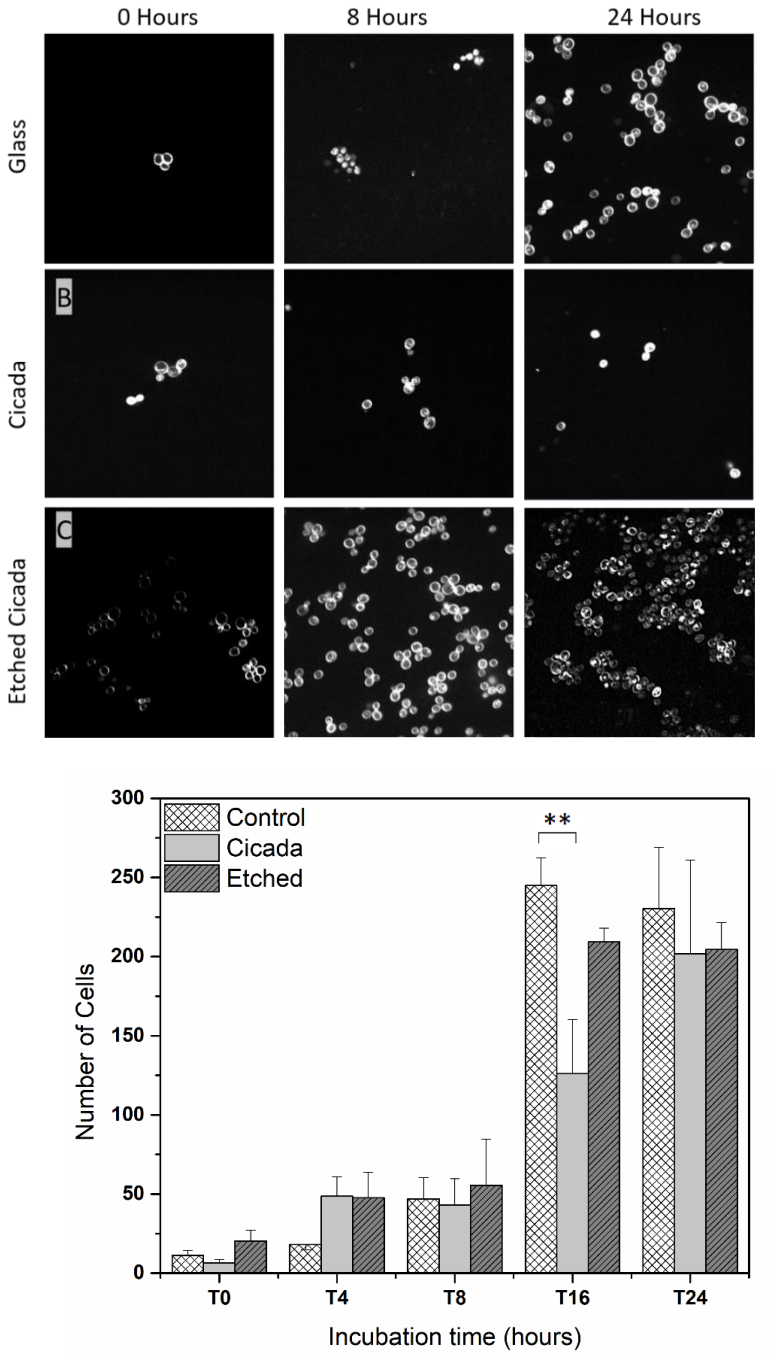


Figure 2.3. Adhesion of *Candida albicans to* different surfaces is observed to have increased with increase in incubation time (A) Flat glass surface (B) Nanostructured cicada wing and (C)Etched cicada wing at different time points (D) Summary of quantification of average number of cells per unit field of view with time. Each bar represents an average of cells per 10 fields of views done in five replicates. *denotes a $P \leq 0.05$ and ** denotes a $P \leq 0.001$

C. albicans polymorphs grown on different nanoscale topographies showed varying degrees of adhesion based on the age of the cells and time of incubation on the surface. On flat surfaces coated with 7nm gold we observed a gradual increase in the number of *C. albicans* cells/FOV as a function of the time of incubation. When compared among all the surfaces the number of cells/FOV were similar for the first eight hours, including the NSS surfaces of the cicada wing, plasma etched surfaces, surfaces coated with Au and native. However, at 16 hrs, we observed significantly fewer cells on the nanostructured cicada wing surfaces when compared to the others. However, at 24hrs growth where most cells reach a stationary phase a decreased adhesion was identified on the Cicada wing and the etched Cicada wing when compared to the flat glass surface. This trend of reduced binding was supported by the results of the MTT assay which demonstrated a reduction of total cell metabolic activity from the yeast cells on the nanostructured cicada wing surfaces when compares to the control surfaces.

2.3.3 Change in the Cell Wall Composition of *C. albicans* on Different NSS

Chitin is a major component of the yeast cell wall and is critical for the mechanical stability of the yeast cell[221]; mechanical challenges to a yeast cell wall has been shown to alter the cell wall chitin biosynthesis[222]. To determine whether contact with a nanostructured surface alters the chitin content of the *C. albicans* cell, we quantified cell wall chitin by labeling chitin using the fluorescent dye CFW and monitoring the change in its intensity using live-cell confocal microscopy. We observed an increase in CFW fluorescence in cells that were in contact with the nanostructured cicada wing when compared to the glass and etched wing surfaces (Fig 2.4). The intensity

of CFW increased with the time of exposure to the nanostructured cicada wing suggesting that the *C. albicans* cells in contact with the wing were responding to a mechanical stress caused by nano-cone arrays. In contrast, from (figure 2.4.) we observed a reduction in CFW fluorescence from *C. albicans* cells cultured on the Au-coated etched wing surfaces when compared to the Au-coated flat glass surface). The Chitin level in the cell wall of *C. albicans* on initial contact were significantly higher and were later reduced with acclimatization to surface.

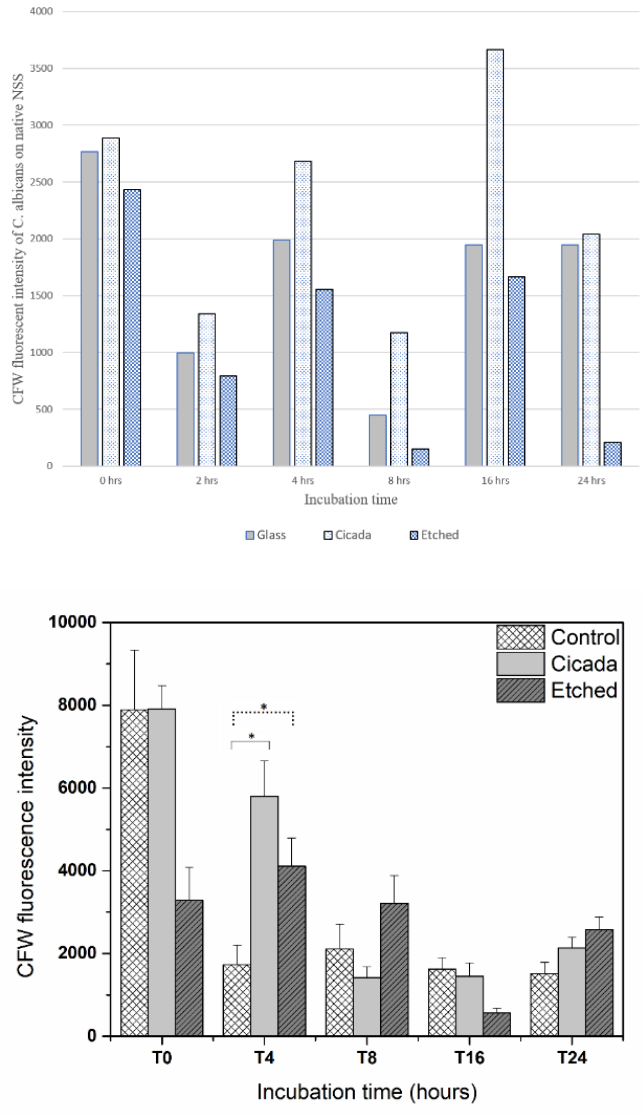
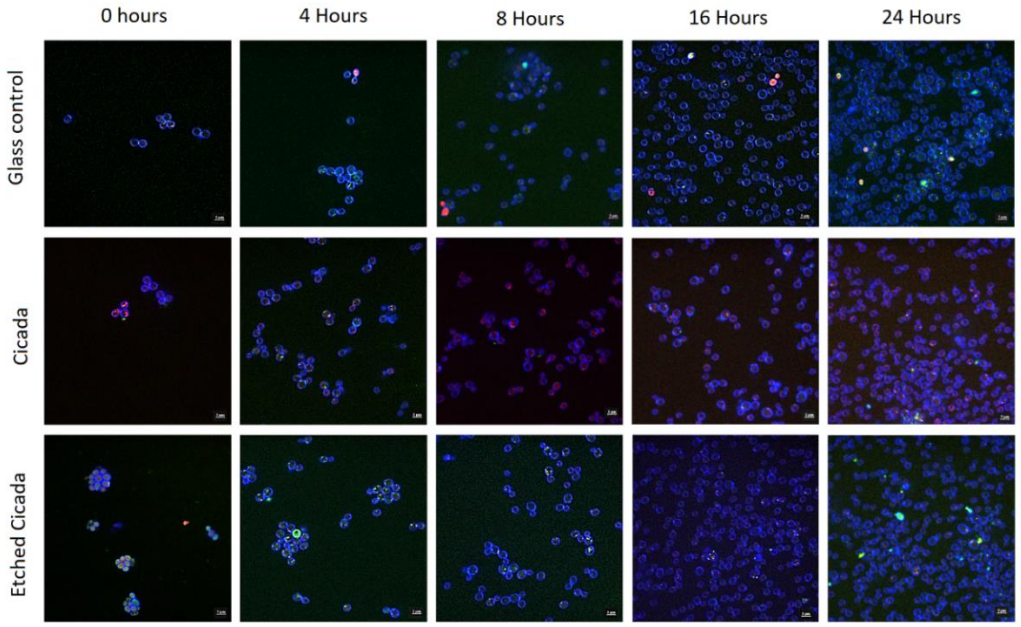


Figure 2.4. Changes in cell wall composition represented by change in chitin in cell wall labelled by CFW fluorescent dye. (a)Native Cicada wing (b)Au-coated Cicada wing. The chitin levels in the *C. albicans* yeast on all the surface decrease with increase in incubation time.
***denotes a $P \leq 0.05$**

2.3.4 *C. albicans* Viability and Rupture of Nanostructured Surfaces

To correlate yeast cell adhesion with the viability of the *C. albicans* cells on our surfaces, we labeled log phase (O.D.~0.6) yeast cells using the vital dye FUN1 and

observed these cells using confocal laser microscopy. FUN1 is a ratio metric cell-permanent vital dye that is processed by living, metabolically active fungal cells; the dye upon cellular uptake fluoresces red upon excitation with a blue light, however, in metabolically active cells the dye is enzymatically processed to a product with green fluorescence[223]. We observed a time-dependent reduction in viability in *C. albicans* cells that have been in contact with the nanostructured cicada wing surfaces both native and Au-coated (Fig 2.5) as indicated by the reduction and/or loss of the red FUN1 fluorescence in these cells. We did not observe significant loss of the green FUN1 fluorescence in cells incubated on flat or etched surfaces (Fig 2.5). The maximal amount of timing of the loss of green FUN1 fluorescence coincided with the reduced adhesion of *C. albicans* cells to the nanostructured wing surfaces, i.e. at 8 hrs. I observed loss of viability of C. albicans cells on the etched wings, but it was not consistent and significant at all the incubation time points (Figure 2.5). To confirm that the loss of metabolic activity as determined by FUN1 staining was in fact due to loss of viability we examined the state of the cells on all surfaces using SEM. Previous work demonstrated that the nanostructured surfaces of the dog day cicada rupture yeast cells from a different species[224].



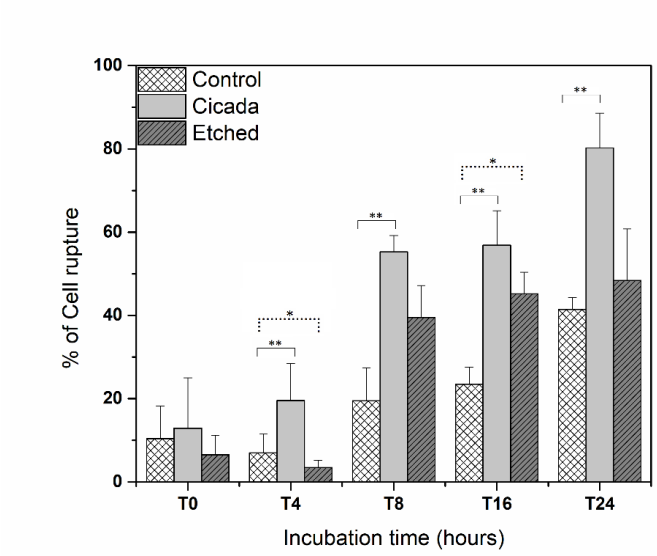


Figure 2.5. Cell viability of W.T. *Candida albicans* stained with fluorescent dye FUN-1. the green cells indicate the live cells and the red cells represent the dead/ruptured cells on different surfaces (A) Flat glass surface (B) Nanostructured cicada wing and (C)Etched cicada wing at different time points (D) Quantification of percentage of cell rupture on different Nano topological surfaces. Each bar represents the average percentage of cell ruptured per 10 fields of views done in five replicates.

***denotes a $P \leq 0.05$ and ** denotes a $P \leq 0.001$**

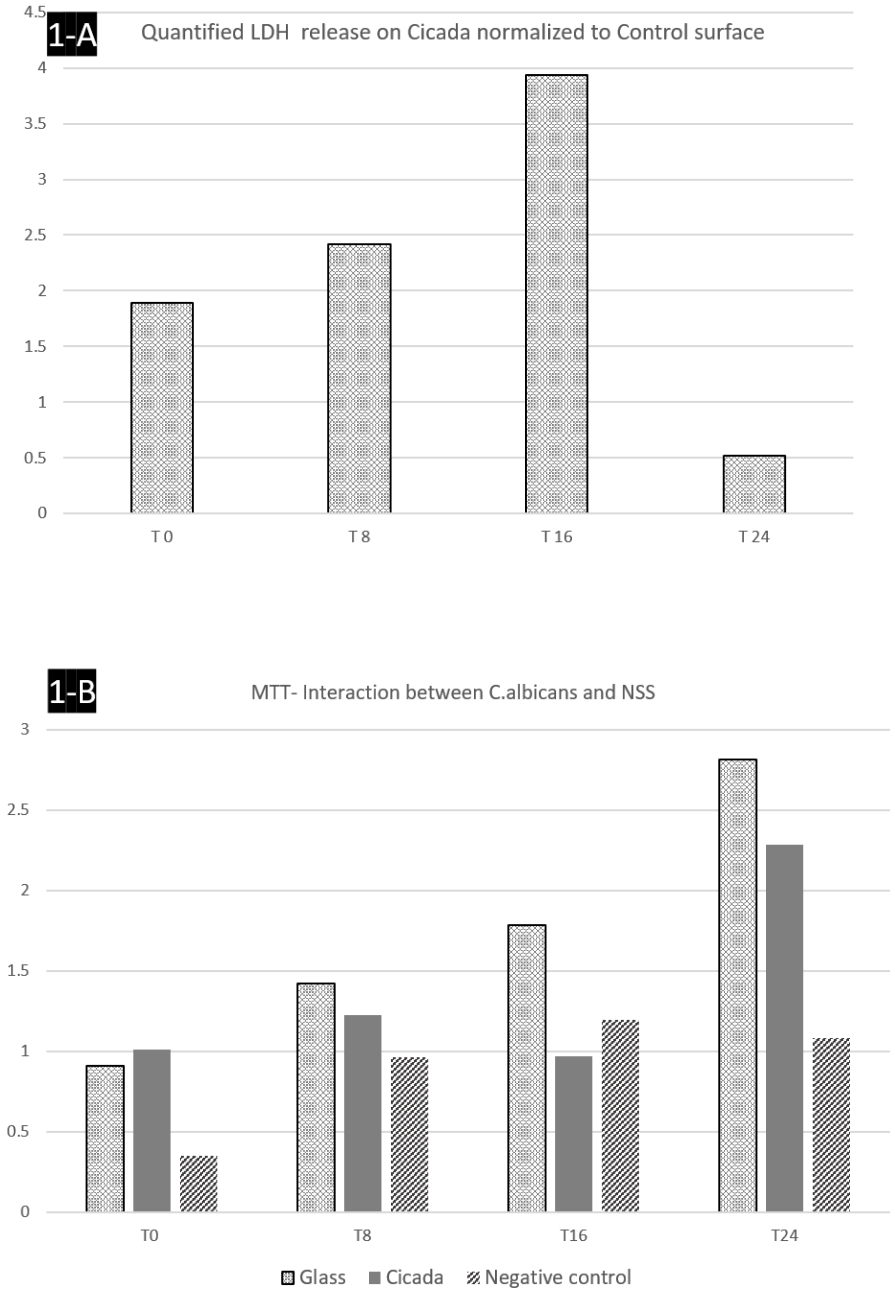


Figure 2.6. LDH and MTT cell toxicity and cell metabolic activity assay on C. albicans on NSS: LDH cytotoxicity assay showing increase in rupture of *C. albicans* up to 16 hours of incubation on nanostructured Cicada wing normalized by Control glass and positive control *C. albicans* treated with antifungal drug. (1-B) MTT assay representing a higher cell metabolic activity of *C. albicans* on control glass surface when compare to nanostructured surfaces indicating these NSS decrease adhesion and cell proliferation

C. albicans cells on the nanostructured surfaces found on the dog day cicada wing also rupture, thus demonstrating that the loss of metabolic activity observed by FUN1 was due at least in part to disruption of cell integrity. Rupture of *C. albicans* cells on the nanostructured surfaces of the cicada wing was further supported by an LDH cell toxicity assay, which measures the results of an intracellular enzyme upon the compromise of cellular integrity[225]. The results from LDH assay have a similar trend with the FUN1 results and show an increased release of LDH from C. albicans cells with an increase in incubation time on the nano-structured cicada wing surfaces up to 16 hours. However, from figure (2.6) we observe a decrease in LDH release from *C. albicans* cells at 24-hour incubation which correlate with enhanced biofilm production at this time.

2.3.5 Surface Structure and Biofilm Formation

Many microbes including cellular yeast respond to contact with different materials by altering their formation of a mature biofilm[60]. To determine whether *C. albicans* produces different production using a Crystal violet assay[226]. From figure (2.7) I observed, crystal violet intensity which is a measure of biofilm formed to be significantly less on the Cicada wing when compared to the control glass surface.

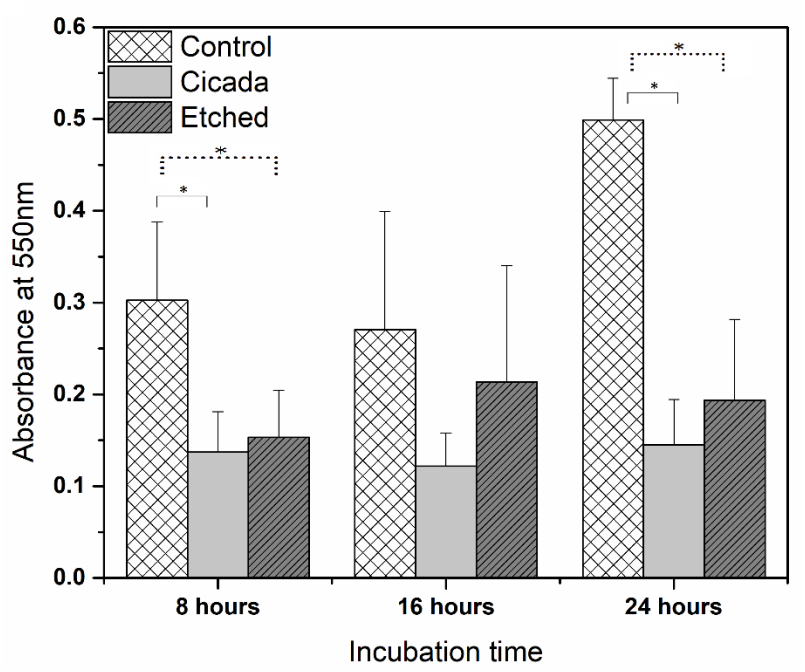


Figure 2.7. Change in biofilm formation of *Candida albicans* quantified by crystal violet assay on different structured surfaces.
***denotes a P ≤ 0.05 and ** denotes a P ≤ 0.001**

2.3.6 Interactions of Hyphal *C. albicans* with Nanostructured Surfaces

C. albicans is a polymorphic fungus that exists in several distinct morphological forms including a hyphal form that is associated with virulence[227]. To determine whether the hyphal form of *C. albicans* behaved differently on the nanostructured surfaces, we differentiated *C. albicans* cells to form hyphae, incubated on our experimental surfaces and observed their behavior. From the scanning electron microscopy images in figure (2.8), I observed no differences in adhesion, viability or cell integrity of hyphal *C. albicans* on any surface suggesting that this morphological forms of *C. albicans* responds differently to nanoscale cues.

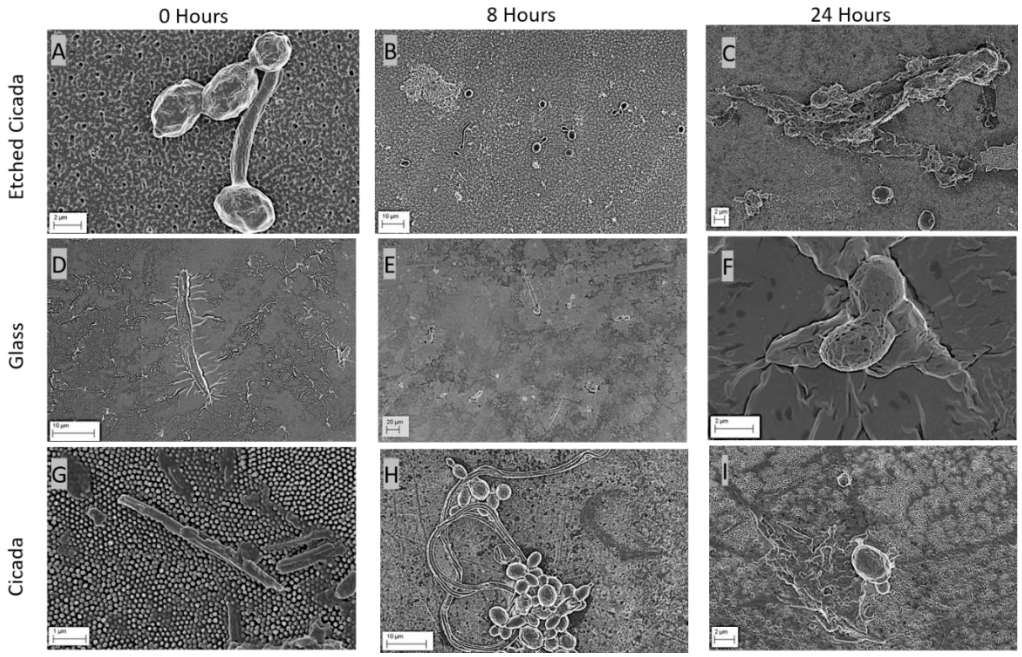


Figure 2.8. Scanning electron micrographs of interaction of *Candida albicans* WT on (A-C) control flat glass surface where the cell structure remained intact even at longer interaction times and increase in colonization of yeast is observed with increase in incubation time (D) Healthy *C. albicans* yeast on Nanostructured Cicada wing at 0-hour incubation (E) ruptured *C. albicans* yeast cell on the cicada wing after 8-hour incubation (f) areas with the remnants of cells and cell debris of ruptured/dead *C. albicans* yeast on Nanostructured Cicada wing after 24-hour incubation (G-I) represents the *C. albicans* yeast on plasma Etched Cicada wing where cells show no damage and also an increased adhesion and colonization with increase in incubation time.

Table 2.2. Effect of surface topography on the morphogenesis of *Candida albicans* on different surfaces.

	Control Glass	**Cicada wing**	**Etched Cicada**
Morphogenesis of *C. albicans* WT	✓	⊘	✓

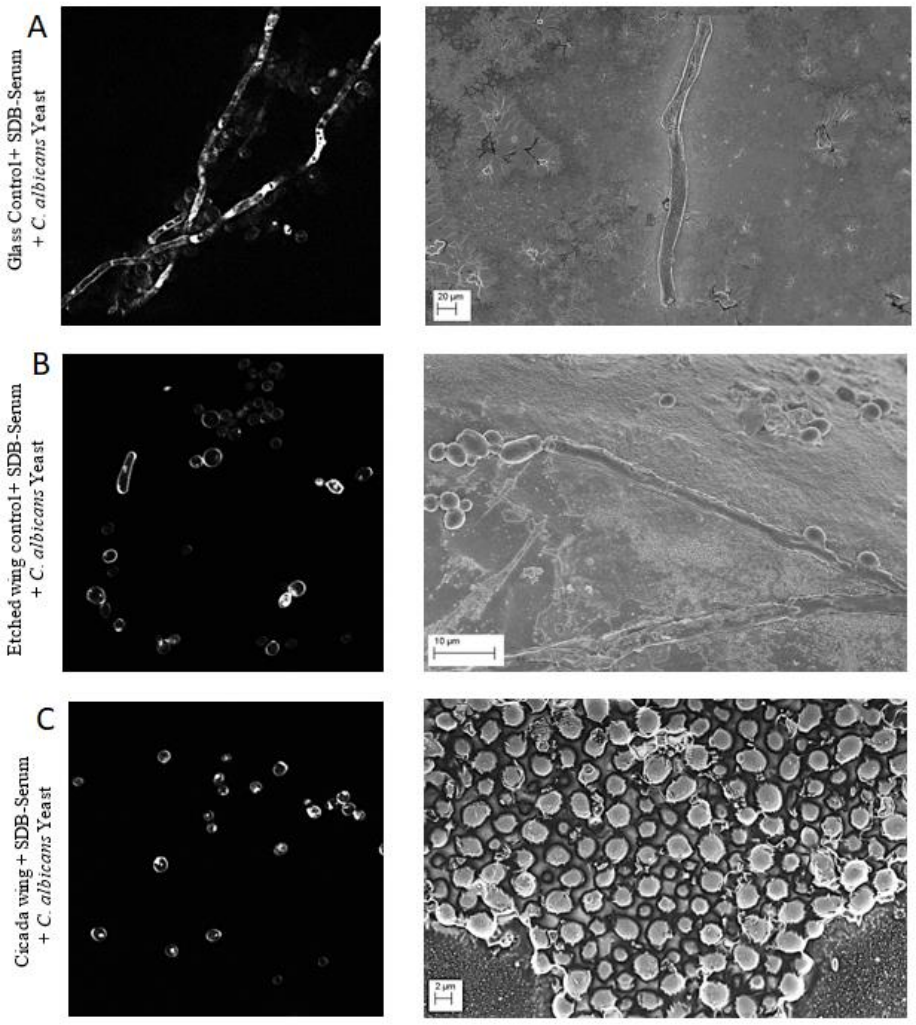


Figure 2.9. Micro colony assay demonstrating the effect of NSS on the Morphogenesis of *C. albicans* from Yeast to Hyphae. From (A) glass surface and (B) etched Cicada surface showed no effect on morphogenesis of *C. albicans*. (C) Cicada wing surface inhibits the differentiation of yeast to hyphae.

Although no changes to viability or adhesion of the hyphal forms of *C. albicans* were observed, I did observe an alteration in the ability of *C. albicans* to change its morphology when in contact with the nanostructured surface of the dog day cicada (Table 2.2, Fig 2.9). The morphogenesis of cellular form to the hyphal form is an important

event of pathogenesis in the life cycle of *C. albicans[228]*. Many different environmental conditions result in the switch from a cellular of *C. albicans* to a hyphal morphology including nutrient levels, temperature, and cell numbers[229]. Experimentally the addition of serum to the culture medium results in a switch to a hyphal form[27]. *C. albicans* microcolonies cultured on flat glass substrates in a modified SDB medium that contained 10% bovine albumin serum exhibited tubular hyphal morphology as demonstrated by confocal laser and SEM micrographs (Fig 2.9 A). Etched wing surfaces with *C. albicans* microcolonies cultured under similar growth conditions also exhibited a similar transformation from yeast to hyphae although not nearly as pronounced as those on flat substrate and primarily presented as pseudo hyphal formation (Fig 2.9 B) . However, the microcolonies of *C. albicans* cultured on the nanostructured arrays found on the wings of the dog day cicada had no hyphal formation (Fig 2.9 C).

2.4 Discussion

The range of microbial cells that rupture on these surfaces supports this conjecture. However, there are two caveats to this model. First, different microbes engage surfaces differently and a purely biophysical model for rupture demands the identification of a common mode of surface engagement by all microbes. Second, if NSS interactions are mechanical then a pure physically incompatible model would need to account for the differences in the mechanical and/or morphological properties of microbes. These two caveats suggest that the mechanism for microbial cell rupture by NSS may be more complex. The mechanism of rupture of pathogenic fungal cells is crucial when designing

antifungal surfaces[230, 231]. The need for novel biocompatible antimicrobial surfaces is gaining importance especially with increasing infections of multidrug resistant strains and failure in indwelling medical devices[232, 233]. While antimicrobial surfaces are not new, rupture inducing NSS operates in a manner that is contrary to the strategies of previously conceived anti-microbial surfaces; that is, NSS promote and require stable cell/substrate adhesion rather than inhibit it[234]. Research on interactions of anti-microbial nanostructured surfaces and bacteria is being widely studied but the interaction of much important pathogenic fungi on the nanostructured surfaces needs to be explored[235, 236]. Although there is a great potential for NSS application, there are no clearly defined limitations of NSS as a mechanical control of fungal growth, or how a cell may develop resistance; by studying the mechanism of NSS rupture, this study will also be the first to study this aspect of NSS in any system.

With an increasing number of drug resistant strains of microbes and emerging superbugs the need for alternate techniques to tackle these deadly microbes is becoming eminent. Fungal super bugs like *Candida auris* of the same family as *Candida albicans* are resistant to most available fungal drugs available in the market either directly or also in combinations[237]. Unlike its other family species C. auris has the ability to proliferate outside the host body and proliferates rapidly in the environment specially on Hospital beds/apparel, heart rate monitors, I-V piping, and sanitation areas, increasing the chances of reinfections[238]. Though spraying antifungal agents and use of harsh chemicals temporarily solves this problem but in a longer time frame in turn leads to the evolution of antifungal species of microbes and other superbugs[52].

Anti-microbial coating on surfaces is an ideal addition to existing anti-microbial techniques as they can be used as coatings on all the surface prone to multi-species microbial fouling and also on indwelling medical devices which significantly raise the lifespan of these implants. Though anti-microbial efficiency of naturally evolved nanostructured anti-microbial surfaces were previously studied on both gram positive and gram-negative bacteria their interaction with a much complex fungal system was only briefly studied. In this study we demonstrate the antimicrobial efficacy of nanostructured surfaces against a harmful pathogenic fungus *Candida albicans*. The adhesion and biofilm formation of *Candida albicans* yeast varied significantly when interacted with different nanostructured surfaces. The inhibition of microbial adhesion displayed by the Cicada wing compared to the control glass surface or etched cicada wing confirmed the role of nano-cones on the surface interaction with microbes. Biofilm formation which is responsible for failure of many indwelling medical devices and a root cause for most *Candida* related infections was also significantly reduced when incubated on the NSS than flat surfaces. This demonstrates the role and need to integrate these NSS as surface coatings on various medical devices and catheters to enhance their life span and reduce further secondary infections. The rupture/death of various bacteria and *S. cerevisiae* on native NSS were previously demonstrated but the in-depth interaction with pathogenic fungi or timing of rupture by these NSS was ignored. The NSS exhibited contact killing abilities by rupturing *C. albicans* yeast on prolonged interaction with the NSS. The interaction time required to rupture the pathogenic yeast was a lot higher than bacterial cells which is attributed to the higher complexity of the fungal cell wall when compared

to bacteria. NSS-*C. albicans* interactions exhibited a change in chitin concentration in the fungal cell wall which is predicted to be a mechanical stress response of the yeast due to the NSS assault.

We observed the role played by the formation of biofilm in the inhibition of rupture and increased adhesion on the substrates. From the (fig 2.6) LDH cell assay performed on the control and the Cicada wing showed an increase in cytotoxicity till 16 hours incubation in the nutrient rich conditions, a sudden decrease in cytotoxicity at 24 hours was observed which is attributed to the excess biofilm formation which block the fungicidal interactions between the cones and *C. albicans.* Though the hyphal structures of *C. albicans* did not exhibit any morphological damage on interaction with the nano structured surfaces we observed an effect on the morphogenesis from yeast to hyphae on these surfaces. The hyphal transformation inhibition on the Cicada wing was observed on both control glass and etched cicada surface confirming that the nano cones have a definite effect on the morphogenesis cycle. We predict the mechanical stress induced by the nano cones on the yeast might impact the cell wall biosynthesis pathways which are essential for the morphogenesis and a detailed genomic analysis is required to confirm it. The nano structured cicada wing exhibited excellent antimicrobial characteristics like inhibiting the adhesion of *C. albicans*, rupturing the cellular yeast on prolonged contact and also controlling the pathogenicity by inhibiting the morphogenesis pathway proving that these nanostructured surfaces if mimicked can be ideal anti-fungal surface.

CHAPTER III

INTERACTION OF DRUG RESISTANCE STRAINS OF *CANDIDA ALBICANS* WITH NANOSTRUCTURED SURFACES

3.1 Introduction

The past decade has witnessed a significant increase in the development of resistance to antibacterial and antifungal agents primarily due to the misuse of antibiotics and evolutionary changes in microbes[239]. Globally, deaths attributed to antibiotic resistance in bacterial infections is more than 700,000 every year and is expected to reach 10 million by 2050[240]. Failure to eradicate a fungal infection from a patient by the use of an antifungal drug which was previously used or demonstrated proven antimicrobial efficacy is called Clinical resistance. Mycological resistance is the in-vitro resistance developed by fungi to the agents which were previously susceptible. Antifungal resistance is a growing concern in the field of medical mycology, predominantly in human commensals like *Candida albicans* which turn pathogenic during sickness or in immune deprived patients like patients suffering from HIV. This is a leading cause of health care associated infections[241]. 7-15% of all *Candida* bloodstream isolates tested were resistant to fluconazole and the drug resistance is higher in other *Candida* species like *C. glabrata* and *C. parapsilosis*. 20–30% of candidemia cases involve species with intrinsic resistance to either fluconazole or echinocandins[242]. In the past few years, significant attention is given to understand the mechanisms of antimicrobial resistance, to improve detection and find alternative methods to tackle antimicrobial resistance[243].

Most of the antimicrobial research is focused on bacterial resistance primarily as they account to a large fraction of human infections. Until recently, fungal species are not identified as serious pathogens but their ability to turn pathogenic causing infections on indwelling medical implants and co-existing with other deadly bacterial species, made the study of molecular mechanisms of resistance and innovation of alternate antifungal techniques against resistant species necessary[244]. Interactions between sterols and phospholipids in the plasma membrane of yeast affect the membrane fluidity and asymmetry and consequently influence the transport of materials across the membranes[243]. A decrease in the amount of drug taken up by the fungal cell may result from changes in the sterol and/or the phospholipid composition of the fungal cell membrane.

Mechanisms by which fungal cells may develop resistance[245]

1) The target enzyme is overproduced, so the drug becomes inefficient to inhibit the biochemical reactions
2) The target drug is altered so the drug cannot bind to the target
3) The drug administered is pumped out by an efflux pump.
4) Fungi develop mechanisms to inhibit the entry of drug at the cell membrane/cell wall
5) Cell develops mechanisms to compensate the loss of function caused by the drug
6) Nonessential enzymes targeted by the fungal drugs are inhibited
7) Fungi can produce enzymes which degrade the drugs before entering the cell.

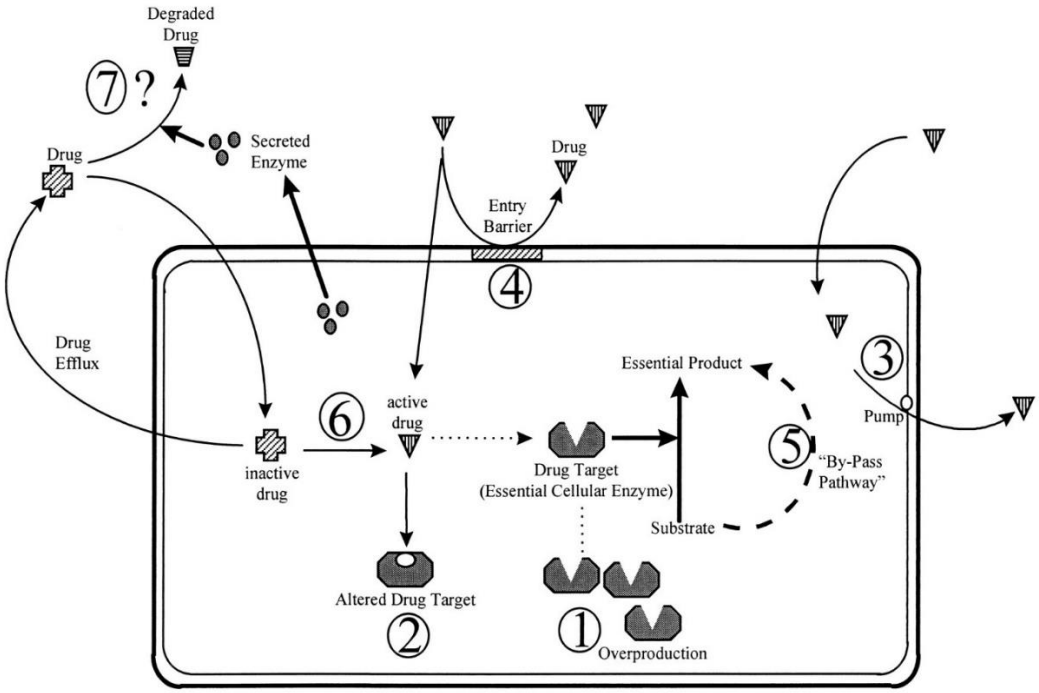


Figure 3.1. Mechanisms by which *Candida albica*ns may develop drug resistance[243]

Therapy for *Candida* infections has been difficult because of the limited number of available antifungal agents. Though Amphotericin B is administered against many infections caused by different *Candida sps.*, it is associated with many nonspecific toxicities and requires intravenous administration[246]. Until recently, Azole drugs were the first choice as they are bioavailable after oral administration but the increase in MIC rates due to developing resistance made it impossible[247, 248]. Azoles treat fungal infections by interfering with the enzyme lanosterol 14-a-sterol demethylase involved in ergosterol biosynthesis, a prominent component of the fungal cell wall, and is a promising antifungal target. This interference causes inhibition of fungal growth by altering the structure and function of the cell membrane[249]. Azole selectively targets fungal cells as their major target components chitin, glucan and mannose are all absent in human cells and the fungal cells also exhibit differently structured ergosterol and

cholesterol compared to the host[250]. Commonly used azole derivatives such as fluconazole, itraconazole, and voriconazole partly target the inhibition of cytochrome P-450-dependent 14α-sterol demethylase[245]. There are no evidences demonstrating modification of azole antimicrobials as a mechanism of resistance. Resistant strains exhibit a modification in the quality or quantity of target enzyme, reduced access to the target, or some combination of these mechanisms. Overexpression of 14α-demethylase which results in the increase in ergosterol synthesis has been implicated as a mechanism of resistance to azole antifungals. *Candida albicans* can become resistant to azoles by increasing the number of efflux pumps in the cell which are simple membrane associated transporters and prevent intracellular accumulation of the drug[107, 251, 252]. Table 3.1. summarizes the biochemical mechanisms of azole resistance in *C. albicans*.

Table 3.1. Biochemical basis of azole resistance[243]

Mechanism	**Caused by:**	**Result**
Alteration in drug target (14α-demethylase)	Mutations which alter drug binding but not binding of the endogenous substrate	Target is active (i.e., can catalyze demethylation) but has a reduced affinity towards azoles
Alteration in sterol biosynthesis	Lesions in the $\Delta^{5(6)}$-desaturase	Results in accumulation of 14α-methyl fecosterol instead of ergosterol
Reduction in the intercellular concentration of target enzyme	Change in membrane lipid and sterols; overexpression of specific drug efflux pumps (CDR1, PDR5, and BEN^r)	Poor penetration across the fungal membrane; active drug efflux

Mechanism	Caused by:	Result
Overexpression of antifungal drug target	Increased copy number of the target enzyme	Results in increased ergosterol synthesis; contributes to cross-resistance between fluconazole and itraconazole

With the increasing resistance of azole class of antifungal drugs in *Candida sps.*, the use of Echinocandins have proven to be effective. Lipopeptidic echinocandins interfere with glucan synthesis of the cell wall[253] by inhibiting the synthesis of β-1-3 D glucan synthase. Most anti-fungal drugs target β-1-3 D-glucan, a critical cell wall polysaccharide as it is present in all yeast cells and acts by selectively targeting the yeasts cells in the host system[254]. Echinocandins cause impaired fungal cell wall formation that can result in osmotic lysis, makes the fungal cell vulnerable to external stresses causing fungal cell death[255]. In addition to being efficacious for the treatment of invasive candidiasis, the echinocandins are also advantageous due to their lack of clinically significant drug-drug interactions and adverse effects[256, 257]. The prolonged use of Echinocandins has led to development of resistance in *Candida albicans* causing recurrent Candidemia specially in immunocompromised patients. First reported cases were from HIV infected patients suffering with esophageal candidiasis due to continuing immunosuppression secondary to their viral infection[258, 259]. In the recent times, Echinocandin resistance in *Candida* sps. is increasingly seen in infections caused in implant recipients and is mostly attributed to the mutations caused in FKS1 and FKS2 genes. The production of biofilm to resist the antimicrobial action of the drugs is

common in fungal species. *Candida albicans* has shown a 1000-fold greater drug resistance in biofilm-forming cells compared with non-biofilm cells in-vitro[260].

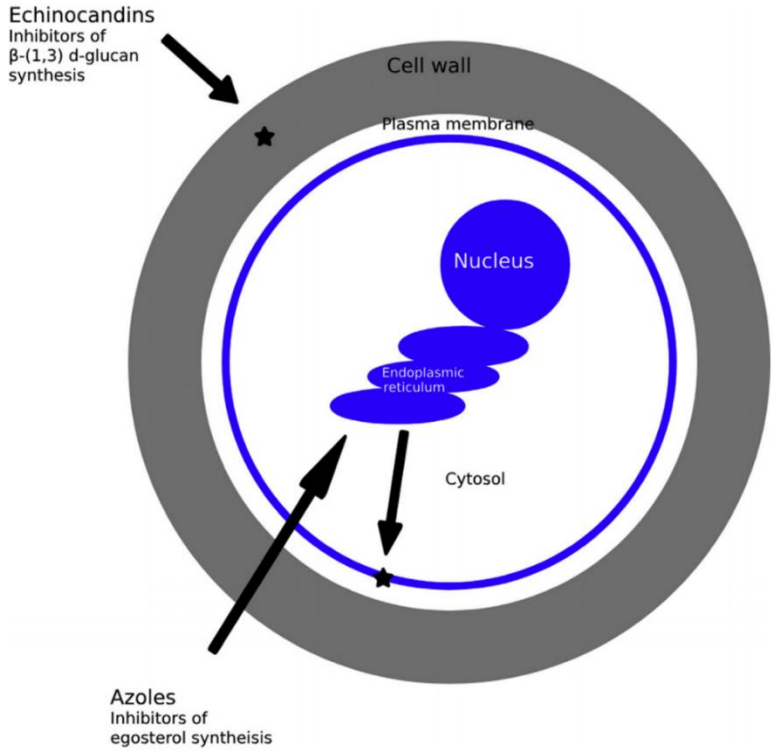


Figure 3.2. Primary targets and mode of action by azoles and echinocandins[251]

With emergence of multidrug resistance in *Candida* sps. affecting hospital settings and patients globally[261], the need for alternate approaches to tackle the antimicrobial resistance is gaining prominence. Antimicrobial surfaces have proven to be effective, even on the drug resistant strains of microbes and there is no known mechanism which can develop resistance to the mechanical stress induced by antimicrobial surfaces. Coating surfaces in healthcare environment with antimicrobial materials made it possible to reduce microbial contamination and also delay

recontamination in a sustained manner[262]. Approaches such as combined therapies to stop emergence of resistant species, where two or more biocides are used simultaneously, have shown to inhibit pathogenic microorganisms as well as their proliferation by promoting further mechanisms for microbe killing[263].

Table 3.2. *Candida albicans* drug resistant strains of ATCC MP-8 panel used in this study

***Candida albicans* strains**	**Resistant Drugs**	**Isolation Source**
Candida albicans WT (ATCC 90028)	None	Blood, Iowa
Candida albicans (ATCC 96901)	Fluconazole	Mouth of HIV-positive patient, Omaha
Candida albicans (ATCC 11651)	Itraconazole	Lung pus, Virginia
Candida albicans (ATCC 76485)	Anidulafungin, Caspofungin	Cerebrospinal fluid of a 6-month-old infant, Dallas
Candida albicans (ATCC 10231)	Anidulafungin, voriconazole, itraconazole, and fluconazole	Man with Broncho mycosis
Candida albicans (ATCC 90029)	5-flucytosine	Blood, Iowa

In the previous experiments, we have demonstrated the efficacy of native nanostructured surfaces as anti-fouling & biocidal and also inhibiting virulence in pathogenic fungi *C. albicans*. We aim to determine the behavior of drug resistant strains of *C. albicans* of ATCC MP-8 panel, listed in table (3.2), which have shown resistance to

one or more antifungal drugs such as anidulafungin, micafungin, caspofungin, 5-flucytosine, voriconazole, itraconazole, and fluconazole.

3.2 Material and Methods

Candida albicans strains listed in table (3.2) purchased from ATCC were stored in -80^0 C freezer. For each experiment, colonies from freshly streaked plates were grown in Sabouraud dextrose broth (SDB) (peptone, dextrose, water) at 25^{0}C in 50 ml conical flasks with shaking ≈ 150 rpm to an early-log phase measured at OD~0.5 where cells are metabolically active. All the work with drug resistant strains was conducted following BSL-2 laboratory guidelines. The cells were later labeled with florescent dyes Calcofluor white and FUN-1 for enumeration on different surface topologies and to determine their viability as previously described in (2.2.3). Anti-fungal susceptibility tests were performed using Fluconazole (Sigma-Aldrich PHR1160) Itraconazole (Sigma-Aldrich PHR 1834) Voriconazole (Sigma-Aldrich PHR 1892) 5-flucytosone (Sigma-Aldrich F0175000) and Anidulafungin (Sigma-Aldrich SML2288) by making drug stocks in DMSO(Sigma-Aldrich 276855) and water, where the final concentration of DMSO was less than 1%. Susceptibility of these resistant strains of *C. albicans* to their respective drugs was done by serial dilution method where the OD_{600} values were measured using BioTek Synergy Mx plate reader. Stock solution for all the drugs were prepared following the CLSI susceptibility range for *Candida albicans[264]*. Tables (3.3) and (3.4) are the standard dilution factors for specific solvents to be used for the drugs for clinical and laboratory use. Biofilm formation of all the drug resistant strains of *C. albicans* on the nanostructured surface was tested by crystal violet method described in

chapter 2. All the control glass surfaces, and nano structured cicada wings used in this study were coated with a 7nm layer of gold using a Leica EM ACE200 with a real time thickness monitoring quartz crystal microbalance (QCM).

Table 3.3. Scheme for preparing dilutions of water-soluble antifungal drugs to be used for susceptibility test against *C. albicans* strains

Antimicrobial Solution								
Step	Concentration (µg/mL)	Source	Volume (mL)	+ Medium (mL)	= Intermediate Concentration (µg/mL)	= Final Concentration at 1:10 (µg/mL)	Log_2
1	5120	Stock	1 mL	7	640 µg/mL	64	6
2	640	Step 1	1.0	1.0	320	32	5
3	640	Step 1	1.0	3.0	160	16	4
4	160	Step 3	1.0	1.0	80	8	3
5	160	Step 3	0.5	1.5	40	4	2
6	160	Step 3	0.5	3.5	20	2	1
7	20	Step 6	1.0	1.0	10	1	0
8	20	Step 6	0.5	1.5	5	0.5	−1
9	20	Step 6	0.5	3.5	2.5	0.25	−2
10	2.5	Step 9	1.0	1.0	1.25	0.125	−3
11	2.5	Step 9	0.5	1.5	0.625	0.0625	−4
12	2.5	Step 9	0.5	3.5	0.3125	0.03125	−5

Table 3.4. Scheme for preparing dilutions of water-insoluble antifungal drugs to be used for susceptibility test against *C. albicans* strains

Antimicrobial Solution								
Step	Concentration (µg/mL)	Source	Volume (mL)	+ Solvent (mL) (eg, DMSO)	= Intermediate Concentration (µg/mL)	= Final Concentration at 1:100 (µg/mL)	Log_2
1	1600	Stock			1600 µg/mL	16	4
2	1600	Stock	0.5	0.5	800	8.0	3
3	1600	Stock	0.5	1.5	400	4.0	2
4	1600	Stock	0.5	3.5	200	2.0	1
5	200	Step 4	0.5	0.5	100	1.0	0
6	200	Step 4	0.5	1.5	50	0.5	−1
7	200	Step 4	0.5	3.5	25	0.25	−2
8	25	Step 7	0.5	0.5	12.5	0.125	−3
9	25	Step 7	0.5	1.5	6.25	0.0625	−4
10	25	Step 7	0.5	3.5	3.13	0.0313	−5

Quantification of Biofilm formed by *Candida albicans* of various strains on nanostructured surfaces was measured using crystal violet technique of biofilm quantification as previously described. Statistical analysis was done using single factor Anova on Microsoft Excel. Scanning electron microscopy was performed on all samples to confirm the results. Same protocols for confocal and scanning electron microscopy described earlier were followed for imaging the drug resistant strains.

3.3 Results

3.3.1 Minimum Inhibitory Concentration of Anti-fungal Drugs to *C. albicans* Mutant Strains

The antifungal action on the *Candida sps*. by the drugs was observed to be dose dependent and was found to vary with the change in number of cells. Susceptibility of *C. albicans* strains to antifungal drugs obtained by serial dilution method was in coherence with the data available from Clinical & Laboratory Standards Institute (CLSI). Fluconazole drug resistant strain *C. albicans* 96901 showed an increase in MIC compared to other antifungal drugs and the wild type. *C. albicans* strains specifically resistant to other azole drugs like voriconazole and itraconazole showed an increase in MIC required for *C. albicans* strains ATCC 11651 and ATCC 90029. The MIC required was quantified for all the *Candida* strains at a concentration of 30,000 cells/ml to all the used antifungal drugs was in table (3.5). Candida mutants exhibiting to specific resistant drug have shown a higher dose requirement of most drugs compared to wild type.

Table 3.5. *In-Vitro* activity of antifungal drugs fluconazole, itraconazole, voriconazole, anidulafungin and 5-flucytosine against various drug resistant *Candida albicans* mutant strains

	C.a WT	C.a 96901 Fluconazole	C.a 11651 Itraconazole	C.a 76485 Echinocandin	C.a 10231 Multi Drug	C.a 90029 Flucytosine
Fluconazole	0.25µg/ml	RESISTANT >256 µg/ml	0.25µg/ml	0.25µg/ml	RESISTANT >32 µg/ml	0.25µg/ml
Itraconazole	0.12µg/ml	0.24µg/ml	RESISTANT >32 µg/ml	0.24µg/ml	RESISTANT >32 µg/ml	0.24µg/ml
Voriconazole	0.64 µg/ml	0.064µg/ml	0.064µg/ml	0.064µg/ml	RESISTANT >32 µg/ml	0.064µg/ml
Anidulafungin	0.081 µg/ml	0.081 µg/ml	0.081 µg/ml	RESISTANT >2 µg/ml	RESISTANT >2 µg/ml	0.081 µg/ml
5-Flucytosine	0.25µg/ml	0. 25 µg/ml	0.25µg/ml	0.25µg/ml	0.25µg/ml	RESISTANT >32 µg/ml

3.3.2 Adhesion and Viability of Drug Resistant Strains of *C. albicans* on NSS of Cicada Wing

Drug resistance developed in C. albicans depends on various crucial mechanisms, most of them related to the cell wall bio synthesis pathways. In these experiments, I aim to determine how different strains of *Candida albicans* with specific resistance developed to anti-fungal mechanisms respond to the mechanical stress induced by the nano cone structures of the Cicada wing.

3.3.2.1 Interaction of Fluconazole Resistant *C. albicans* 96901 with NSS

Candida albicans yeast resistant to fluconazole, the most commonly used antifungal drug of the class azoles, is interacted with nano structured surface of the Cicada wing and a glass cover slip as control to study adhesion and cell viability of the drug resistance mutants. From the confocal microscopy images (figure 3.3), I observe an

increase in the number of cells on the surface with an increase in the incubation time, primarily as the cells replicate and increase in number with time. Quantifying the number of cells, figure (3.3) shows no significant change in the initial adhesion of the *C. albicans* cells on the nano structured surface of the Cicada wing at incubation time 0 hours and 8 hours compared to the NSS. But the adhesion of Fluconazole resistant *C. albicans* at incubation time of 16 hours is significantly ($P \leq 0.05$) lower on NSS compared to the control glass.

The cell viability data from the Fun-1 fluorescent assay showed an increased percentage rupture of fluconazole resistant yeast on the NSS when compared to the flat surfaces. At 16 hours of incubation, we observed a significant ($P \leq 0.001$) mortality of cells on the NSS compared to the control surface. Though the rupture of these resistant cells is comparatively less than the wild type, it is higher than the control surfaces, indicating that these NSS can be used against drug resistant fungi.

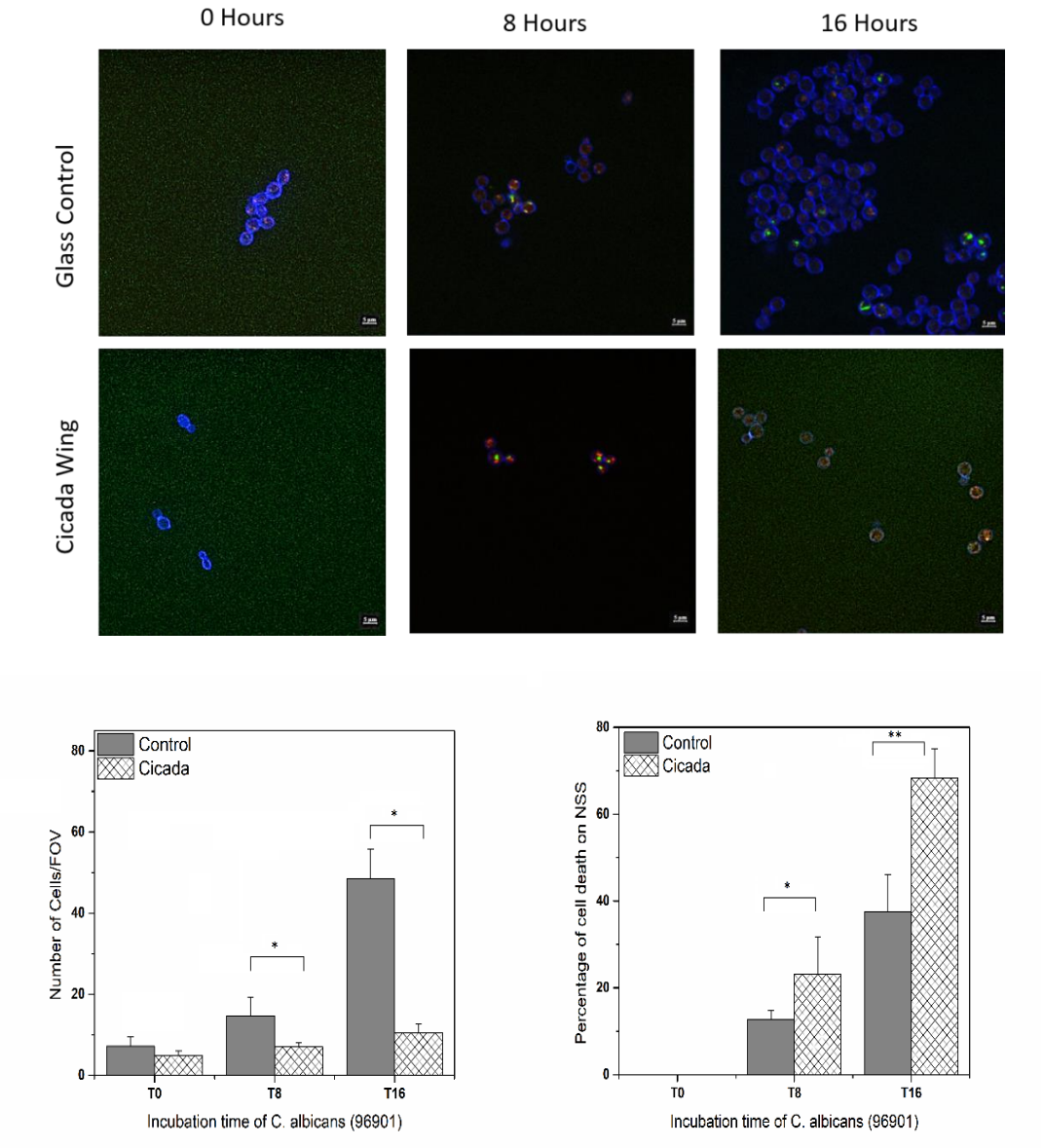


Figure 3.3. Confocal microscopy studies of fluconazole resistant *C. albicans* 96901 on NSS of Cicada wing. Each bar graph represents average of data collected from 10 field of views.
***denotes a $P \leq 0.05$ and ** denotes a $P \leq 0.001$**

3.3.2.2 Interaction of Itraconazole Resistant *C. albicans* ATCC 11651 with NSS

Itraconazole is also a member of the azole class of antifungal drugs and acts by acting on the 14α-sterol demethylase involved in ergosterol biosynthesis yielding changes to the plasma membrane. The adhesion behavior of these *C. albicans* 11651 which have developed resistance by acting on the cell wall biosynthesis pathway like *C. albicans* 96901 was very different. From the figure (3.4) itraconazole resistant fungi showed very little adherence to both flat and structured surfaces but for the adhered cells, though not significant, the cells on the NSS showed higher cell death compared to the flat surface.

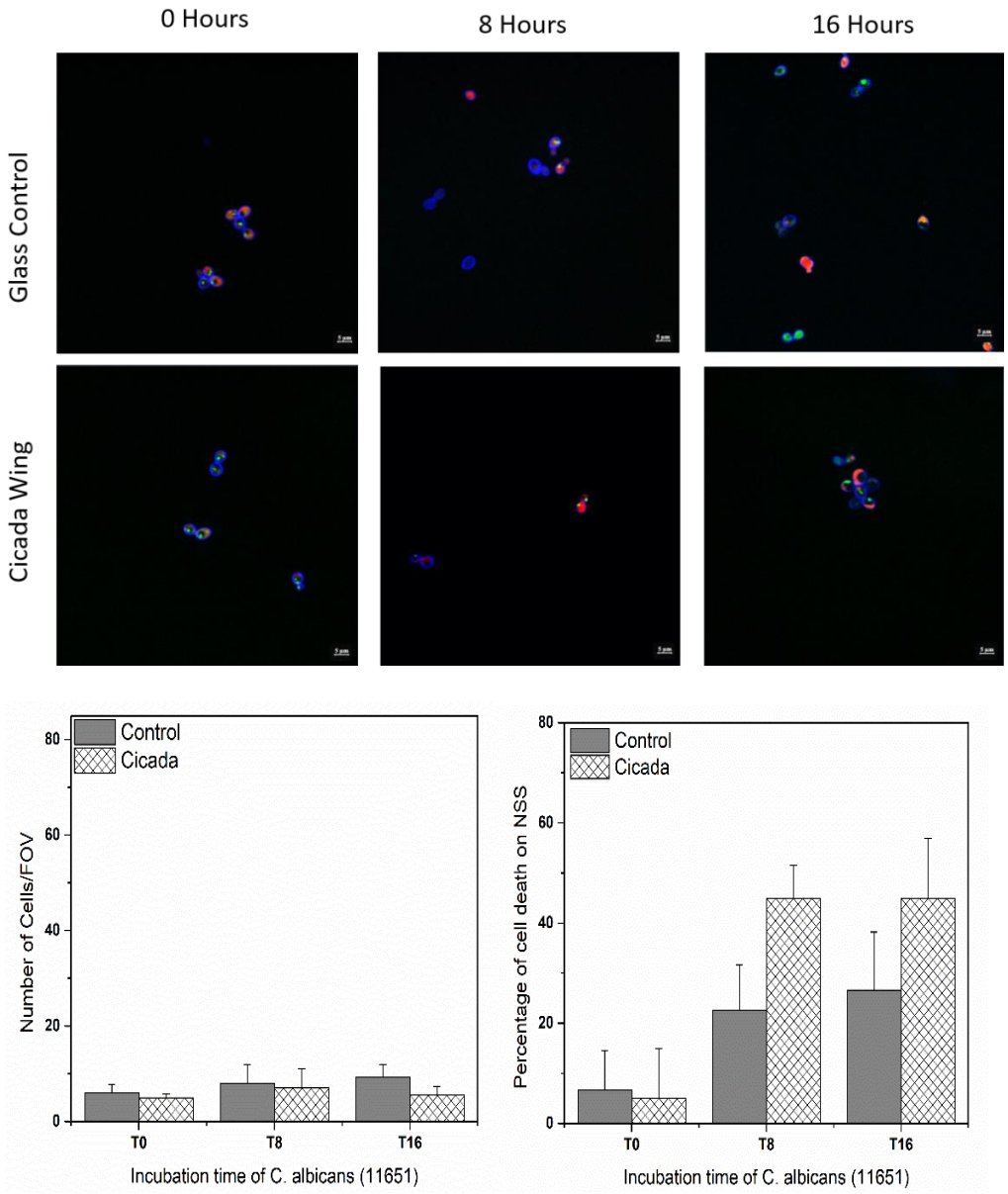


Figure 3.4. Confocal microscopy studies of Itraconazole resistant *C. albicans* 11651 on NSS of Cicada wing. Each bar graph represents average of data collected from 10 field of views.
***denotes a $P \leq 0.05$; ** denotes a $P \leq 0.001$**

3.3.2.3 Interaction of Caspofungin and Anidulafungin Resistant *C. albicans* ATCC 76485 with NSS

Candida albicans mutants have developed resistance to echinocandin class of antifungal drugs which acts by reducing the cell wall rigidity. When these yeast cells were inoculated on the NSS of the Cicada wing and flat glass surface figure (3.5), a reduced adhesion of these cells to all the surfaces was observed. Echinocandin resistant cells at 16 hours of incubation showed a significant decrease in number of cells adhering to the control and NSS of Cicada compared to the wild type *C. albicans* strain.

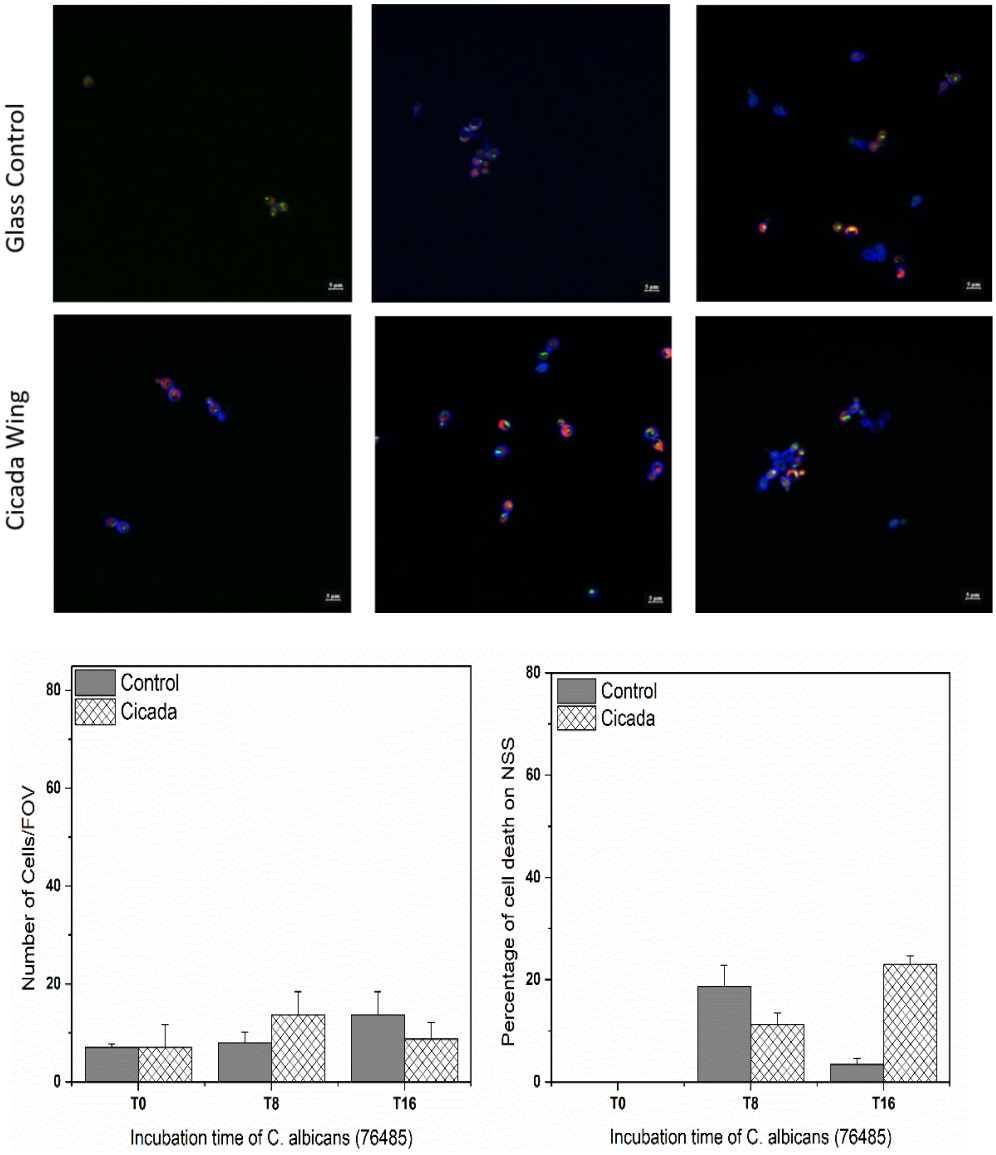


Figure 3.5. Confocal microscopy studies of anidulafungin and caspofungin drug resistant *C. albicans* on NSS of Cicada wing. Each bar graph represents average of data collected from 10 field of views.
***denotes a $P \leq 0.05$; ** denotes a $P \leq 0.001$**

With fewer cells adhering to the surface, the cell viability on the control and test surface did not show any significant change in cell viability with increase in incubation time of yeast on the surfaces. I predict that the echinocandin resistant mutant cells of *C.*

albicans have a thicker cell wall compared to the wild type strains as a physiological adaptation by which antifungal resistance is developed, which is responsible for the anomalous behavior on these surfaces.

3.3.2.4 Interaction of Multidrug Resistant *C. albicans* ATCC 10231 with NSS

Candida albicans yeast 10231 are mutants that have developed resistant to multiple azole and echinocandin class of drugs like fluconazole itraconazole, voriconazole anidulafungin and caspofungin were inoculated on flat and NSS to study their cell viability and adhesion behavior. From the confocal microscopy images, an overall low adhesion tendency of mutants compared to wild type strains was observed. The adhesion of these mutant cells was found to be significantly ($P \leq 0.001$) low at 0-hour and 8-hour incubation on NSS of Cicada wing when compared to the flat glass surfaces. A slight increase in the percentage of cell death was observed on the NSS at all time points compared to the controls but the cell rupture efficiency towards these mutants is below par compared to the wild type *C. albicans.*

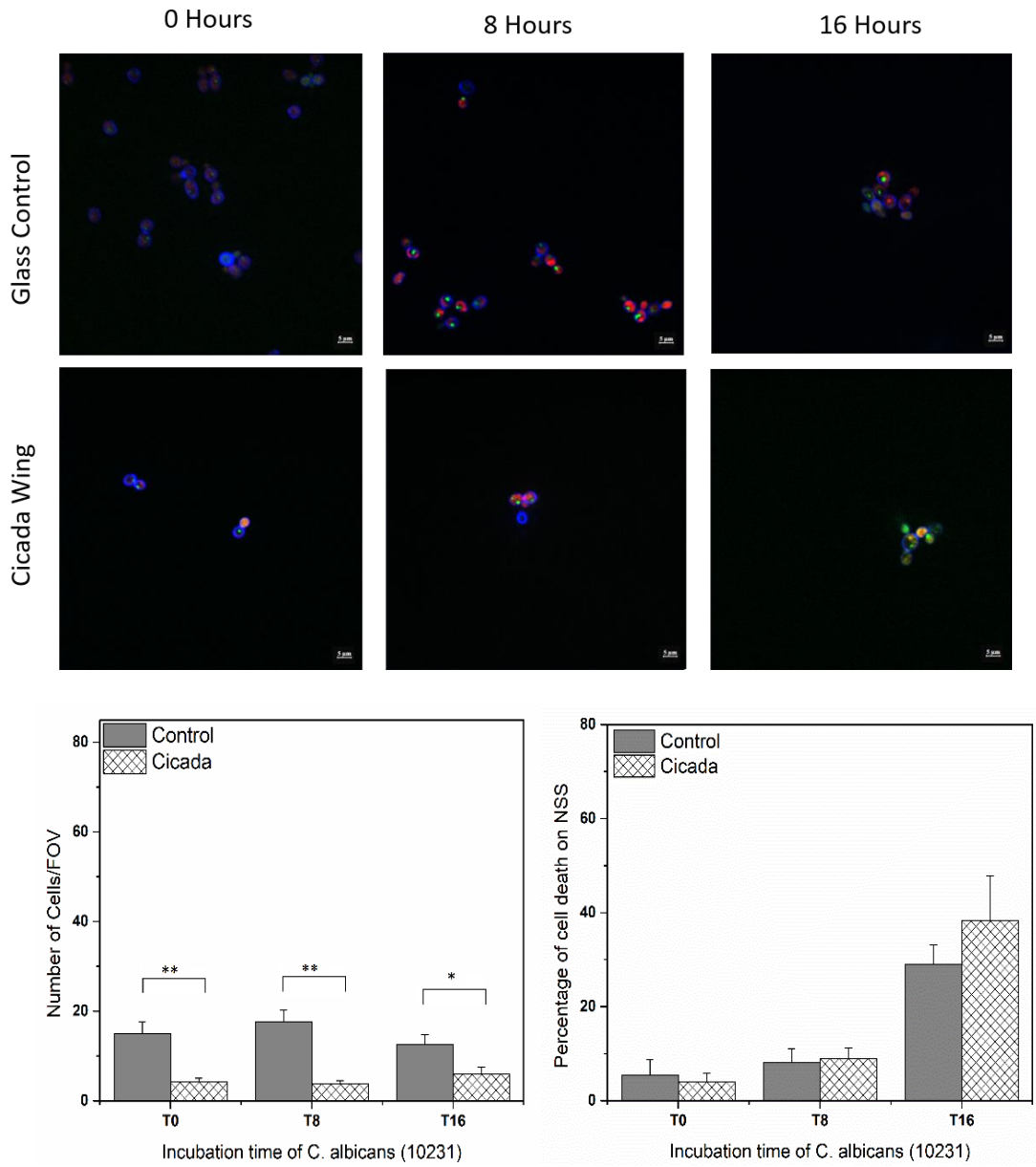


Figure 3.6. Confocal microscopy studies of azole and echinocandin class drug resistant *C. albicans 10231* on NSS of Cicada wing. Each bar graph represents average of data collected from 10 field of views.
***denotes a $P \leq 0.05$; ** denotes a $P \leq 0.001$**

3.3.2.5 Interaction of Flucytosine Resistant *C. albicans* ATCC 90029 with NSS

Of all the *C. albicans* drug resistant mutants we worked on, flucytosine resistance strains differ in the mechanism of development of resistance. Flucytosine drug is an effective antifungal by affecting the nucleic acid of the fungi and these mutants develop resistance by blocking the nucleic acid damage. Flucytosine resistant *C. albicans* strains showed an increase in the number of cells adhering with an increase in incubation time on the substrates but the adhesion of *C. albicans* cells on the flat glass surface versus NSS of cicada wing showed no significant change. However, the cell viability assay of these strains of fungi on the substrates confirmed an anti-microbial response of the NSS with a significant number of cells showing cell damage compared to those on the control glass surface.

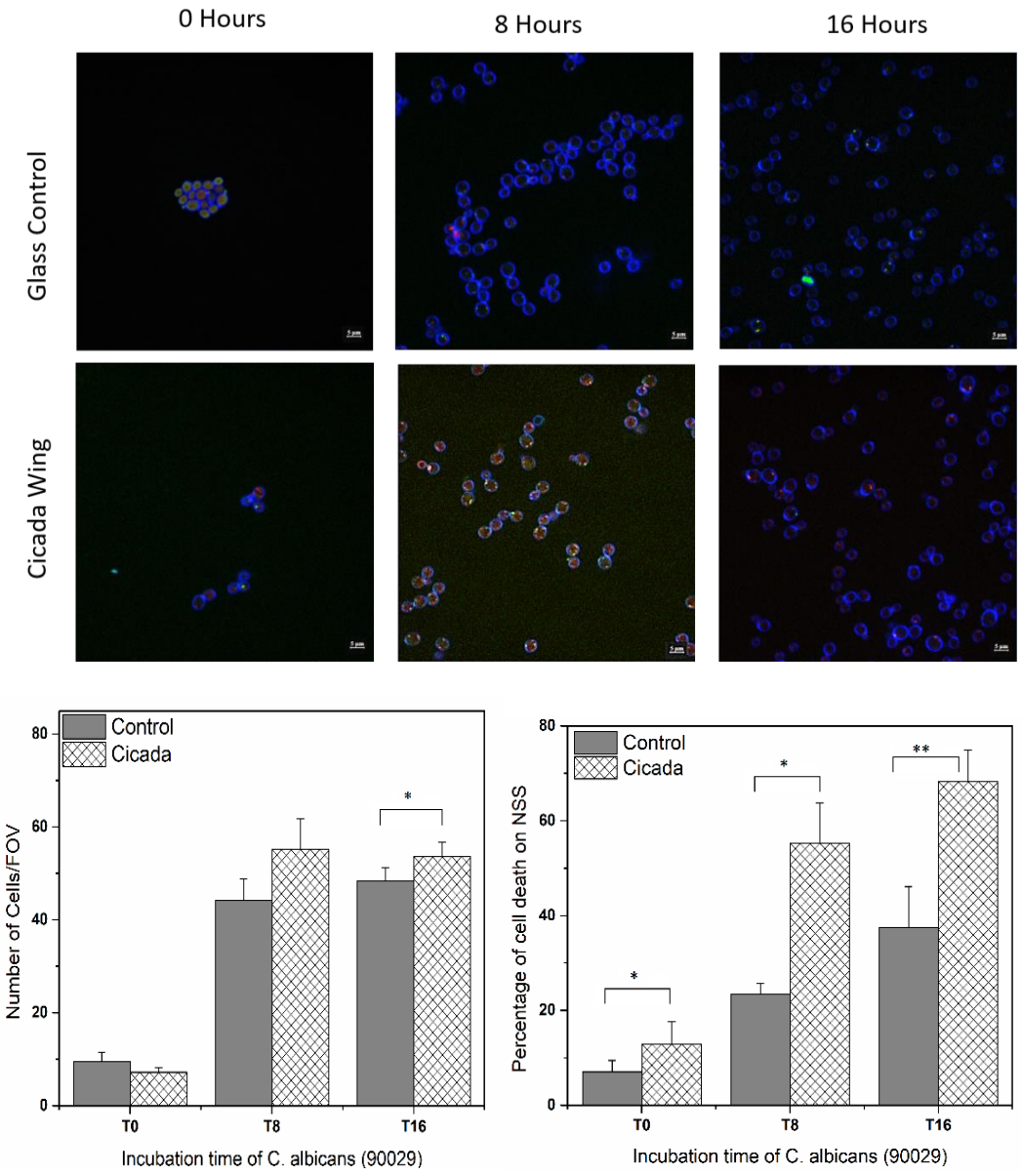


Figure 3.7. Confocal microscopy studies of 5-flucytosine drug resistant *C. albicans* 90029 on NSS of Cicada wing. Each bar graph represents average of data collected from 10 field of views.
***denotes a P ≤ 0.05; ** denotes a P ≤ 0.001**

3.3.3 Biofilm Formation of Drug Resistant Strains of *C. albicans* on Nanostructured Surface of the Cicada Wing

Biofilm formation is a crucial step in the evolution of resistance to specific antifungal drugs and for the virulence in *Candida albicans.* The first step for biofilm formation is the adhesion of microbes to the surface. Change in adhesion of different drug resistant strains of *C. albicans* ultimately affects the biofilm formed on the surface. The biofilms formed by all the mutant yeast strains of *C. albicans* grown on the control glass surface and NSS of Cicada are stained with crystal violet dye and absorbance at 550nm is measured. From the figure (3.8) I observe an inhibition in the biofilm formation on native NSS Cicada wing by wild type and drug resistant strains of *C. albicans.*

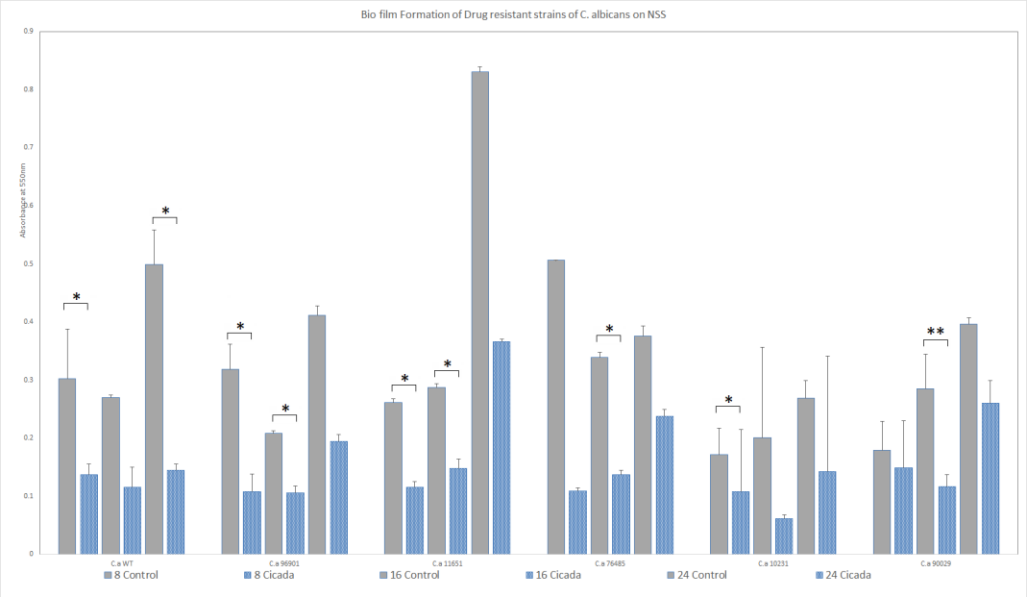


Figure 3.8. Biofilm formation of various drug resistant and wild type strains of *C. albicans* on flat glass surface and NSS of Cicada wing.
***denotes a P ≤ 0.05; ** denotes a P ≤ 0.001**

3.3.4 Effect of NSS on the Morphogenesis of Drug Resistant Strains *C. albicans*

Candida albicans turns pathogenic by transforming from cellular yeast to tubular hyphal form. This morphogenesis is modulated by many factors including a thigmotropic response where the hyphal orientation and formation is dependent on the adhesion of yeast to the substrate. The micro colony assay performed on these specific drug resistant strains of *C. albicans* showed altered responses in the morphogenesis on the NSS and flat surface (Table 3.6). The morphogenesis in resistant strain 96901 showed an inhibition of morphogenesis when inoculated on Cicada wing but showed hyphal structure formation on flat glass cover slip. A similar response was observed in *C. albicans* 11651 which has developed resistance to an azole class drug itraconazole. The morphogenesis experiments in anidulafungin and caspofungin resistant 76485 showed no effect of NSS on the morphogenesis and formed hyphae. The multi drug resistant strain also showed similar response on the NSS and control and formed hyphae. *C. albicans* 90029 had an influence of the nano structured surface in the inhibition of hyphal formation but transformed into hyphae on the flat surface.

Table 3.6. Effect of NSS on the morphogenesis of yeast to hyphae in drug resistant strains of *C. albicans*. ✓ indicates the formation of hyphae and ⊘ indicates the inhibition of hyphal formation on the surface

Morphogenesis	*Candida albicans* WT	C.a 96901 Fluconazole	C.a 11651 Itraconazole	C.a 76485 Fungin	C.a 10231 Multi-Drug	C.a 90029 Flucytosine
Glass	✓	✓	✓	✓	✓	✓
Cicada Wing	⊘	⊘	⊘	✓	✓	⊘

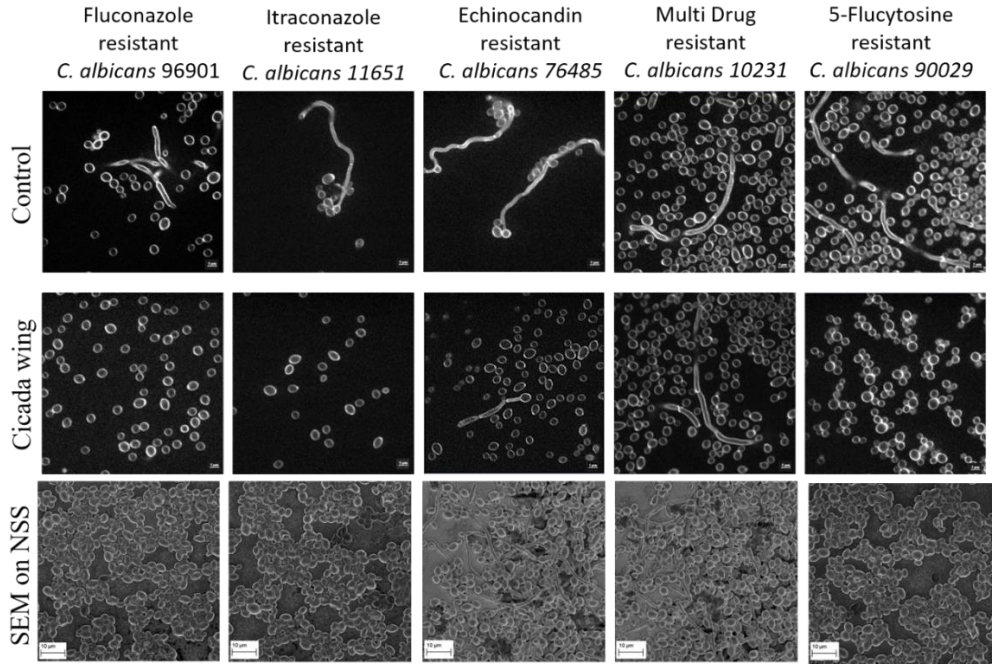


Figure 3.9. Confocal micrographs showing the effect of NSS on the morphogenesis on drug resistant strains of *C. albicans*

3.4 Discussion

Drug resistance in microbes is a growing concern specially in commensal fungi like *Candida albicans* which turn pathogenic and is a serious concern in implant recipients and immunocompromised patients. In this proposed study of alternative antifungal approach using nanostructured surfaces to tackle drug resistance, I focused on three important phases of *Candida* life cycle i.e. adhesion, maturation (biofilm formation) and virulence. The azole class of antifungal drug acts by inhibiting the 14α-demethylase involved in ergosterol, an important component of fungal cell. The anti-fungal resistance developed in *C. albicans* mutants retain the ergosterol biosynthesis and maintain homogeneity in the fungal cell wall. When Fluconazole, an azole class drug resistant strain was exposed to NSS assault, I found similarity in cell rupture and adhesion

behavior of these strains and the wildtype. However, when mutants exhibiting resistance to another azole drug itraconazole was interacted with NSS, a dramatic inhibition in adhesion to all the substrates compared to fluconazole resistant or the wild type strains was observed, indicating the complexity in behavior of *Candida albicans*. Echinocandins on the other hand owe their antimicrobial activity by decreasing the cell wall rigidity and damaging cell integrity. I predict the mutants which exhibit a developed resistance in echinocandins compensate the loss of cell wall thickness and have a thicker cell wall. NSS interaction of the fungin drug *C. albicans* exhibited decreased adhesion on all the substrates like the itraconazole resistant and no effect on the cell viability by the NSS induced stress, demonstrating failure of these nanostructured surfaces to induce anti-microbial effect to these thicker cell-walled pathogens. The behavior of multidrug resistant strain of *C. albicans* was in coherence with the echinocandin resistant strain. Flucytosine resistance is developed by blocking the nucleic acid damage caused by the drug and there was no proven mechanism demonstrated that brings in changes to the cell wall composition or structure in the evolution of fluconazole resistance. Rightfully so, the flucytosine resistant strain exhibited similar response in cell adhesion and viability to the wild type strains of *C. albicans*.

Biofilm formation is the key step in *Candida albicans*, protecting the cells from external shocks, action of drug efflux pumps, adhesion to surface and proliferation of the microbial community. The biofilms caused by drug resistance are an important health concern as they are always mis diagnosed which can lead to systemic infections in host. The drug resistance in biofilms formed by *C. albicans* on the implants and indwelling

medical devices highlighted the need to control and tackle biofilms formed to reduce infections. Nature of substrate plays a key role in the microbial adhesion and research pertaining to the changes in adhesion and biofilm formation by different microbes on various surfaces was well studied. The inhibition of biofilm formed by drug resistance strains of *C. albicans* on the Cicada wing compared to the flat surface is accounted to various responses of the yeast to surfaces like decreases adhesion to structured surfaces compared to flat surface, rupture of cells leading to a decrease in number of cells capable of secreting biofilm matrix.

Nanostructured surface of Cicada wing also proved to have an effect on the morphogenesis on the drug resistant mutant strains of *Candida albicans*. The thickness and composition of cell wall play a very important role as the echinocandin resistant strains of *C. albicans* predicted to have a thicker cell wall as a drug resistant response, this did not show any inhibition of morphogenesis on NSS of Cicada wing like the wild type strain. Multidrug resistant mutant of *C. albicans* showed no effect of NSS in the yeast to hyphae transformation. There are no confirmed mechanisms in flucytosine antibiotic activity that involve any changes in cell wall biosynthesis pathways and *C. albicans* resistant to flucytosine showed an inhibition in hyphal formation when incubated on the NSS of Cicada wing. *C. albicans* mutants resistant to most azole class of antifungal drugs when inoculated on the NSS showed inhibition in the morphogenesis from yeast to hyphae yielding to decrease in virulence of the organism.

CHAPTER IV

NANO STRUCTURE MODIFICATIONS OF SILICONE CATHETER TO IMPROVE THE ANTIMICROBIAL ACTIVITY AND TO TACKLE DRUG RESISTANCE IN FUNGI

4.1 Introduction

Nosocomial infections caused by *Candida albicans* are very common causing urinary tract infections like Candiduria[265]. 10 to 15% of nosocomial urinary tract infections are reported in hospitalized patients with indwelling devices[266]. Indwelling bladder catheters are implicated in ~90% of the 1 million episodes of urinary tract infection that occur each year in US hospitals. The last decade has witnessed a rapid growth in these infections primarily accounted to resistance developed in microbes to commonly used drugs[267]. Candiduria prevalence is associated to immunosuppressive therapy, aggressive use of antibiotics, prolonged hospital stays, poor sanitation and conditions like diabetes[268].

Of the 30 million catheters implanted annually in the United States 10 to 30% are affected by infections[269]. *Candida albicans* is responsible for 26% of the urinary tract infection in implant recipients[270] and most of these are due to the biofilms formed on the surface of catheters which encloses microcolonies of hyphae, pseudo hyphae embedded in the protein and polysaccharide matrix yielding to resistance to drugs like amphotericin-B and fluconazole rendering them ineffective during the treatment of Candiduria[271]. The first step in biofilm formation on a urinary catheter is deposition of

a conditioning film of host urinary components, including proteins, electrolytes, and other organic molecules, which can transform the surface of the urinary catheter and neutralize any antiadhesive properties. The chemical nature of the catheter surface is attributed to the magnitude of biofilm formation[272]. The architecture of *C. albicans* biofilm depends on the adhering surface indicating that it may depend on highly specific contact-induced gene expression[273].

Encrustation of catheters is a major problem in patients undergoing long term bladder catherization[274]. Encrustation is defined as formation of crystalline deposits mainly calcium and magnesium phosphates cling to the internal lumen or exposed walls of the catheter causing blockage and leading to failure of the implant[275]. Encrustation occurs as the result of a 3-step process that incorporates colonization of the catheter with bacteria, formation of a biofilm, and crystalline formation[276]. A number of strategies have been evaluated but have proved to be ineffective in preventing encrustation. Traditionally catheters we made using DEHP and DINP which were recently marked to be carcinogenic. Many modern commercial catheter manufacturers like DOVER, MEDLINE use silver ions and alloys as surface coating with proven resistant to bacterial colonization of the urine this effect only last for the first few days.

Treatment of encrustation is a painful process which involves frequent catheter replacement of acid irrigation through the catheters which digests the blocks[277]. Surfaces embedded silver nanoparticles used as coatings on catheters have shown to resist bacterial infections but the antimicrobial effect disappeared after a week and is predicted to be because of the biofouling of the surface by multiple species of

microbes[278]. Silver nanoparticles have also been coated with dopamine on catheter materials to create anti-microbial surfaces[279].

C. albicans wild type cells have shown to develop 100-fold increase in tolerance to fluconazole exposure over that of logarithmic-phase planktonic cells[75]. Fluconazole resistance in silicone adhered cells is partially related to surface-induced upregulation of drug efflux pumps and is irrespective of biofilm formation[280]. The biofouling activity of common fungal commensal *Candida albicans* involved in more than 50% of catheter related urinary tract infection (CRUTI) and approaches to improve antifouling and antimicrobial properties are studied.

Nanostructured surface on the wings of Cicada proved to show the antimicrobial potential of NSS against *Candida* sps. These native nano cone structures were biomimetically synthesized by Nowlin et al using polystyrene nanosphere employing a nanosphere lithography process by an anisotropic etch using a plasma reactor. Most silver nanoparticles synthesis techniques involves solution based processes involving complex synthesis condition involving toxic precursor material and harsh biproducts as experimental waste[281]. In this study I adopted a green synthesis method of silver nanoparticles synthesis described by Chevva et al using a natural biopolymer chitosan[282] and filter out the nano particles from formed mixture and use it as coating on a commercially available catheter to test the anti-microbial and resistance developing efficacy of pathogenic fungi *C. albicans*.

4.2 Material and Methods

4.2.1 Synthesis of Nano Cone Structures Mimicking the NSS of Cicada Wing

Nanospheres of 400 nm (Polysciences) were resuspended in equal volume of water and ethanol and is applied on to glass surface and transferred by inserting the glass into a dish with deionized water at a shallow angle. The nanospheres were transferred on to a polystyrene surface (petri dish-Fisher scientific) by reversing the previous process. Polystyrene surfaces masked with nanospheres were etched using an anisotropic etch in South Bay Technology Model PC-2000 Plasma Cleaners in the presence of oxygen at 100 W forward power and 200mT pressure.

4.2.2 Green Synthesis of Silver Nanoparticles

500mg of Chitosan (448869 Sigma-Aldrich) was dissolved in 10ml of deionized water with 1% wt of pure glacial acetic acid in the presence of 0.2M sodium chloride in 5mL solution with continuous stirring on a magnetic stirrer. 5ml of Silver nitrate solution in water at 10g/l is added slowly and the mixture is ultra-sonicated overnight. The mixture is then centrifuged in an ultra-centrifuge at 14000prm for 30 mins and the supernatant with nanoparticles is extracted and used.

4.2.3 Modification of Catheters for Interaction with *C. albicans*

Urinary catheters obtained from Cure medical LLC, California (2024-03-28) were used to study the behavior of *C. albicans* in the catheter environment. Silicone tubing manufactured for aquarium use purchased from Walmart was sterilized and used as controls. Polyethylene glycol 4000 (81240 Sigma-Aldrich) was sterilized and mixed with 10% v/v of AgNPs from chitosan mediated AgNP synthesis. The mixture was flowed

through the catheters to coat the surface and immobilized by heat treatment at 60^0C. Scanning electron microscopy was performed to see the interaction of *C. albicans* on nano cone surface.

4.2.4 Interaction of *C. albican*s on Modified Catheters

Development of fluconazole resistance in *Candida albicans* wild type was tested by taking *C. albicans* WT cells from a fresh plate and growing to an OD~0.5 in SDB media (dextrose, peptone) diluting them at 1:1 concentration with fresh media and inoculating 100 µl of cells on a 10 cm^2 etched nano cone surface and 10 cm^2 silicone surface of the catheter. After 8 hours of interaction on the silicone and synthetic nano surface the cells were serial diluted and plated on to an SDB plate with fluconazole drug at 0.25 µg/ml, 2 µg/ml,8 µg/ml, 25 µg/ml and 64 µg/ml. The plates were incubated at 30^0C overnight and colony forming units were enumerated the next day.

The antimicrobial activity of the AgNP-PEG modified catheters was tested in comparison to control untreated catheters by performing a flow assay of *C. albicans* wild type cells at 10 µl/min through both the tubes of 40 cms length. A serial dilution series was performed on the treated cells collected and incubated at 30^0C overnight and colony forming units were enumerated the next day.

4.3 Results and Discussion

4.3.1 Synthesis of Nano Cone Structures Mimicking the NSS of Cicada Wing

Nano structured surfaces created by nano sphere lithography and anisotropic plasma etching process were similar in structure and aspect ratio as nano cones on Cicada wing(Figure 4.1).

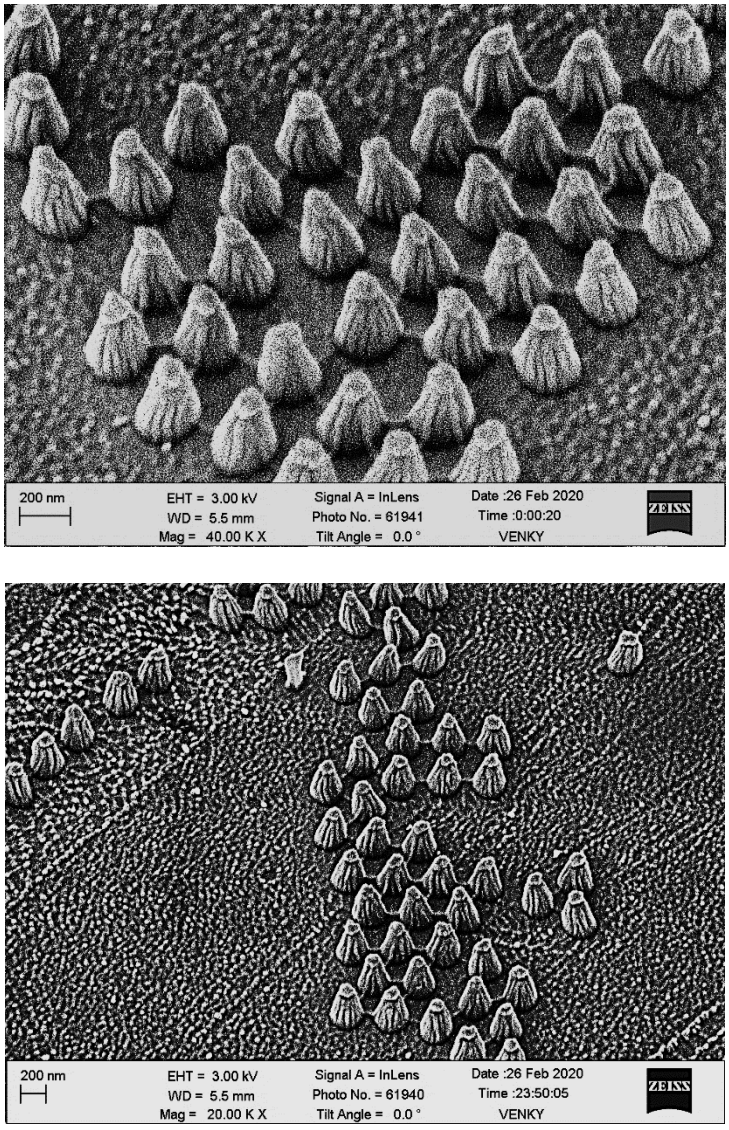


Figure 4.1. Scanning electron microscopy images of nano cones etched on polystyrene substrate

4.3.2 Interaction of WT *C. albicans* Cells on Bioinspired Nano Cone Surfaces

Nano cone surfaces on the polystyrene showed a decreased in cell adhesion compared to the bulk flat surface polystyrene. The imperfections in the nano sphere lithography facilitated the study of adhesion behavior of yeast. Scanning electron

micrographs (Figure 4.2) show the adherence and colonization of *C. albicans* on flat patches than zones with nano cones etched on them. Though there I found a few ruptured cells we predict as it is not significant and not uniform all through the surface.

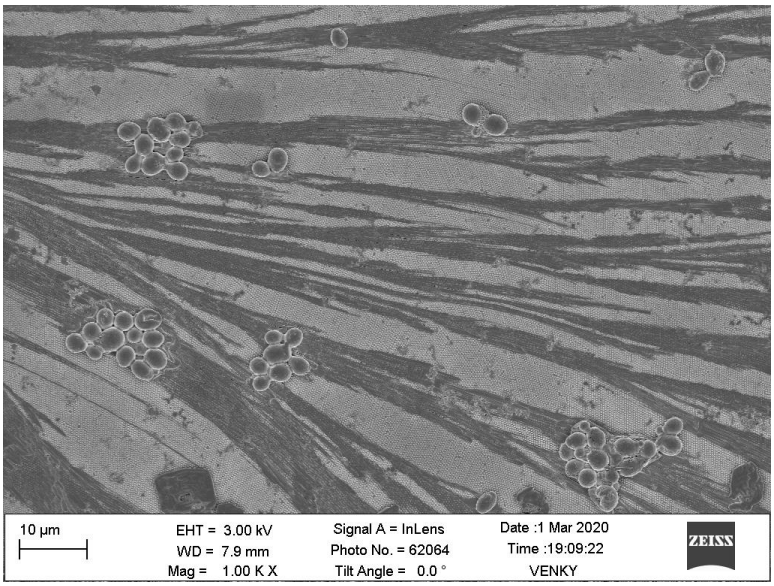


Figure 4.2. Scanning electron microscopy image of adhesion behavior of WT *C. albicans*

Silicone surfaces developed fluconazole resistance in wild type *Candida albicans* when the cells adhered to the surface and this effect can be confirmed from the spot plate serial dilution studies (figure 4.3)where there was significant growth of *C. albicans* on SBD plate at all dilutions from 10^{-1} to 10^{-4} concentrations whereas cells grown on nano cone surfaces displayed an inhibition in the growth of cells indicating no drug resistance was developed on these surfaces.

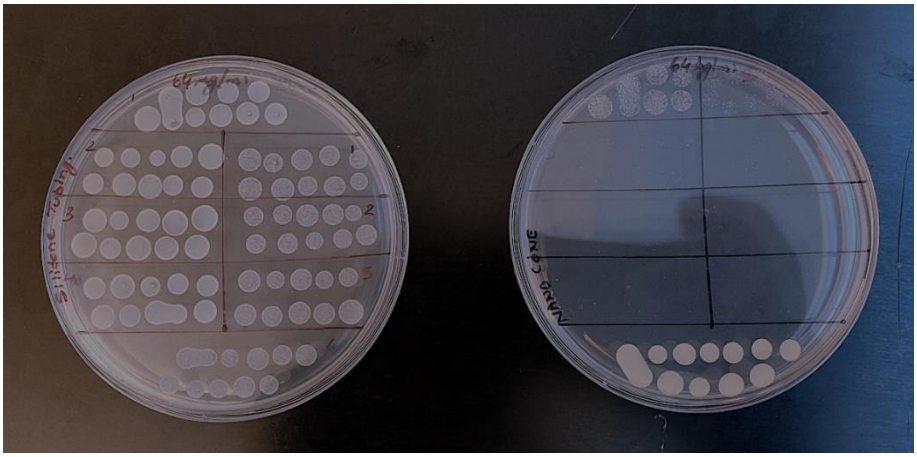


Figure 4.3. Development of fluconazole resistance in *Candida albicans* wild type. Plate 1 shows cells inoculated on silicone tubing Plate 2 cells inoculated on nano cone surface. Development of fluconazole resistance can be observed by growth of WT *C. albicans* on 64 µg/ml fluconazole

4.3.3 Interaction of *C. albicans* on Modified Catheters.

AgNP-chitosan mixture was centrifuged, and the supernatant was aspirated, and further analysis was done. DLS analysis from Figure (4.4) of AgNP in ag/chitosan was quantified and the average size of nanoparticles synthesized was confirmed as 180 nm.

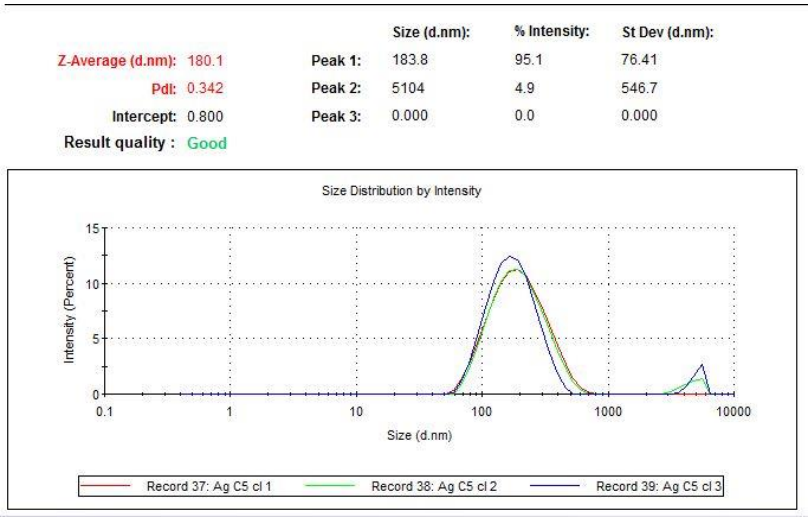


Figure 4.4. DLS Analysis of AgNPs in Ag/Chitosan Solution

The results from flow assay showing the anti-microbial efficacy of these catheters modified with AgNPs can be read as colony forming units W.T. *C. albicans* on fluconazole plate were enumerated. The acceptable CFU was found at 10^{-2} concentration and number of colonies forming unites were enumerated and tabulated. A significant difference in CFU in the cells flown through modified and unmodified catheters can be observed.

Table 4.1. CFU of *C. albicans* flowed through modified and unmodified silicone catheters

Concentration of fluconazole in SDB plate	CFU of *C. albicans* flowed through control catheter tube	CFU of *C. albicans* flowed through modified catheter
64 μg/ml	87	13
25 μg/ml	92	11
8 μg/ml	96	12
2 μg/ml	102	16

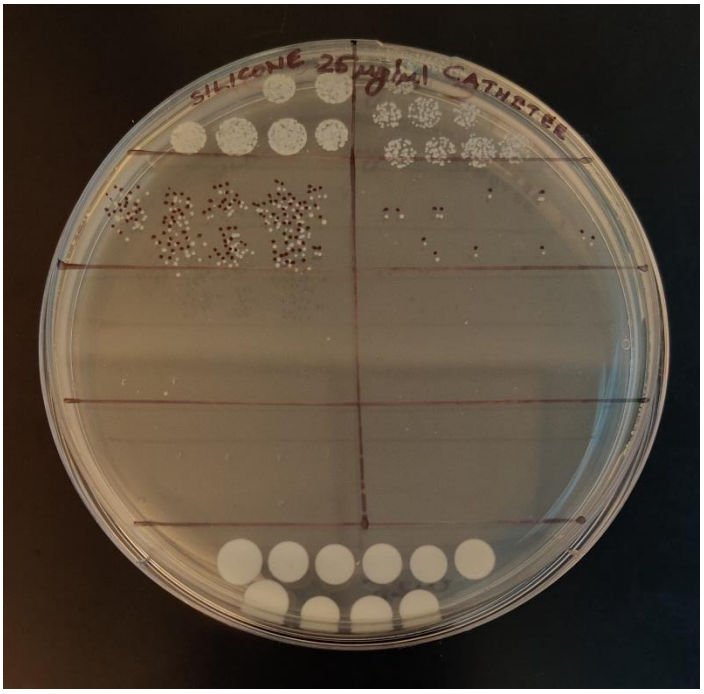


Figure 4.5. CFU of serial diluted cells flowed through silicone tubing and AgNP modified catheter. Acceptable CFU was observed at 10^{-2} dilution and number of cells flown through modified catheter were significantly lower than cells on control.

4.4 Discussion

The use of indwelling urinary catheters is becoming more common specially to treat organ failure, surgical process and rate of CRUTI is also increasing considerably leading to 8% of nosocomial infections. With intensive research in past few years new age catheters coated with silver alloys were developed to combat raising infections. Silver-coated catheters significantly reduce clinically significant infections such as blood steam and kidney infections arising from a UTI[283] by changing the microenvironment to prevent biofilm formation[284]. Encrustation of ammonium and phosphate salts and their interaction with coated metallic alloys has made encrustation severe causing blockage of catheters.

In this study, I have established PEG-AgNP coatings to urinary catheters which had a significant effect on the survival of *Candida albicans* a major cause for the biofilm related infections in medical implants and catheters. The use of silver nanoparticles with hydrophilic compounds like polyethylene glycol ensures prevention of encrustation by reduced the accumulation of phosphate salts and cumulatively provide antimicrobial effect against infectious pathogens[285].

The evolution of commonly used fluconazole in *C. albicans* adhering to silicone was demonstrated earlier[75]. To tackle this problem, I study bio inspired nano cone structures of the cicada wing created by nanosphere lithography to use as coating on medical devices, implants and hospital equipment to reduce the biofouling and also provide antimicrobial characteristics to surfaces. Nanosphere lithography gives the flexibility to mast any surface with nanostructures without any limitations of size shape

and roughness of the material. Nanostructured surfaces are an excellent alternative to existing anti-microbial antibiotics and techniques specially proven ability to combat not just gram positive and negative bacteria but also fungal species like *Saccharomyces* and pathogenic *Candida albicans.*

CHAPTER V
CONCLUSION

Candida albicans is a common commensal fungus assisting in digestion, nutrient absorption and also to maintain the microbial balance in human gut. *C. albicans* have a dimorphic nature and its ability to cause virulence when in the microbiome is disturbed or in immunocompromised patients, causes systemic infections with a high mortality rate. Though many drugs are available for the treatment of candidiasis, drug resistance in *C. albicans* is a growing concern. In chapter 2, we have established the antimicrobial efficacy and antifouling capabilities of nanostructured surface of the Cicada wing towards wild type *Candida albicans.* Timing of rupture of *Candida albicans* on Cicada wing was confirmed at 8 hours. NSS of Cicada have shown to resist microbial adhesion creating a model to develop synthetic nano structured surfaces with excellent anti biofouling characteristics owing to the surface topology to inhibit biofilm formation. These surfaces can have many applications including coatings on catheters and indwelling medical devices, dentures and in the areas of sanitation.

With the increasing resistance in the microbes since the last century and with the emergence of super bugs like *Candida auris,* which is resistant to most available antifungal drugs and also has the ability to proliferate outside the human body, the need

for alternate antimicrobial agents to existing therapies is needed. In chapter 3, I have studied the susceptibility of NSS of the Cicada wing on the drug resistant strains of *Candida albicans* and also studied their interactions in terms of adhesion, biofilm formation and morphogenesis. Studies confirmed that NSS have a definite effect on the adhesion of *C. albicans* mutants compared to flat surfaces. The mechanism of development of resistance to certain drugs also proved to play a role on the fate of *C. albicans* on NSS. Azole resistant *C. albicans* mutant showed a decrease in cell viability on the NSS and also showed the stress induced by these NSS can inhibit the morphogenesis from yeast to hyphae. Echinocandin resistant strains which have developed resistance by making modification in cell wall against drugs that weakens cell wall rigidity showed no significant cell rupture on the NSS and this is predicted to be because of a thick cell wall of the mutant strain which protect the cell from NSS assault. There was no effect of NSS of Cicada wing on the morphogenesis of these nano cone surfaces on the morphogenesis of mutant *Candida* from yeast to hyphae. These experiments confirmed the role of cell wall thickness and rigidity in relation to the mechanical strength and stress induced by the nano cone structures. We propose a further AFM force spectroscopy study to measure the mechanical modulus of cell wall of various mutant strains of *Candida albicans* to create surfaces of desired aspect ratio and strength to use as coatings for various biomedical applications.

Biofilms associated to *Candida* species are a great health concern and are responsible for about 60% of nosocomial infections. Biofilms produced by drug resistant strains are difficult to treat and exponentially increase health hazards in implant recipients

by housing other deadly microbes like MRSA. We have observed significant decrease in biofilm formation in all the three stages i.e., early, matured and late phases on the NSS of Cicada wing compared to the flat surfaces. This is primarily attributed to a decreased adhesion, cell rupture on NSS and also inhibition of hyphal formation which binds the biofilm colony together. This effect boosts the idea of using nanostructured coatings in the areas of sanitation specially to reduce bacterial and fungal infections.

Use of catheters is becoming more common in modern day medicine in people suffering from cardiac diseases, organ removal due to cancer, paralysis of organ systems and in most indwelling medical devices. Urinary catheters traditionally are simple sterile silicone tubing which are used mostly in paralyzed or mentally/physically challenged patients. *Candida albicans* is related to 50% of CRUTI and a need to tackle this is increasing rapidly. Though many commercial companies have used metal ions and alloys to coat catheters with silver to use its innate antimicrobial property for microbial growth inhibition, the problem of encrustation where the catheters are blocked by deposited salts is persistent. We demonstrate an AgNP dispersed in PEG as coatings to commercially available urinary catheters to solve the problem of biofouling and also encrustation. A decrease in cell viability on fluconazole-SDB plate was observed confirming the action of AgNP-PEG coating as an effective antifouling and antimicrobial agent.

Fluconazole is the most commonly used over the counter drug for fungal infections and increase in resistance will make it ineffective. While most catheters are made of silicone, the evolution of silicone adhered *Candida* cells by 100-fold to fluconazole was demonstrated earlier. In chapter 3 we demonstrate the effect of

synthetically created nano cone surfaces by using nano sphere lithography to inhibit the resistance developed in silicone adhered *C. albicans* to fluconazole drug.

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