Adhesion and subsequent biofilm formation of Candida albicans on chemically different surfaces as investigated using confocal scanning laser microscopy

by Karen Emma Wesenberg

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemical Engineering

Montana State University

Abstract:
Candida albicans comprises part of the normal human flora whose growth is usually restricted by the normal flora bacteria and the host's immune system. C albicans is an opportunistic fungal pathogen which causes infections in immunocompromised individuals, mechanical trauma victims, and iatrogenic patients. C. albicans can ingress the human host by adhering to a plastic surface (i.e., prosthetic devices, catheters, artificial organs, etc.) and forming a protective biofilm which provides a continuous reservoir of yeast to be hematogeneously dispersed. In order to battle device-related infections, the mechanisms of adhesion and biofilm formation of C. albicans must be recognized. A well-defined culture surface allows the initial adhesion and biofilm development to be studied. There has been some skepticism as to whether the initial adhesion events have any relationship to subsequent biofilm formation. Thus, to better comprehend the relationship between the initial adhesion rates and the long term growth rate and mature biofilm formation, these events were studied on two different culture surfaces, native polystyrene and Pluronic F127-conditioned polystyrene. The adhesion studies determined that Pluronic F127 adsorption dramatically reduced the adhesion of two strains of C. albicans of different serotypes to polystyrene. The biofilm growth studies, analyzed by confocal scanning laser microscopy, revealed that Pluronic F127 decreased the biofilm surface coverage, cluster group size, thickness, and the presence of hyphal elements over the untreated polystyrene. These findings indicate that the effect of a material's surface chemistry on the initial adhesion process has a direct influence on subsequent biofilm formation.

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APPROVAL

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Karen Emma Wesenberg

This dissertation has been read by each member of the dissertation committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Candida albicans comprises part of the normal human flora whose growth is usually restricted by the normal flora bacteria and the host's immune system. *C. albicans* is an opportunistic fungal pathogen which causes infections in immunocompromised individuals, mechanical trauma victims, and iatrogenic patients. *C. albicans* can ingress the human host by adhering to a plastic surface (i.e., prosthetic devices, catheters, artificial organs, etc.) and forming a protective biofilm which provides a continuous reservoir of yeast to be hematogeneously dispersed. In order to battle device-related infections, the mechanisms of adhesion and biofilm formation of *C. albicans* must be recognized. A well-defined culture surface allows the initial adhesion and biofilm development to be studied. There has been some skepticism as to whether the initial adhesion events have any relationship to subsequent biofilm formation. Thus, to better comprehend the relationship between the initial adhesion rates and the long term growth rate and mature biofilm formation, these events were studied on two different culture surfaces, native polystyrene and Pluronic F127-conditioned polystyrene. The adhesion studies determined that Pluronic F127 adsorption dramatically reduced the adhesion of two strains of *C. albicans* of different serotypes to polystyrene. The biofilm growth studies, analyzed by confocal scanning laser microscopy, revealed that Pluronic F127 decreased the biofilm surface coverage, cluster group size, thickness, and the presence of hyphal elements over the untreated polystyrene. These findings indicate that the effect of a material's surface chemistry on the initial adhesion process has a direct influence on subsequent biofilm formation.

INTRODUCTION

Fungi and bacteria have been shown to adhere to biological and non-biological surfaces with the subsequent formation of biofilms. These biofilms provide a refuge from the effect of antimicrobial agents and molecules of the immune system while also serving as a source for seeding further biofilm development. Pluronics, poly(ethylene oxide) (PEO) containing triblock copolymers, have been shown to minimize adhesion, an initial stage of biofilm formation, of host proteins, bacteria, and fungi on inert surfaces. This introduction is meant to provide insight into the findings of previous investigators in the areas of

1) *Candida* ecology, epidemiology, virulence, morphology, and structure,
2) fungal and bacterial attachment, growth, and differentiation into mature, recalcitrant biofilms, and
3) surface modification with Pluronics or PEO polymers, generating highly hydrophilic surfaces.

Candida albicans

Candida is a normal commensal of the human gastrointestinal and genitourinary tracts and mucosa.(36,43) Candidiasis is an infection caused by species of *Candida* with *C. albicans* being the major etiologic agent. Since *Candida* is a part of the normal human flora, this is an opportunistic fungal infection. Generally the growth of *Candida* is kept in check by the body's immune defenses and normal flora bacteria, however, overgrowth can

occur in immunocompromised individuals (e.g., diabetes, lymphoma, leukemia, DiGeorge Syndrome, AIDS), mechanical trauma victims (e.g., burn patients), and as a result of iatrogenic factors including drugs that affect the normal human flora or immune system (e.g., broad spectrum antibiotics, corticosteroids, antitumor chemotherapy), catheters and other medical devices, and surgical procedures (e.g., abdominal, heart, and transplant surgery).(8,43)

Proposed virulence factors of *C. albicans* include hyphal formation, adhesion properties, toxin production, and the dynamic cell surface. *C. albicans* can exist in one of three different morphological states, namely as blastoconidia, hyphae, or pseudohyphae. The form of the yeast depends on environmental conditions such as pH, incubation temperature, inoculum size, and composition of the growth medium.(8,43,49,81) The budding phenotype is typically observed at low pH or low temperature whereas the hyphal phenotype is generally associated with high pH and high temperature. Stationary phase yeast cells inoculated into medium at 37°C and a pH of 4.5 grow solely in the yeast form. If stationary phase yeast cells are introduced to medium at 37°C and pH of 6.7, the yeast will grow solely as hyphae as long as the pH remains above 6.0. At a pH between 5.5 and 6.5, the stationary phase yeast cells will initially form elongated daughter cells and later revert to the budding form and intermediate phenotypes.(81) The change from yeast to hyphal form is accompanied by changes in the chemical and structural makeup of the cell wall components.(86) The morphological changes may occur as a result of modifications in gene regulation in response to contact with a surface.(32)

S. cerevisiae has been proposed as a model system for *C. albicans*. However, there are important differences in the regulation of genes responsible for hyphal formation. Thus, although *S. cerevisiae* gene homologs in *C. albicans* influence morphology, they still fail to provide a clue as to the relationship between hyphal formation and virulence.(49)

The cell wall of *C. albicans* plays a role in adhesion to host tissues and plastics, in procuring nutrients from its environment, resisting drugs and products of the immune system, and in eliciting an immune response.(37) The cell wall of blastoconidia is composed of chitin, glucan, mannan, and lipids. The total glucan and mannoprotein content remains fairly constant in the transition from the yeast to filamentous form, whereas the chitin and lipid levels increase. The cell wall of *C. albicans* has been described in terms of regions of enrichment.(14,61,78) The outermost fibrillar layer consists primarily of mannoproteins. The yeast to hyphal transition is associated with an increase in adhesion and cell surface hydrophobicity (CSH). The increase in CSH is due to changes in the outer fibrillar layer mannoproteins which are thought to be the primary adhesins.(19,24,38)

The adhesion of stationary phase yeast cells is greater than that of log-phase cells to tissues, but adhere primarily to splenic tissue. (Note: humans are naturally exposed to endogenous stationary phase yeast cells.) Adhesion to host tissues, however, is not in and of itself responsible for the onset of disease since *S. cerevisiae*, a nonpathogenic yeast, binds to host tissues. In otherwise immunologically normal mice, *C. albicans* binds to macrophage-rich regions of lymph nodes and spleen and kidney tissue. The kidney is the

only organ that consistently supports fungal growth, thereby contributing to disease.(9,19) Hydrophilic yeast cells bind to the macrophage rich regions of lymph nodes and spleen; whereas hydrophobic yeast cells adhere to all tissues and in regions that are void (or nearly so) of macrophages. Hydrophobic yeast cells are more virulent than hydrophilic yeast cells. Hydrophobic yeast cells seed kidney tissues, germinate at a faster rate than hydrophilic cells, and display decreased killing by phagocytoses.(36)

The hydrophobicity/hydrophilicity of *C. albicans* depends on the growth form and environmental conditions. Excessive and limited carbohydrate levels in the growth medium result in increased cell surface hydrophobicity (CSH). Yeast cells grown on solid or liquid culture exhibit increased CSH over those grown within a liquid.(32,37) Although temperature alone does not dictate CSH, stationary phase yeast cells grown at 28°C are typically more hydrophobic than cells grown at 37°C. Actively growing yeast cells display modest levels of CSH, pseudohyphae display variable amounts of CSH, and hyphae are highly hydrophobic.(19,24,36,37) Due to the dynamic nature of the cell surface, *C. albicans* can quickly change from hydrophilic to hydrophobic. Within 60 minutes of exposure of yeast cells to tissue culture medium or fresh growth medium, *C. albicans* can change from hydrophilic to hydrophobic. Low concentrations (10^8 cells/ml) of yeast in suspension on ice can convert rapidly from hydrophilic to hydrophobic. If pelleted on ice, however, the cell surface hydrophilicity can be maintained for four hours.(36,37)

The fibrils of hydrophobic cells are blunt and aggregated whereas those of the hydrophilic cells are long, distinct, thin, and tightly packed. The fibrils of hydrophilic cells

consist of high molecular mass mannoproteins. Hydrophobic and hydrophilic cells have similar hydrophobic proteins. The hydrophobic proteins are small, poorly glycosylated, and are tightly associated with the cell wall. In hydrophilic cells, the hydrophilic proteins mask the hydrophobic proteins. The hydrophilic proteins are large and loosely associated with the cell wall and are synthesized and shed throughout cell growth. Hydrophobic proteins are present during the various stages of growth regardless of temperature or medium composition. Exposure of the hydrophobic proteins, however, depends on the growth phase, growth form, and temperature. Adhesion of *C. albicans* to low surface charge substrata involve nonspecific hydrophobic interactions, whereas adhesion to host tissues requires specific hydrophobic adhesins.(37,38,39)

The adhesion of *C. albicans* to host tissues and plastics is influenced by the type and quantity of sugars. Adhesion to polystyrene (PS) is blocked by amino sugars and enhanced by increasing glucose concentrations up to 50mM. Above this concentration, however, adhesion to PS is prevented. Adhesion of *C. albicans* strain 51 was shown to be augmented by galactose, glucose, and divalent ions (e.g., Ca^{2+}, Mg^{2+}) and hindered by Fe^{3+}. At 37°C, adhesion of *C. albicans* strain 51 was greater than at 20° and 25°C and attained a maximum at 40°C. Binding to host tissues is precipitated by the interaction between host proteins and *C. albicans* surface molecules. *C. albicans* has cell surface receptors specific for host proteins including fibrinogen, fibronectin, laminin, and type I and type IV collagen which promote adhesion to conditioning films that form on surfaces exposed to host fluids.(19)

C. albicans can exist as either serotype A or serotype B, which differ in the composition of the phosphomannan (PM) complex. The PM complex of serotype A is similar to that of serotype B with the exception of the presence of some non-phosphodiester-linked β-(1,2)-oligomannosyl side chains in the phosphomannan acid stable portion (PM-AS). The monoclonal antibodies (MAb) B6.1 and B6 are of the IgM isotype with the former conferring increased resistance against experimentally disseminated candidiasis. Both MAb B6.1 and B6 are specific for yeast cell wall mannan. MAb B6.1 epitope is part of the cell wall PM complex, namely fraction III and fraction IV of phosphomannan acid labile (PM-AL) portion. Fraction III comprises the greatest portion of the B6.1 epitope and consists of isomers of mannotriose. The B6.1 epitope is a β-(1,2)-mannotriose. Fraction IV is composed of mannotriose and mannotetraose. MAb B6 epitope is part of the PM-AS portion and is mannan in nature.(29)

Mannan adhesins can be extracted from the fungal cell wall with 2-mercaptoethanol (2-ME). This extract inhibits the adhesion of *C. albicans* to lymph node and spleen tissue, independent of strain or serotype. Fraction IIa and fraction IIb, obtained from fractionation of the 2-ME extract, displayed greater adhesion than the other three fractions. Fraction IIa prohibited adhesion to lymph node and spleen and is comprised primarily of mannose (98-99%). The mannose portion of mannoproteins is responsible for the adhesion of hydrophilic yeast cells to tissue such as spleen and lymph node. Adhesion of hydrophobic yeast cells to tissue is mediated through the cell wall proteins.(45)

An antigen (10G Ag) was located within *C. albicans* cell wall surface and plasmalemma. The 10G Ag epitope was determined to be a β-(1,2)-linked tetramannose and is thought to be part of the acid labile region of the phosphomannan complex. Both the 10G Ag and the 10G Ag epitope bind to mouse spleen marginal zone macrophages and block binding of *C. albicans*.(70)

Prevention of candidiasis precipitated through the application of catheters and other medical devices requires the generation of a non-fouling surface. Production of a non-fouling surface necessitates not only the blocking of *Candida* adhesion but also that of host proteins. Upon exposure of surfaces to host fluids, proteins and other components adhere to the surface, forming a conditioning film. *C. albicans* has cell surface receptors specific for host proteins, enabling it to bind to surfaces coated with a conditioning film. The problem then is how to generate such a non-fouling surface.(19) Generation of a surface that completely precludes binding of yeast and host proteins may prove difficult, but it should be possible to produce a surface that limits adhesion. By limiting adhesion, we minimize one of the virulence factors associated with *C. albicans.* By reducing the presence of the hyphal form of *C. albicans*, another proposed virulence factor can be controlled. Pseudohyphae and hyphae are associated with higher levels of CSH than the yeast form of *C. albicans*.(19,24,36,37) This, along with the observation that *C. albicans* adheres to low surface charge substrata through hydrophobic interactions (37,38,39), leads to the question of whether a highly hydrophilic surface could limit both the interaction of *C. albicans* with the surface and the presence of the hyphal phenotype.

Fungal Adhesion and Biofilm Formation

C. albicans can adhere to a variety of biomedical implants including contact lenses, prosthetic devices, pacemakers, artificial joints, and urinary, central venous, and peripheral catheters. Following adhesion to a surface, yeast can develop microcolonies sheathed within a polymeric matrix, forming a biofilm which acts as a protective barrier. A biofilm provides a setting where the yeast can proliferate and release cells into the surrounding fluids and tissues, contributing to the onset of acute disseminated infections. Although slow to develop, the infections are relentless as the microorganisms are unreachable by host defenses and antibiotics. Thus, suppressing the infection usually requires removal of the implant.(32,92)

The preponderance of manifestations of candidiasis are associated with the formation of biofilms on inert or biological surfaces and in mucosal and systemic sites.(66) Enhanced adhesion of *Candida* species in an oral environment is influenced by sucrose- and glucose-rich diets, acidic pH, cell surface hydrophobicity and cell surface mannoproteins. *C. albicans* and *C. dubliniensis* have been acquired simultaneously from the mouths of immunocompromised individuals. Under planktonic conditions in Sabouraud liquid broth modified antibiotic medium 13 (SDB), *C. albicans* prevails over *C. dubliniensis*. When exposed to consistent conditions of pH, temperature, nutrient levels, and waste removal, the phenotype or organism with the enhanced growth rate will predominate. *C. albicans* also prevails over *C. dubliniensis* under sessile conditions in

SDB, however, not to as great an extent. Population differences due to variations in growth rates are not as notable in biofilms as they are in suspension cultures.(48)

The ability of *S. cerevisiae* to demonstrate the initial stages of biofilm formation has led to its indicated use in ascertaining the role of *C. albicans* cell surface proteins in pathogenesis and in evaluating the capacity of compounds to block fungal adhesion. Flo11p is a *S. cerevisiae* cell surface protein which is required for cell-cell adhesion and cell-surface adhesion. The ortholog of Flo11p in *C. albicans* is a proposed virulence factor since expression in *S. cerevisiae* led to adhesion to mammalian cells.(71) However, as indicated earlier, adhesion does not by itself account for the onset of disease.(9)

The emergence of candidiasis is attributed in large part to the formation of a biofilm (66), and therefore prevention requires circumventing biofilm formation. The question is then whether limiting the initial adhesion event can lead to diminished growth and thwart the appearance of a mature differentiated biofilm.

Bacterial Adhesion and Biofilm Formation

Biofilms are found in association with ship hulls, oil drilling pipes, food fermentors, and dental plaque. Biofilms can be a contributing factor in upper respiratory infections, kidney stones, prostate infections, urogenital infections, periodontal disease, Legionnaire's disease, peritonitis, and middle ear infections. Approximately 10 million infections that occur in the U.S. each year are precipitated by biofilm formation on permanent medical implants. These surfaces which are not adequately protected by the host immune system provide binding sites for microorganisms. In North America, more than 100 million

urethral catheters and urinary stents are employed each year. In the absence of antibiotic treatment, up to 28% of urethral stents and up to 100% of catheters are prone to infection which can lead to death. Upon exposure to body fluids (e.g., saliva, blood, urine) surfaces are coated with a conditioning film consisting of host proteins and other substances. Host proteins including serum albumin, fibrinogen, fibronectin, and collagen can serve as sites for bacterial adhesion. The existence of a conditioning film is thought to be an initiating stage for infectious biofilm development. An effort to compromise or eliminate the link between the conditioning film and the biofilm, such as through fluctuating shear forces or surfactants, could hinder biofilm formation or allow for removal through sloughing.(68)

The recalcitrance of bacterial biofilms is thought to be due to a multicellular endeavor. For example, degradation of hydrogen peroxide by catalase produced by bacterial cells, including nonviable cells, requires a concerted effort by a group of cells. A single cell could not produce enough catalase to overcome the debilitating effects of hydrogen peroxide. Similarly the activity of some antibiotics requires oxygen. The cells on the perimeter of the biofilm consume the oxygen and thereby protect their deeper neighbors. Again, a single cell could not deplete enough oxygen from its surroundings to prevent the antibiotic's activity. The presence of various metabolic states within a biofilm also provides for protection from chemical and physical assaults. The majority of cells are in an active growing state which leaves them prone to the effects of antimicrobial agents. However, some of the biofilm cells are in a static, spore-like state and are protected, allowing them to reseed the biofilm. Thus, the symptoms of an infection may subside only to flare up later.(59,82)

The presence of various metabolic states represents a physiologically based mechanism of resistance. Another potentially contributing mechanism to bacterial biofilm recalcitrance could be a transport-based mechanism. The reduced susceptibility of biofilm bacteria can not be explained by reversibly sorbing, nonreacting solutes or by stoichiometrically reacting solutes. In contrast, irreversibly sorbing, nonreacting solutes or catalytically reacting, nonsorbing solutes could account for the increased resistance of biofilm bacteria provided the reaction is fast enough in the latter instance. However, there is no indication of extensive irreversible sorption of antibiotics to biofilms, and the vast majority of antibiotics do not react rapidly enough to account for the increased tolerance.(83)

Bacterial biofilms consist of microcolonies shrouded in extracellular polysaccharide (EPS) matrix or glycocalyx and demarcated by water channels. These sessile communities are physiologically and morphologically distinct from their planktonic counterparts. The exterior cells of microcolonies receive sufficient nutrients and waste removal. The aerobic conditions allow for the growth and activity of bacteria that require oxygen for these functions. These bacteria produce toxins and other substances that produce deleterious effects on the host. However, this active state also contributes to their destruction by drugs such as penicillin which act on replicating cells. The interior cells of microcolonies experience limited nutrient levels and waste removal and depend on the diffusion of these molecules. These cells are exposed to reduced oxygen levels which leads to an inactive state. Thus, these cells pose little threat to the host. This state also provides resistance to

drugs such as penicillin, leaving these cells to consume those cells that expire and to restore the biofilm.(6,10)

Biofilm bacteria generate signal transduction proteins which gather information from the environment and relay it to chromosomal elements. This form of communication allows for a group virulence response.(68) *P. aeruginosa* has two known cell-to-cell signaling processes including the *lasR-lasI* and *rhlR-rhlI.* The *lasR-lasI* and *rhlR-rhlI* gene products are homoserine lactones which attain, with sufficient population sizes, concentration levels necessary for gene activation. This type of gene regulation is termed "quorum sensing and response". Both the wild-type (PAO1) and the *lasI-rhlI* double mutant biofilms attained steady-state within two weeks. The *lasI-rhlI* double mutant formed thin, dense biofilms and failed to produce quorum sensing signals. The *rhlI* mutant generated biofilms with similar thickness and cell packing as the wild-type. The *lasI* mutant developed biofilms that had similar thickness and cell packing to the double mutant and unlike the wild-type was sensitive to sodium dodecyl-sulfate (SDS). In the presence of synthetic signal molecules, however, the *lasI* mutant formed biofilms which appeared normal. Thus, the quorum sensing signal $3OC_{12}$-HSL, the gene product of *lasR-lasI*, is necessary for normal biofilm differentiation. The initial stages of biofilm development, adhesion, and growth are normal for the *lasI* mutant, but it fails to form a mature differentiated biofilm. The wild-type and planktonic counterparts generate similar amounts of EPS but differ in the glycocalyx appearance which is "compressed and incomplete" in planktonic systems. This may explain the close packing observed in the

mutant biofilms. Thus, an ability to block cell-to-cell signaling provides a means of preventing mature biofilm formation.(10)

A chemical being developed to target bacterial communication was spurred by findings that the red marine alga *D. pulchra* does not contain biofilms on its fronds. The chemicals responsible for this antibacterial behavior are substituted furanones which act by binding to locations on bacteria that are normally occupied by signal molecules. Thus, the bacteria do not receive a signal to amass and create a biofilm. Substituted furanones have been shown to not only prevent biofilm formation but to disperse existing biofilms. They are also nontoxic and fairly stable in the human body and bacteria have not developed a tolerance over the years of exposure to them in the oceans.(6,59)

Another potential way to limit biofilm formation is to employ drugs directed against the production of extracellular matrix and against adhesion to surfaces, preventing subsequent biofilm formation. Upon adhering to a surface, the production of many proteins not found in planktonic cultures is initiated. For example, *Pseudomonas aeruginosa* expresses the algC gene which is required for the production of alginate, a principle component of the extracellular matrix.(6)

In nature, biofilms are a multispecies conglomeration.(48) Dental plaque biofilms have over 500 species. Gram-positive bacteria, primarily streptococci, are the first to appear. They are followed by gram-negative anaerobic bacteria such as *P. gingivalis* which represents a transition from a commensal to a pathogenic entity. *P. gingivalis* adheres to oral surfaces through fimbriae. The *fimA* gene product, FimA, is a primary protein subunit of fimbriae. Expression of the *fimA* gene is influenced by environmental

factors and signaling molecules. *S. cristatus* produces a signal molecule which reduces *fimA* gene expression and thereby prevents *P. gingivalis* biofilm formation. *S. gordonii*, in contrast, provides a binding site for *P. gingivalis* through an adhesin-receptor interaction with FimA and *S. gordonii* surface molecules.(89)

Host proteins that makeup the conditioning film that forms on surfaces exposed to body fluids provide sites for the adhesion of bacterial cells. The presence of a conditioning film is considered one of the initiating stages for infectious bacterial biofilm development.(68) Thus, generating a surface that repels the adhesion of host proteins would eliminate not only one element of *Candida* adhesion, but also bacterial adhesion. The recalcitrance of bacterial biofilms is described as being a multicellular endeavor (59, 82), studies indicate that this is also true for *Candida* biofilms (3,5,33). Therefore, the ability to produce a surface which limits adhesion and obstructs the appearance of multicellular structures would also increase the sensitivity of those cells that attach to the surface. Thus, the cells that adhere to the surface would be subject to the effects of antimicrobial agents and antiseptics. The thin, dense biofilms produced by *P. aeruginosa lasI* mutants were sensitive to SDS and failed to form mature differentiated biofilms, in contrast to the wild-type.

Fungal and Bacterial Biofilm Formation

Biofilm development is influenced by nutrient supply, hydrodynamic flow, cell movement, and interactions between organisms. Interactions between bacteria and fungi can either promote or hinder adhesion. The presence of bacteria decreased the

susceptibility of fungi to antimicrobials. The initial adhesion event is a random process which depends on the surface free energy and proximity to the surface. Following adhesion, cells multiply and differentiate into a heterogeneous multilayer community that allows for the presence of various metabolic states. The biofilm presents a diffusion barrier which cannot necessarily be attributed to the EPS.(19,42)

Poly(ethylene oxide) and Pluronic

Highly hydrophilic surfaces are non-thrombogenic due to a low blood-material interfacial tension which results in a low driving force for adsorption. The grafting of hydrophilic polymers such as poly(ethylene oxide) (PEO) onto polymer surfaces produces a hydrophilic polymer. The propinquity of protein molecules to the surface is limited as a consequence of excluded volume effects and decreasing configurational entropy of mobile PEO chains. The adsorption of amphiphilic PEO block copolymers onto glass, polystyrene, and polyethylene diminished adhesion of albumin, fibrinogen, and blood platelets as compared to the unmodified surfaces.(21)

Poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) copolymers are surfactants which are commercially available as Pluronics (produced by BASF) or Poloxamers (produced by ICI). Pluronics are utilized by the pharmaceutical industry as drug solubilizers, in controlled release systems, and as a burn covering. The conjugation of Pluronic copolymer micelles containing drugs to a vector allows for efficient transport of the drugs into tissues, including the brain. In bioprocessing, Pluronics serve to guard microorganisms against mechanical and chemical stress.(1)

The maximum adsorption of Pluronic to surfaces occurs at a concentration greater than the apparent critical micelle concentration (CMC). The PPO segment makes contact with surfaces such as polystyrene while the PEO segments extend into the bulk fluid (Figure 1). The PEO segments prevent fibrinogen and platelet adhesion through steric repulsion.(1) PEO chains grafted to particle surfaces prevents binding of blood components through steric stabilization. The thickness of the barrier increases with increasing PEO chain length. The steric stabilization is the result of osmotic and elastic contributions on overlap of polymer chains and an elastic contribution due to loss of configurational entropy between neighboring polymer chains.(30)

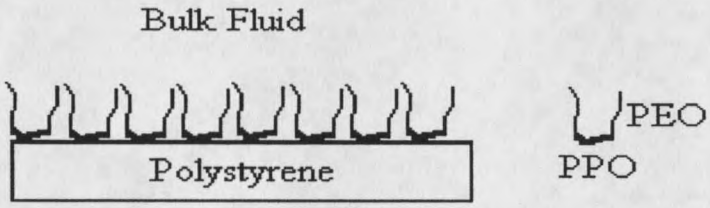


Figure 1. Schematic representation of adsorbed Pluronic F127 surfactant on PS.

The protection conferred by Pluronic F68 to insect and animals cells against shear stress is thought to be the result of decreased membrane fluidity. Pluronic F68 has been found to alter membrane permeability. The presence of Pluronic F68 extended the lag phase of *Saccharomyces cerevisiae* without significantly affecting the biomass concentration. A 1% Pluronic F68 solution did not affect the growth or flocculation of *Saccharomyces cerevisiae*.(20,41) In adhesion studies of human epithelial cells (HepG2) to hydrophobic surfaces, the presence of Pluronic F68 prevented collagen adsorption and

thereby HepG2 binding to the more hydrophobic BGPS but not to the less hydrophobic TCPS.(13)

Highly hydrophilic surfaces which are non-thrombogenic can be produced by adsorption of PEO-PPO-PEO triblock copolymers. The use of Poloxamers has also been shown to significantly limit the adhesion of *S. epidermidis* to polystyrene (4) and the adhesion of *P. aeruginosa* to hydrophilic contact lenses (64). Thus, the question for this thesis to consider is whether Pluronics are also capable of significantly inhibiting the adhesion of *C. albicans*.

Studies were performed to examine the following questions:

1) Does adsorption of Pluronic F127 onto the PS surface significantly limit the adhesion of *C. albicans*?
2) Does the ability to limit adhesion translate into limited biofilm formation of *C. albicans*?
3) Is the effect of Pluronic F127 on *C. albicans* adhesion and biofilm formation strain or serotype dependent?

EXPERIMENTAL PROCEDURES

Construction of a Well-Defined Culture Surface

The desire to generate a well-defined culture surface stems from the need to be able to definitively relate the substratum chemistry with the characteristic adhesion and the variation in biofilm structure of *C. albicans* that is observed. One can not positively relate the effect of different surface chemistries on the adhesion and biofilm structure of *C. albicans* without knowing the exact makeup of the substratum. The following describes the preparation process that is followed in an attempt to develop a surface whose chemistry is reproducible: that is, to generate a surface whose chemistry is consistent from one sample to another as verified using surface analysis techniques.

Polymer

Polystyrene acts as the substratum in the adhesion and growth experiments. These experiments were performed using either clean, untreated polystyrene or surface-treated polystyrene. The polystyrene was chemically altered using oxygen plasma, oxygen beam, or Pluronic F127. The polystyrene was prepared as half-inch coupons for the adhesion experiments and was spin-coated onto silanized glass coverslips for growth experiments. Polystyrene (PS) has a melting point between 150° and 243°C, which becomes important when considering sterilization techniques.(80)

Preparation of Polystyrene Surfaces. The half-inch PS coupons were cleaned prior to experimentation or manipulation. The PS was cleaned by placing a coupon in hexane and swirling for approximately five seconds. The PS sample was then laid on a chemwipe and allowed to dry. After drying, the PS sample was set in a beaker containing methanol and sonicated on ice for approximately five minutes. The PS sample was removed from the methanol and placed in a clean Pyrex glass container.

The glass coverslips (43 × 61 mm) were initially silanized in a 5% (v/v) dimethyldichlorosilane (DMDCS) solution in toluene. The glass coverslips were exposed to the silane solution for a minimum of three hours and then rinsed with methanol followed by nanopure water. The coverslips were then allowed to air dry within a covered glass container. The dry, silanized glass coverslips were spin-coated with 5 wt% PS (Mw ≈230,000) in toluene. The coverslips were spin-coated at approximately 3000 to 5000 rpm. The PS spin-coated coverslips were prepared at least 24 hours prior to experimentation or manipulation.

Preparation of Oxygen Plasma Treated PS Surface. A clean PS sample was placed on a stage in the glass barrel of a plasma reactor (Figure 2). The forward power of the 13.56 MHz RF generator was set to obtain 60 watts. The amount of reflected light was minimized using the matching network control box. The reactor chamber was evacuated to a pressure of ~100 millitorr prior to oxygen admission. The working pressure ranged from ~110 to ~250 millitorr. An oxygen flowrate of 5.0 cm^3/min was utilized. The PS sample was exposed to an oxygen plasma for approximately fifteen minutes. The treated

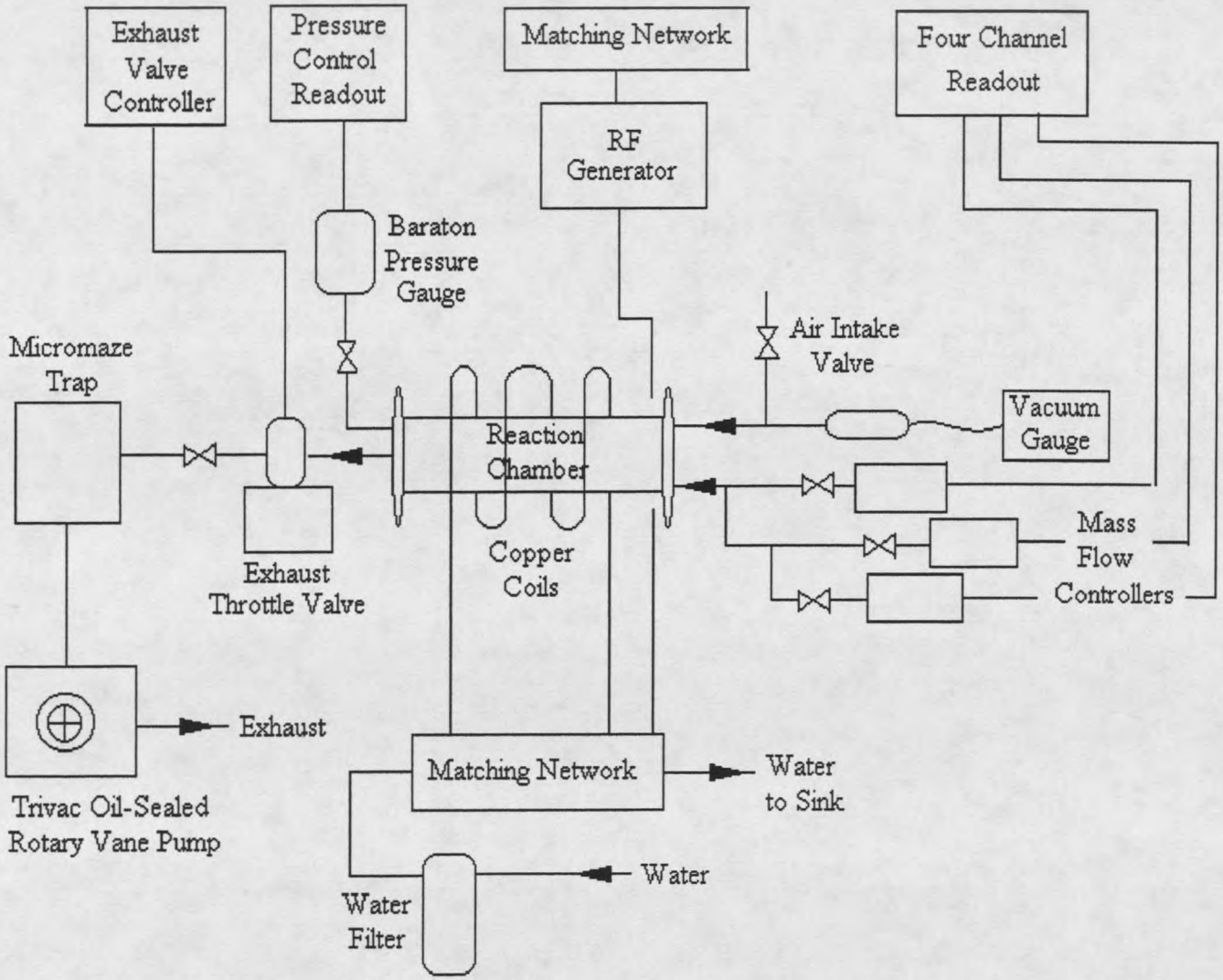


Figure 2. Schematic of oxygen plasma reactor.

sample was stored in clean Pyrex glass container for no more than two weeks.

Preparation of Pluronic Treated PS Surface. A clean PS coupon or coverslip was immersed in a sterilized 4% Pluronic F127 conditioning solution in phosphate buffered saline (PBS) and allowed to sit at room temperature for a minimum of three hours. The PBS solution was 0.01M with a pH between 7.15 and 7.25. Following the treatment period, the PS coupons were rinsed twice with ten milliliter aliquots of PBS followed by rinsing three times with ten milliliter aliquots of nanopure water. The PS coverslips were rinsed with PBS during the setting of the flow rate and the removal of the air bubbles. The Pluronic F127 treated PS coupons were stored in a covered ten milliliter beaker for no more than one week. The Pluronic F127 treated PS coverslips were used immediately due to concerns over potential contamination.

Pluronic F127 is an A-B-A block copolymer where A is poly(ethylene oxide) (PEO), the hydrophilic segment, and B is poly(propylene oxide) (PPO), the hydrophobic segment. Pluronic F127 is believed to adhere to the PS surface via the hydrophobic PPO segment such that the hydrophilic PEO segments extend into the bulk fluid. Pluronic F127 has an average molecular weight of 12,500. The graphic formula for Pluronic F127 and the repeating chemical structural unit for polystyrene are shown in Figure 3.

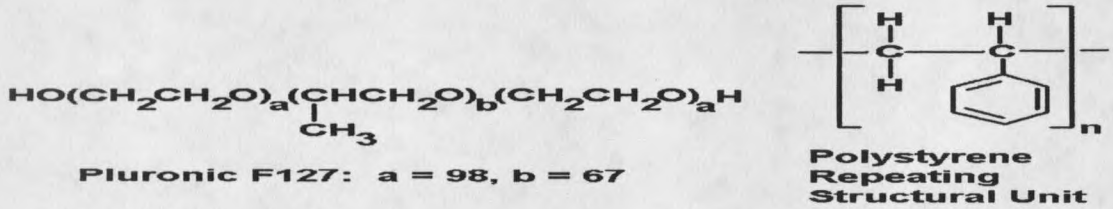


Figure 3. Pluronic F127 and PS structural units.

A disadvantage of adsorbing Pluronic F127 to the PS is that over time the Pluronic F127 may be removed from the surface. Others have generated a more stable surface by incorporating the PEO-containing triblock copolymer into a polymer matrix. The polymer matrix that was used was a polyurethane (PU). The trapped copolymers were shown to accumulate at the polymer surface.(21,54) Based on these results, I attempted to generate a more stable Pluronic F127 treated PS surface by integrating the surfactant into the PS matrix. The methods utilized incorporated those applied by others in creating the PEO additive-containing PU surfaces.(21,54)

PS pellets were dissolved in tetrahydrofuran (THF) to generate a 15 wt% solution. Pluronic F127, 10 wt% dry, was added to the PS solution and then mixed. This final solution was then spin coated onto silanized glass coverslips. The surface was allowed to dry to eliminate the residual solvent. The surface was analyzed by X-ray Photoelectron Spectroscopy (XPS) and compared to a surface developed by adsorption of Pluronic F127 onto a PS spin coated surface. The oxygen content of the blended surface was similar to that for untreated PS. This may have been due to not storing the surface in PBS for 24 hours prior to examination. The surface was not stored in PBS due to concerns about out-gassing during XPS analysis. The surfaces that were generated were quite hazy. This presents a problem when trying to visualize adhesion and biofilm formation and therefore, no further investigation of these surfaces was conducted.

Characterization of the Chemically Modified Surface

Surface Analysis Techniques

Surface analysis techniques were utilized to verify the presence and location of desired surface chemistries. These techniques confirmed that the procedures employed in the generation of the substratum surfaces were a reliable and consistent means for developing the desired surface chemistries.

XPS Analysis. Once the PS surface was chemically altered, verification of the presence of the desired chemical species was accomplished using XPS (X-ray Photoelectron Spectroscopy or Electron Spectroscopy for Chemical Analysis, ESCA). The XPS spectra were obtained using a PHI 5600 XPS system equipped with standard (Al Kα and Mg Kα) and monochromatized x-ray (Al Kα) sources. The monochromatized x-ray source was used. An Al mono 2mm filament was utilized as the x-ray anode. The surface charge compensation was achieved by turning on the neutralizer which floods the sample with a monoenergetic source of low-energy (<20 eV) electrons. The electron energy was adjusted to get the narrowest width of the hydrocarbon component of the C1s peak. The maximum counts were also made as large as possible. The hydrocarbon component of the C1s peak served as the internal reference with the binding energy set at 285.0 eV. Generally, a survey scan and a multiregion scan were performed. From these scans, a table of the atomic concentration (AC) ratios was acquired.

ToF SIMS Analysis. Once a chemically patterned polymer surface had been created, Time-of-Flight Secondary Ion Mass Spectrometry (ToF SIMS) was used to provide information as to the spatial location of chemical species immobilized on the surface based on specific fragment ions. This information was translated into an image. ToF SIMS spectra supplied molecular information about the unmodified and modified PS surfaces from the atomic and molecular ions that were ejected from the surface and detected by a mass analyzer.

Identification and Serotyping *Candida albicans* Strain 662

Experiments were conducted on two strains of *C. albicans*, namely CA1 and CA662. As indicated in the introduction, *C. albicans* can exist as either serotype A or serotype B. The two serotypes differ in the composition of the PM complex expressed on the cell surface. Since the characteristics of the cell surface are important in adhesion to surfaces, there may be significant differences in the behavior of the two serotypes when exposed to the surfaces under examination. CA1 was originally given in the literature as being a serotype A strain as determined by Hasenclever's original antiserum.(17,27,28,45, 70) However, more recent literature reports indicate that the IATRON Candida Check characterizes CA1 as being a serotype B strain.(17,28). The serotype of strain 662 was unknown.

Identification and Serotyping Tests

Four marketed tests for the identification and serotyping of *C. albicans* were utilized. They included api 20 C AUX Yeast Identification System, *C. albicans* screen, IATRON *Candida* check, and CHROMagar *Candida.*

Candida albicans Adhesion Studies

In order to study the influence of materials' surface chemistry on the adhesion of *C. albicans*, adhesion assays were carried out. The adherence of the yeast to the surface was quantified by counting the number of cells that adhered to an area during a 60 minute exposure period. The experiments, which were conducted in a parallel-plate flow chamber, were carried out in triplicate to verify that the activity that was observed was representative of what could be expected to take place.

Cells and Culture Media

C. albicans CA1 and CA662 isolates were maintained in a sub-zero freezer (- 67°C). Every month new subcultures were generated using the streak plate method of isolation. Following a 48 hour incubation period at 35°C, a single colony was removed from the isolation plate and transferred to a slant tube containing Sabouraud dextrose agar. The inoculated slant tubes were incubated for 48 hours at 35°C and then kept at ~ 4°C. This culture of *C. albicans* was used to perform adhesion and growth experiments. Each inoculum of *C. albicans* for experimentation was grown in GYEP (5% glucose, 0.3% yeast extract, 1% bacto-peptone) broth at 35°C and 160 rpm for 24 hours. The first

inoculum was removed from a slant tube and placed in 100 milliliters of sterile GYEP broth. The second inoculum was obtained from the initial broth culture of which 0.25 milliliters were placed in 100 milliliters of sterile GYEP broth.

The *C. albicans* cells used in the growth and adhesion experiments were obtained from the second inoculum following a 24 hour incubation period. Two four-milliliter portions of cells were removed from the broth culture and spun down for 90 seconds. The cells were rinsed three times with two milliliters of 0.01M phosphate buffered saline (PBS) which was refrigerated prior to use and then kept on ice during the rinsing process. Following the final rinse, two milliliters of PBS were added to each portion of cells. The cells were combined to generate a little more than four milliliters of cells in PBS. From this, 100 microliters were removed and placed in 900 microliters of 0.01M PBS and the remaining cell solution was spun down for 90 seconds and kept on ice until it was used to generate the seeding solution. Twenty microliters were then removed from this new one milliliter cell solution and transferred to 980 microliters of 0.01M PBS. From this final cell solution, ten microliters were loaded onto a hemacytometer and the remaining portion was kept on ice. The cells were counted in the four squares. The hemacytometer cell count was used to determine the concentration of cells in the approximate four milliliter solution that had to be added to PBS to generate 140 milliliters of solution with a concentration of $\sim 1 \times 10^7$ cells/ml, the seeding solution. The PBS for the seeding solution was at ambient conditions for the adhesion experiments and at ~37°C for the biofilm growth experiments.

The hydrophobicity/hydrophilicity of the cells used for the adhesion studies and for seeding the flow cell for the biofilm studies was determined using the microsphere hydrophobicity assay (HMA) that was briefly discussed in the introduction. The procedure utilized was outlined previously by Hazen et al. (34,35,40).

Buffer.

Phosphate-buffered saline (PBS) was used as the suspension media in the flow cell experiments. A 0.01M PBS solution was generated by combining twenty-five milliliters of a 0.2M monobasic sodium phosphate (NaH_2PO_4) solution with seventy-five milliliters of 0.2M dibasic sodium phosphate (Na_2HPO_4) solution in 1.9 liters of nanopure water. The pH of the final solution was adjusted to be between 7.15 and 7.25 by adding additional 0.2M NaH_2PO_4 or 0.2M Na_2HPO_4 as necessary. The PBS used in the adhesion and growth experiments was sterilized by autoclaving for fifteen to twenty minutes.

Cell Adhesion Experiments.

Cell adhesion experiments were conducted using either untreated or treated PS. The PS coupon was set in the well of the Teflon flow cell. The flow cell was assembled as shown in Figure 4. The cell solution in PBS with a concentration of ~1 x 10^7 cells/ml was employed in the cell adhesion experiments. Initially, the PBS solution was pumped through the flow cell in order to establish the desired flow rate of 1.5 ml/min, focus the Olympus microscope, and remove air from the flow cell assembly. After achieving this end, the cell solution was pumped with a peristaltic pump through the flow cell and the adhesion of yeast was observed on a monitor connected to the Olympus microscope, and

images were recorded using Imaging Program for Windows. The adhesion process was followed for an hour at which time the experiment was terminated. The experiment was concluded early if air bubbles entered the flow cell. The experimental setup is shown in Figure 4.

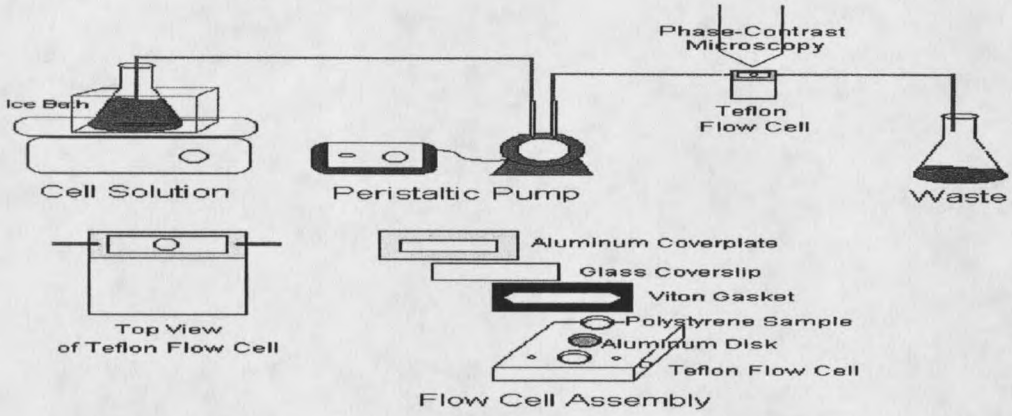


Figure 4. Adhesion experimental setup including flow cell assembly.

The adhered cells were counted and recorded manually. The adhesion experiments were conducted at room temperature. A shear rate of 16 sec^{-1} was selected based on arterial wall shear rates which range from 10 to 1000 sec^{-1}. A flow rate of 1.5 ml/min was necessary to achieve the specified wall shear rate as determined by the design of the flow cell. Other individuals have shown that the adhesion of *C. albicans* to PVC and FEP increased with decreasing shear rate.(87)

The above procedure was followed for CA1, but the procedure for CA662 differed in the use of the new Kynar flow cell and a flow rate of 3.4 ml/min. The cell solution was of the same concentration, but the volume had to be increased two fold in order to compensate for the increased flow rate. Also, the adhesion was observed at one location

(i.e., location A in Figure 5) over the 60-minute adhesion process, but at the end of 60 minutes, six other locations were examined (i.e., locations B, C, D, E, F, G in Figure 5). The adhesion of CA662 was observed on the top coverslip and not the bottom coverslip due to difficulties in focusing to that depth.

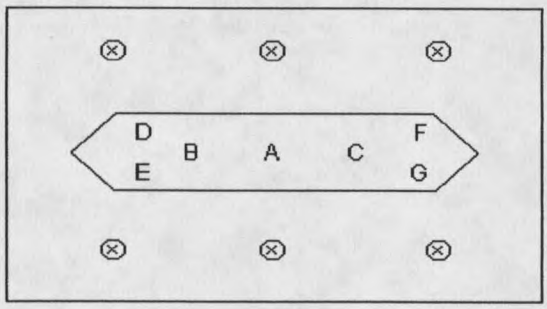


Figure 5. Observed regions in the adhesion and biofilm studies for CA662.

The adhesion experiments for CA1 were also observed in a central location over the 60-minute adhesion period, but after that time, the locations observed were random and were not specified as being from any certain location on the surface. Fluid-flow and mass transfer characteristics of the Kynar flow cell system are given in Table 1.

Candida albicans Biofilm Studies

In an attempt to influence the structure of the biofilm, the substratum that the yeast encounters was modified. It was hypothesized that a difference in the adhesion observed on the various substrata could be translated into an commensurable effect on the structure of the biofilm that develops on this surface. The *C. albicans* biofilm was allowed to materialize after seeding the flow cell with yeast cells through a thirty-minute adhesion

Characteristics of the Kynar Flow Cell System	
Dimensions of Region Exposed to Flow:	Length: 5.1 cm Width: 1.0 cm Height: 1/16 inch
Flow Cell Volume:	0.81 ml
Flow Rate:	3.44 ml/min
Mean Residence Time:	14 seconds
Reynolds Number at 25 degrees C:	6.67 (Laminar Flow)
Entry Length:	0.05 cm
Yeast Cell Diameter:	5 microns
Reynolds Number for a Particle in a Fluid at 25 degrees C:	0.02
Terminal Settling Velocity, Spherical Rigid Particle, Reynolds Number < 0.1:	6.4 x 10^-6 cm/sec
Liquid Diffusion Coefficient:	1.02 x 10 ^-9 cm^2/sec
Diffusion Distance (Drift):	1.2 microns (14 sec) 19 microns (60 min)

Table 1. Fluid-flow and mass transfer characteristics for the Kynar flow cell system.

process. The adhesion period for the biofilm studies was reduced from that used in the adhesion studies due to problems with plugging of the flow cell.

In order to establish some association between what was found in the lab and what was occurring during the initiation of an infection associated with a medical device or implant, the biofilm experiments which were initially conducted at ambient temperatures were conducted at 37°C.

Polystyrene Coverslip Preparation

Glass coverslips (43 mm × 61 mm) were prepared for spin-coating by soaking in 5% dimethyldichlorosilane (DMDCS) in toluene for a minimum of three hours and then rinsed with methanol to remove the excess silanizing agent, then rinsed with nanopure water, and then allowed to dry at ambient conditions. The silanized coverslips were then spin coated with 5wt% polystyrene (Average M_W 230,000) in toluene. The PS spin-coated coverslips were prepared 24 hours prior to experimentation or manipulation.

Growth Experiments

Problems associated with the use of the Teflon flow cell, namely plugging and adhesion to surfaces other than that of interest, prompted the design of a new flow cell. The new flow cell design incorporated the need for limited material types, materials that resisted adhesion of *C. albicans*, and a larger flow channel. To meet these ends, the material Kynar was selected based on information (Eldon James Fittings) that the material was resistant to fungal adhesion - although the specific fungal types were not specified. The body of the flow cell was constructed out of Kynar, and the flow channel was delineated by two PS surfaces separated by a Kynar wall (1/16 inch thick).

After assembling the flow cell, a 5% bleach solution was injected into the flow cell and allowed to sit for 15 minutes in an attempt to disinfect the flow cell. The bleach was then removed and the flow cell was aseptically connected to the tubing. Since temperatures in the autoclave can exceed 121°C and the melting temperature of PS is between 150° and 243°C, autoclaving was not a viable option due to the structural

integrity of the polymer being compromised and the surface becoming cloudy when exposed to extreme temperatures.

The *C. albicans* biofilm growth experiments were initially conducted in the manner described for the adhesion experiments. After the half-hour adhesion experiment, the introduction of the cell solution to the flow cell was discontinued and replaced by the initiation of reduced GYEP broth (0.05% glucose, 0.03% yeast extract, and 0.1% bacto-peptone). The flow rate was 3.4 ml/min in the new flow cell. The composition of the feed was established as a result of several biofilm studies on *C. albicans* strain 1. The makeup of the GYEP broth used in the suspension cultures was reduced 100 fold in order to develop a feed which brought the quantity of biofilm to a manageable level, i.e., until plugging of the flow cell was minimized.

The cells were fed and allowed to grow for 48 hours. In the early phases of this research, the growth was monitored with the phase-contrast microscope. However, in addition to the development of a new flow cell came other changes, including the belief that monitoring the growth was more disruptive than it was informative. The monitoring required the movement of the incubator in and out of the microscope room which meant not maintaining a constant temperature and the turning on and off of the pump and the heat, not to mention the movement of the flow cell in and out of the incubator. Images were recorded using Imaging Program for Windows. Following the 48-hour biofilm formation period, the cells were stained with acridine orange and then visualized using confocal scanning laser microscopy (CSLM). A Leica DMRXE microscope and Leica TCS NT imaging program were utilized. The experimental setup is shown in Figure 6.

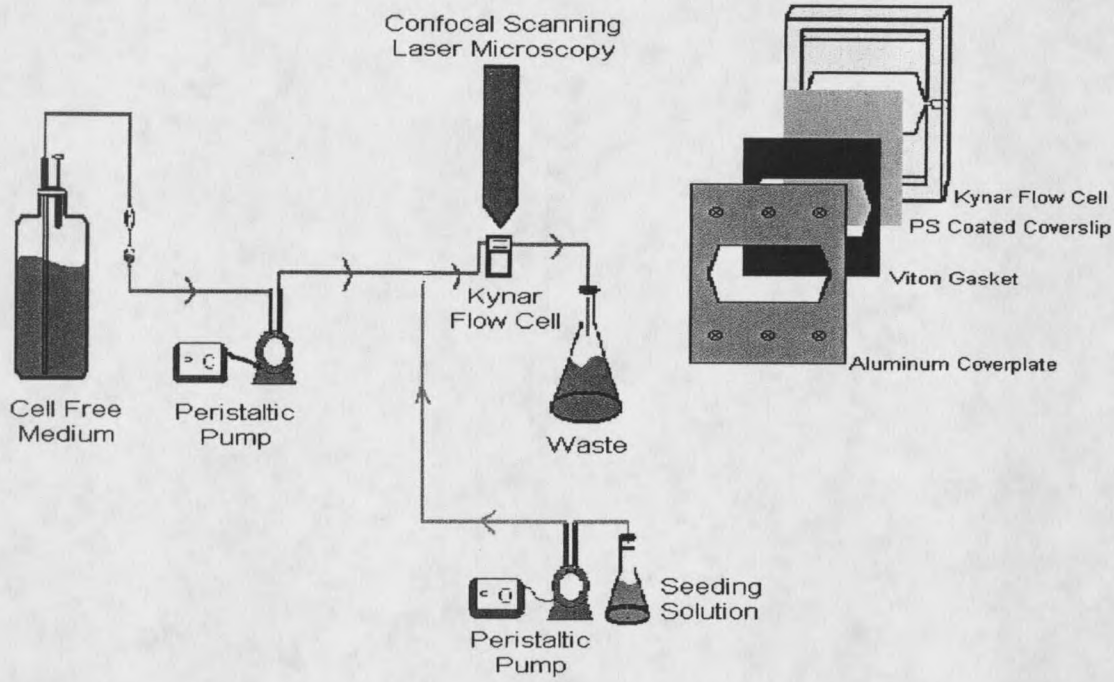


Figure 6. Biofilm experimental setup including flow cell assembly.

C. albicans biofilm formation has been described previously (3,5,31,33,60). However, in these experiments *C. albicans* was grown at 37°C in either wells of tissue culture plates or in Erlenmeyer flasks with shaking.

Toxicity tests were conducted by generating growth curves for CA1 and CA662. The growth curves were carried out in suspension cultures at 37°C in 100 ml of reduced GYEP medium with or without the addition of 1 ml of 4% Pluronic F127 solution. Growth curves were also generated for suspension cultures at 37°C in 100 ml of GYEP medium with the addition of 10 µl or 1000µl of 4% Pluronic F127 solution or in the absence of Pluronic F127.

However, since the effect of chemicals on planktonic cells is different from that on their sessile counterparts, the impact of Pluronic F127 adsorbed to the surface on cell

viability was examined through the use of propidium iodide (PI) stain. The reliability of PI in identifying non-viable cells of *C. albicans* was tested by staining "live" cells and "dead" cells. The "live" cells were obtained in the usual manner as for adhesion studies . A dilution series was performed as if for a hemacytometer count and then 20 μl from the final dilution was combined with 955 μl of PBS and 25 μl of a 1 mg/ml stock solution of PI. The "dead" cells were obtained by combining 20 ml of PBS with 1 ml of cells from a 4 ml cell solution created in the same way as that used for generating the seeding solution. This cell solution was then autoclaved for 20 minutes. Twenty microliters of the heat "killed" cell solution was mixed with 955 μl of PBS and 25 μl of PI. The PI stained cells were loaded into a hemacytometer and the cells in one of the four corner blocks were counted. The compromised cells fluoresce red under UV light as observed using an Olympus microscope fitted with a 20 x objective (Olympus DplanApo 20 UV) under x15 magnification.

Characterization of *Candida albicans* Biofilm Structure

Biological Stain

An acridine orange (AO) solution was prepared using PBS to generate a final solution concentration of 0.05% AO. The solution was syringe-filtered using a sterile 0.2μm filter immediately before use. The biofilm cultivated after the 48-hour growth period was stained with the AO solution for 15 to 30 minutes, depending on the length of time needed for penetration of the biofilm. The length of time was determined by observing that the cell mass was stained orange. The excess AO was removed by

pumping PBS through the flow cell until the effluent ran predominantly clear. AO is a suspected mutagen and carcinogen and was handled with care. All glassware, filters, and tubing were rinsed thoroughly, and the rinse water along with any solid waste was placed in marked containers for pickup by Hazardous Materials Management.

Live-Dead Fluorescent Stain

A stock solution of 1 mg/ml of PI in nanopure water was prepared. A working solution with a concentration of 25 μg/ml in PBS was prepared for all staining procedures employing PI. The nanopure water and the PBS were both sterilized in the autoclave prior to use. PI is also a suspected mutagen and was handled with care. All glassware and tubing were rinsed thoroughly, and the rinse water was placed in marked glass containers for pickup by Hazardous Materials Management.

Confocal Scanning Laser Microscopy

Once the biofilm was stained, it was visualized using a confocal scanning laser microscope. The flow cell was oriented in a vertical position during the seeding and biofilm formation processes. Therefore, the side of the flow cell examined should not make a difference. The biofilm was examined using a Leica DMRXE microscope with a 63x dry objective 0.70 PL Fluotar having a working distance of 2.0 mm, a 20x dry objective Nplan with a working distance of 2.52 mm, dual Mitsubishi Diamond Pro 91 TXM monitors, and the Leica TCS NT imaging program. The CSLM was fitted with an argon ion laser, 488 nm wavelength, that was used to excite the AO and PI. AO bound to DNA has an excitation and emission wavelength of 500 nm and 526 nm, respectively. The

excitation and emission wavelength for AO bound to RNA is 460 nm and 650 nm, respectively. The excitation and emission wavelength for PI bound to nucleic acids is 535 nm and 637 nm, respectively. In 1947, Strugger noted that living yeast cells stained with acridine orange fluoresced green to yellow-green while those that were dead fluoresced orange to red. These findings were later supported by Schwartz et al. in 1977.(77)

Images were acquired at 4 to 5 μm z-intervals with the pinhole setting at 1.00. A z-interval of 5 μm was always specified, but the program would often times return a value for use that was slightly less than 5 μm. An average of seven to ten regions on CA1 biofilms were examined during each of three experiments for the two materials (PS and Pluronic F127 treated PS). An average of four to twelve optical sections having an area of 158.7μm x 158.7μm were collected depending on the thickness of the biofilm. Each optical section was averaged two times in an effort to eliminate visual noise. The last visible layer next to the surface was the last image that was obtained. That is, an image of the surface itself was not procured. This point becomes important when discussing the location of the thickest portion of the biofilm.

Image acquisition was the same for biofilms of CA662 except that six regions were selected for observation for each experiment performed. The six regions selected are shown in Figure 5. A point was randomly selected during each experiment within each region of interest. This was done to allow for comparison of specific regions from one experiment to the next and between the two different surfaces (PS and Pluronic F127 treated PS).

Quantitative Analysis

Quantitative analysis of the biofilm was performed using the UTHSCSA ImageTool program (developed at the University of Texas Health Science Center at San Antonio, Texas and available from the Internet by anonymous FTP from maxrad6.uthscsa.edu). The percent surface coverage as a function of position within the biofilm was determined by taking the images obtained by the Leica TCS NT imaging program and converting the colored image to black and white pixels such that the cells appeared black and the void area appeared white. Any pixels over an intensity of 35 on a scale of 0 to 255 were converted to black pixels. The percent of black pixels was then calculated and used as the value for the percent surface coverage. An image for each section of the biofilm taken at ~5μm intervals was analyzed in this manner. A plot of percent surface coverage versus the distance beyond the first cell layer was then generated. The data points were connected by performing a cubic spline interpolation between the data points in order to produce a smooth curve. The graph showing the average percent surface coverage versus position was obtained by using the cubic spline method to interpolate the data and estimate the percent surface coverage at 5μm, 10μm, and on up to 40μm by 5μm intervals. Since the data points were not all exactly at 5μm increments apart, it was necessary to find a common distance from the first cell layer. The values for the percent surface coverage were then averaged for the different regions of the biofilm that were observed.

The cell clusters were organized into different groups based on area. The smallest cluster size consists of single cell clusters with a diameter up to 5μm or budding cell clusters. The cluster groups were organized to comprise $20\mu m^2$ increments. The images were converted into black and white pixels as for the percent surface coverage. Then the black objects in the image can be classified into groups by area which is specified by the user. The ImageTool program then counts the number of objects that are within each classification group. This classification was performed for each layer within each region of the biofilm. The fraction of each cluster group in each layer of the biofilm was then determined by dividing the area of each cluster group by the total cluster area in each layer of the biofilm.

Statistical Analysis

In order to assess whether there was a significant difference in the number of cells that adhered to Pluronic F127 treated PS (PL-PS) in comparison to native PS at 60 minutes at location A, a randomization test was performed using Matlab®. A relationship between location on the surface and the number of cells per unit area on native PS and PL-PS was established through regression analysis using Minitab®. The maximum percent surface coverage on PS versus PL-PS at each of the seven locations on the surface was analyzed by counting using combinations. In order to determine whether there was an effect due to strain or Pluronic F127 on the log number of cells per unit volume in a suspension culture grown in reduced medium, the data was analyzed by balanced ANOVA using Minitab®.

Prior to performing the statistical analysis on the growth curve data, the concentrations were adjusted to take care of the disparity in the initial inoculum size. That is, the concentrations were scaled so that the initial concentration of cells was zero. Since the quantity of cells was not determined for inoculation, but rather 0.25 milliliters of stationary phase yeast cells at 37°C was the size of the inoculum used, the concentration of cells in the growth medium following inoculation was not exactly the same.

PRELIMINARY RESULTS

Adhesion to Oxygen Plasma Treated Polystyrene

Adhesion of *C. albicans* strain 1 to oxygen plasma treated PS surfaces (768 ± 448 cells/mm^2) was found to be greater than adhesion to Pluronic F127 treated PS surfaces (116 ± 31 cells/mm^2), but still markedly reduced in comparison to the untreated PS surface (16,241 ± 540 cells/mm^2) as shown in Figure 7. Problems with the functioning of the plasma reactor made continued research of these surfaces difficult.

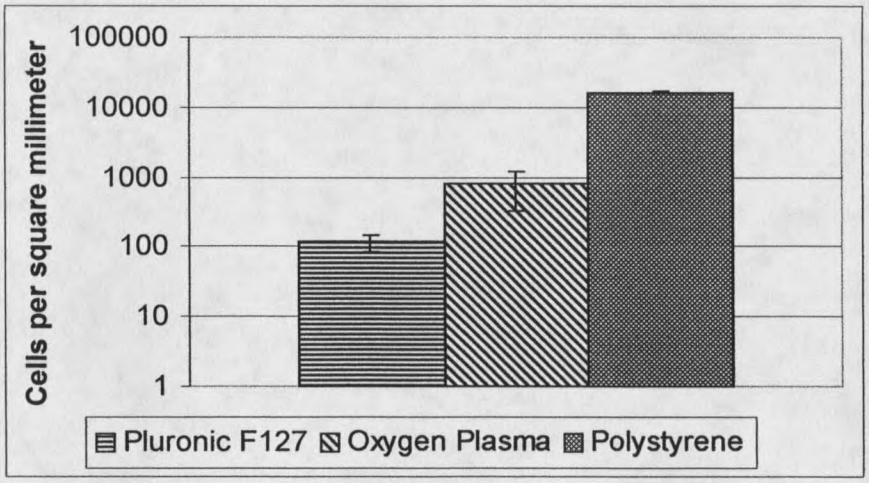


Figure 7. Adhesion of C. albicans to treated and untreated polystyrene after 60 minutes of exposure to a cell concentration of ~ 1×10^7 cells/ml.

Patterned Surface on Polystyrene

It was attempted to generate a patterned surface with oxygen rich regions and oxygen poor regions. A copper grid (400 mesh, 3.05 mm o.d.) was placed over the PS

surface and then exposed to an oxygen beam. Table 2 contains the atomic concentration levels obtained using XPS for native PS and PS exposed to an oxygen beam. The oxygen beam treated sample was also analyzed using ToF SIMS. A ToF SIMS image (Figure 8) was obtained showing the spatial locations of carbon and oxygen on the surface which were organized in a pattern. An adhesion experiment was then conducted on a patterned surface. *C. albicans* demonstrated organized adhesion on the chemically patterned surface following an hour long adhesion experiment. Figure 9 shows the patterned adhesion of *C. albicans* on the perimeter of the PS coupon where the chemical pattern existed. The oxygen beam was able to get under the copper screen, except at the perimeter, due to difficulties in obtaining close contact with the screen and the PS coupon.

	Concentration (%)	
Element	Pristine Polystyrene	Oxygen Beam Treated Polystyrene
C1s	98.40	83.06
O1s	1.60	16.94

Table 2. XPS atomic concentrations.

Growth Curves for CA1 and CA662 in Nonreduced Medium

Growth curves were generated for CA1 and CA662 suspension cultures at 37°C in 100 ml of GYEP medium (5% glucose, 0.3% yeast extract, 1% bacto-peptone) with the addition of 10 μl or 1000 μl of 4% Pluronic F127 solution or in the absence of Pluronic

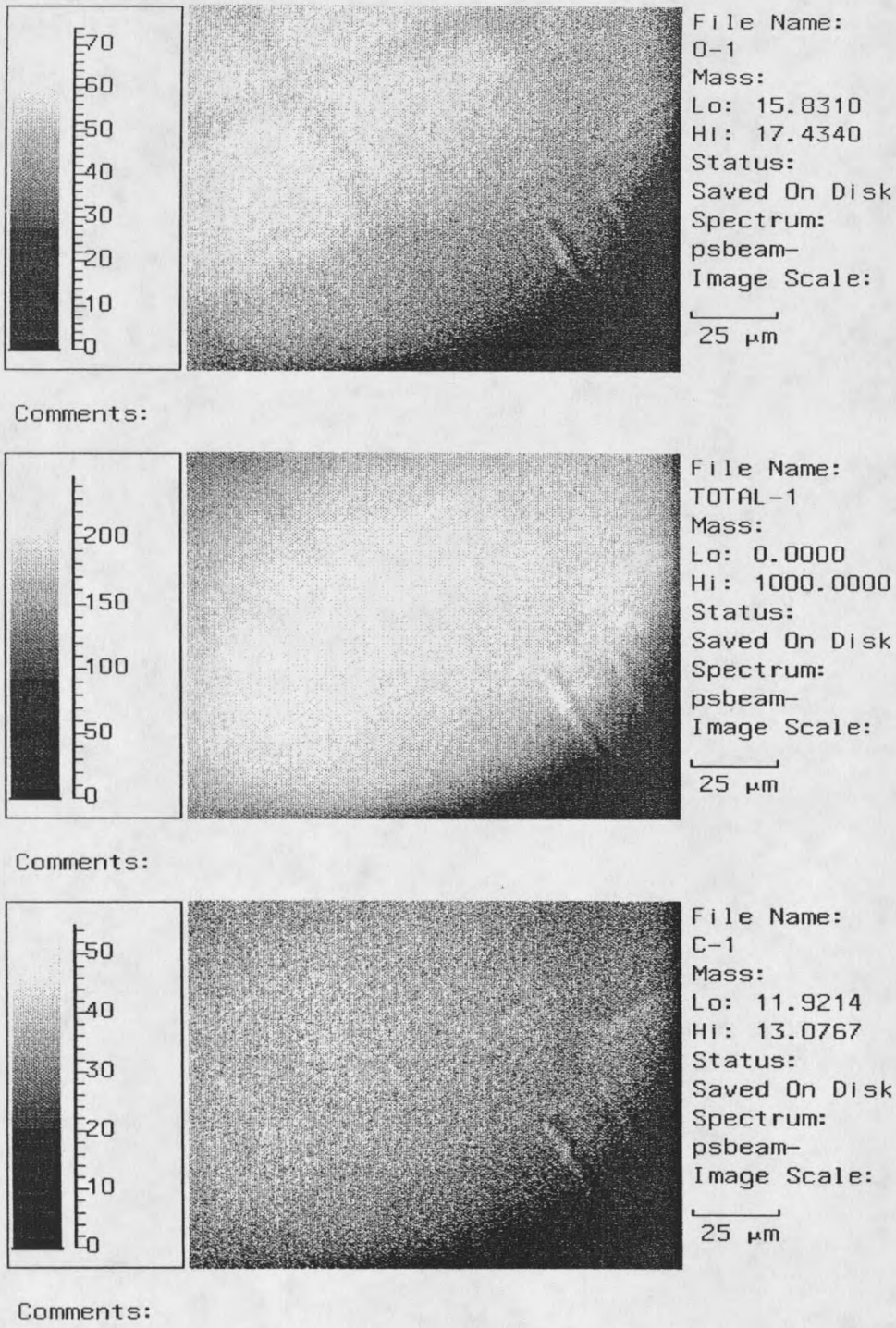


Figure 8. ToF SIMS image showing the spatial locations of carbon and oxygen on the surface.

F127. The growth curves for CA1 (Figure 10) and CA662 (Figure 11) reveal that the presence of Pluronic F127 did not limit the growth of CA1 or CA662 compared to growth in the absence of Pluronic F127. Based on these findings and those for growth in reduced GYEP medium, the effect of Pluronic F127 on growth of *C. albicans* depends on the concentration of the growth medium.

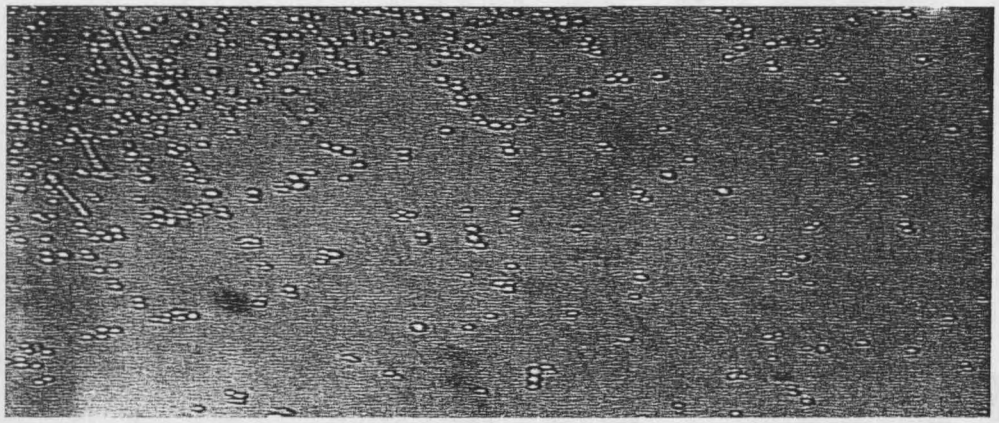


Figure 9. Patterned adhesion of *C. albicans* strain 1 on the perimeter of the PS coupon exposed to an oxygen beam through a copper grid.

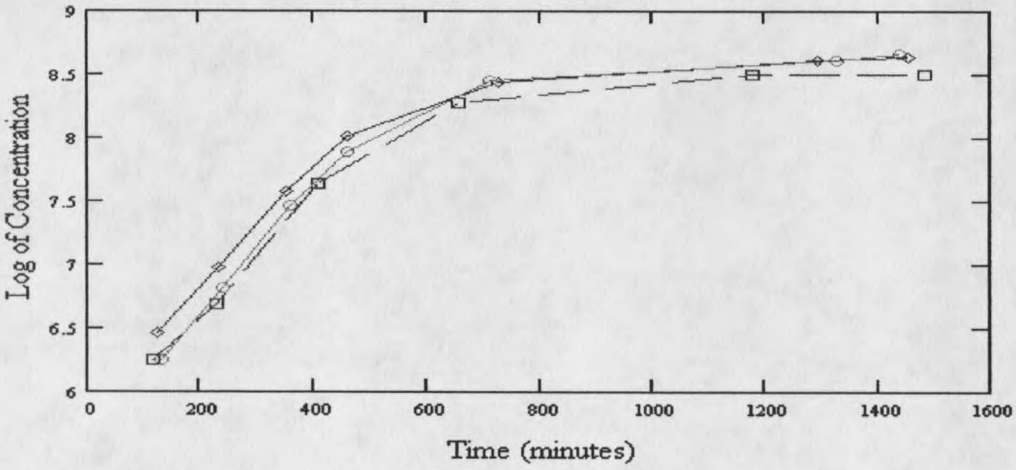


Figure 10. Growth curves for CA1 in GYEP medium with the addition of 10µl (-◇-) or 1000µl (-○-) of 4% Pluronic F127 solution or in the absence of Pluronic F127 (-□-).

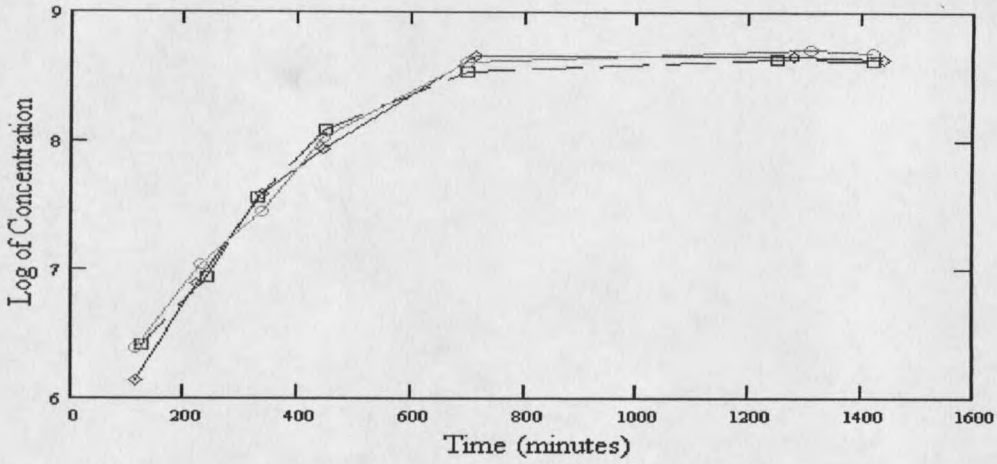


Figure 11. Growth curves for CA662 in GYEP medium with the addition of 10µl (-◇-) or 1000µl (-○-) of 4% Pluronic F127 solution or in the absence of Pluronic F127 (-□-).

INFLUENCE OF SURFACE CHEMISTRY ON THE ADHESION AND BIOFILM FORMATION OF *CANDIDA ALBICANS*

Introduction

The motivation for studying the adhesion and biofilm formation of *Candida albicans* is spurred by the need to inhibit infections caused by the yeast. Candidiasis, the infection caused by species of *Candida* with *Candida albicans* being the primary etiologic agent, represents an opportunistic disease. *Candida albicans* exists as a normal commensal of the human gastrointestinal and genitourinary tracts and mucosa. The infection stems from an overgrowth of this normal human flora. Groups susceptible to the disease include immunocompromised individuals, mechanical trauma victims, and those undergoing iatrogenic procedures.(51,61)

Several possible factors are thought to contribute to the virulence of *Candida albicans*. Those relevant to this study include hyphal formation, adherence properties, and variable characteristics such as the dynamic cell surface. Hyphal formation is one of three different morphogenetic processes that *Candida albicans* can undergo. The other processes include blastospore formation and pseudohyphal formation. The morphological state depends on the pH, incubation temperature, inoculum size, and the composition of the growth medium.(51,61)

Candida albicans can invade the human host by adhering to a plastic surface (i.e., prosthesis, catheter, prosthetic valve, etc.) with subsequent formation of a protective biofilm and then disperse by means of the vascular system. Combating candidal infections

requires a strategy that combines the use of antifungal agents along with blocking of adhesion and biofilm growth. By themselves, antifungal agents are not capable of preventing and controlling yeast bloodstream infections.(32,92) In order to prevent adhesion and biofilm growth of yeast, these processes must be understood. A means of studying adhesion and biofilm structure is through the use of a well-defined culture surface. Control over the chemical features of the surface allows the influence of chemistry on cell behavior to be studied.

Approximately one in five Americans has a long-term implanted medical device. One of the most common complications associated with implanted medical devices is infectious biofilms. Most studies of infectious biofilms have been performed with bacteria. These device-centered infections are resistant to the body's immune system, antibiotics, and antifungal agents. Since one-quarter of the implant infections are caused by the yeast *Candida albicans*, the necessity for gaining insight into the initial adhesion process and ensuing biofilm development becomes apparent.

Other investigators have shown that Pluronic inhibits the adhesion of bacteria (4,63) and proteins (1,21,30) to polymer surfaces. Our investigations, reported here, have shown that Pluronic F127 adsorption dramatically reduces the adhesion of *Candida albicans* to polystyrene (PS). However, the relationship between these initial adhesion rates and the long-term growth rates and biofilm formation are still highly controversial. Therefore, the objective of this work was to determine how the initial adhesion pattern and the material's surface chemistry influence the growth of *C. albicans* on the surface. The biofilm development was investigated using confocal scanning laser microscopy.

Materials and Methods

Culture Surface Preparation

The well-defined culture surfaces utilized included native polystyrene and Pluronic F127 conditioned polystyrene. The PS surface employed was either a cleaned PS coupon or a PS surface formed on a silanized glass coverslip by spin coating. The PS coupons were half-inch diameter disks punched from a sixteenth of an inch thick sheet. The coupons were cleaned prior to experimentation or manipulation. The PS coupons were cleaned by placing each coupon in HPLC grade hexane with swirling for approximately five seconds. The coupons were then allowed to dry. After drying, the PS coupons were set in a beaker containing methanol and sonicated on ice for approximately five minutes. The PS coupons were removed from the methanol and placed in a clean Pyrex glass container.

Glass coverslips (43 mm × 61 mm) were prepared for spin-coating by soaking in 5% (v/v) dimethyldichlorosilane (DMDCS) in toluene for a minimum of three hours and then rinsed with methanol to remove the excess silanizing agent, then rinsed with nanopure water, and then allowed to dry at ambient conditions. The silanized coverslips were then spin coated with 5wt% polystyrene (Average M_W 230,000) in toluene. The PS spin coated coverslips were prepared 24 hours prior to experimentation or manipulation.

The PS coupons were utilized in the adhesion experiments while the spin coated PS coverslips were utilized in the biofilm growth experiments. The two different PS surfaces were needed as a result of modification of the flow cell design for the biofilm

growth experiments. The new flow cell design minimized problems associated with plugging and with analyzing the biofilm with confocal scanning laser microscopy (CSLM).

The Pluronic F127 treated PS surfaces were generated by soaking clean PS coupons or coverslips in a 4% Pluronic F127 solution in phosphate buffered saline (PBS) for a minimum of three hours. The PBS solution was 0.01M with a pH between 7.15 and 7.25. The conditioned PS samples were either used directly or stored for no longer than one week.

Pluronic F127 is an amphiphilic A-B-A triblock copolymer. A is poly(ethylene oxide) (PEO), the hydrophilic segment, and B is poly(propylene oxide) (PPO), the hydrophobic segment. Pluronic F127 consists of 98 PEO segments and 67 PPO segments. Pluronic is thought to adhere to the plastic surface via the hydrophobic portion in such a manner that the hydrophilic portion extends into the bulk fluid, imparting a hydrophilic character to the plastic surface. Pluronic F127 is a trade name given to industrial and pharmaceutical grades of Poloxamer 407.

Surface Composition

X-ray photoelectron spectroscopy (XPS) was used to confirm the surface chemical composition. XPS analysis was performed with a PHI 5600 XPS system. The operating pressure in the analysis chamber was in the 10^{-9} torr range. A monochromatized x-ray source (Al Kα anode, 1486.6 eV) was operated at 15 kV and 300 watts. The aperture was set to four, corresponding to analysis of a spot 800μm in diameter. Charge neutralization was achieved using a low energy electron gun. All binding energies were

referenced to the hydrocarbon component (C-C / C-H) of the C1s peak which was set to 285.0 eV. A pass energy of 58.7 eV was used.

Cells and Culture Media

Candida albicans CA1 isolates were maintained in a sub-zero freezer. Every month a new subculture was generated using the streak plate method of isolation. Following a 48 hour incubation period at 35°C, a single colony was removed from the isolation plate and transferred to a slant tube containing Sabouraud dextrose agar, generated following the manufacture's instructions. The inoculated slant tubes were incubated for 48 hours at 35°C and then kept at approximately four degrees Celsius. This culture of *Candida albicans* was used to perform adhesion and biofilm growth experiments. Each inoculum of *Candida albicans* for experimentation was grown in GYEP (5% glucose, 0.3% yeast extract, 1% bacto-peptone) broth at 35°C and 160 rpm for 24 hours. The first inoculum was removed from a slant tube and placed in 100 milliliters of sterile GYEP broth. The second inoculum was obtained from the initial broth culture of which 0.25 milliliters were placed in 100 milliliters of sterile GYEP broth. The GYEP broth was sterilized by autoclaving for 15 to 20 minutes.

The *C. albicans* cells used in the growth and adhesion experiments were obtained from the second inoculum following a 24 hour incubation period (stationary phase). Two, four milliliter portions of cells were removed from the broth culture and spun down for 90 seconds at ambient conditions. The cells were rinsed three times with two milliliters of sterile, refrigerated 0.01M PBS. Following the final rinse, two milliliters of sterile PBS

were added to each portion of cells. The cells were combined to generate a little more than four milliliters of cells in PBS which were pelleted and kept on ice prior to use. The concentration of cells in this solution was determined using a hemacytometer. This solution of cells was then used to generate a new solution of cells, with a concentration of $\sim 10^7$ cells per milliliter, to be utilized in the flow cell experiments.

Adhesion and Biofilm Growth Experiments

The adhesion experiments were performed in a Teflon flow cell device containing the material of interest. A Pluronic F127 treated PS coupon or native PS coupon was set in the well of the Teflon flow cell. Initially, PBS was pumped through the flow cell using a peristaltic pump in order to establish the desired flow rate, focus the Olympus microscope, and remove air from the flow cell assembly. The yeast, at a concentration of $\sim 1\times 10^7$ cells/ml, were then introduced into the flow cell. A shear rate of 16 sec^{-1} was selected based on arterial wall shear rates which range from 10 to 1000 sec^{-1}. A flow rate of 1.5 ml/min was necessary to achieve the specified wall shear rate as determined by the design of the flow cell. The adhesion process was conducted at room temperature and observed for an hour. The progress of the experiment was monitored using an Olympus microscope (phase contrast microscopy in the reflected light mode) fitted with a 20x objective under x10 or x15 magnification and recorded through Imaging Program for Windows®.

The biofilm growth experiments on the culture surface were conducted in a Kynar flow cell device that was maintained at approximately 37°C. The sample to be studied

was placed in the flow cell and then disinfected with a 5% bleach solution for 15 minutes followed by rinsing with PBS for 15 to 30 minutes, which also allowed for the creation of the required flow rate. Following a half-hour seeding (adhesion) period, the cells within the flow cell were fed a sterile, cell free 0.05% glucose medium solution, reduced GYEP broth (0.05% glucose, 0.03% yeast extract, 0.1% bacto-peptone), for approximately 48 hours to allow for the development of a biofilm. The biofilm cultivated after the 48 hour growth period was stained with a 0.05% acridine orange (AO) solution in PBS for 15 to 30 minutes depending on the length of time needed for penetration of the biofilm. The excess AO was removed by pumping PBS through the flow cell until the effluent ran predominantly clear. AO, a cationic dye, binds to DNA and RNA.

Biofilm Imaging

CSLM was then used to obtain a 3-D image of the *Candida albicans* biofilm. The biofilm was examined using a Leica DMRXE microscope with a 63x dry objective 0.70 PL Fluotar or with a 20x dry objective Nplan having a working distance of 2.0 mm and 2.52 mm, respectively, dual Mitsubishi Diamond Pro 91 TXM monitors, and the Leica TCS NT imaging program. The CSLM was fitted with an argon ion laser, 488 nm wavelength, that was used to excite the AO. AO bound to DNA has an excitation and emission wavelength of 500 nm and 526 nm, respectively. The excitation and emission wavelength for AO bound to RNA is 460 nm and 650 nm, respectively. Images were acquired at 4 to 5μm z-intervals with the pinhole setting at 1.00. An average of seven to ten regions were examined during each of three experiments for the two materials. An

average of four to twelve optical sections having an area of 158.7μm x 158.7μm were collected depending on the thickness of the biofilm. Each optical section was averaged two times in an effort to eliminate visual noise. The last visible layer next to the surface was the last image that was obtained. That is, an image of the surface itself was not procured. This point becomes important when discussing the location of the thickest portion of the biofilm. Quantitative analysis of the biofilm was performed using the UTHSCSA ImageTool program (developed at the University of Texas Health Science Center at San Antonio, Texas and available from the Internet by anonymous FTP from maxrad6.uthscsa.edu).(79)

Results and Discussion

To confirm the presence of the desired chemical species, the native PS and Pluronic F127 conditioned PS were subjected to XPS analysis. The surfaces were prepared 24 hours prior to examination to ensure that the samples were dry, preventing out-gassing of the sample, and to minimize the possibility for contamination. Table 3 presents the carbon and oxygen surface concentrations. The surface oxygen on native PS was the result of oxidation of the surface upon exposure to air. The increase in the surface oxygen content of PS upon treatment with Pluronic F127 was mainly due to the presence of ether-type carbon (C-O-C) as evidenced by an C1s component near 286.5 eV (Figure 12).

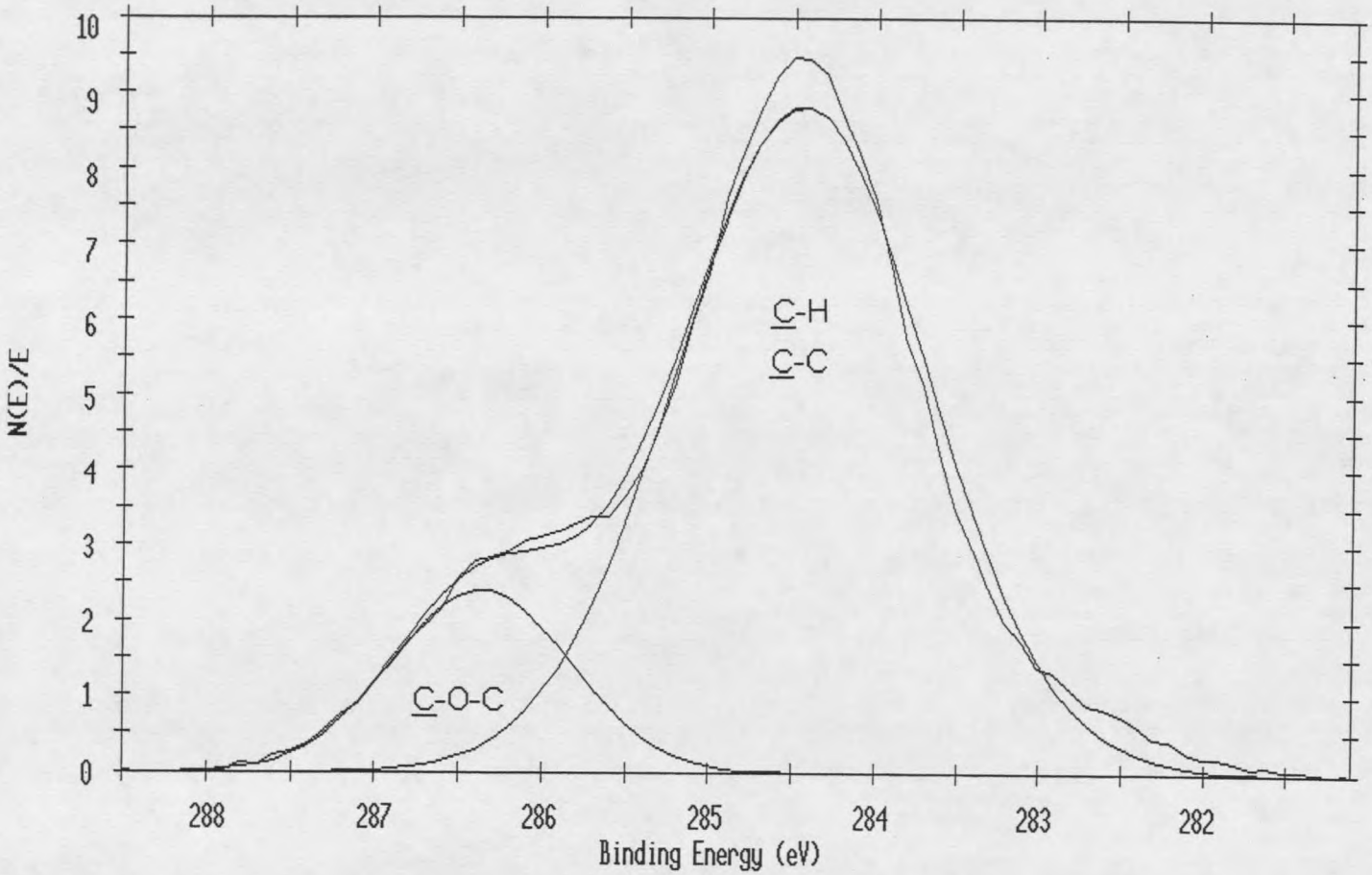


Figure 12. The C1s spectrum (resolved into component peaks) for Pluronic F127 treated polysytrene.

Element	Concentration (%)	
	Pristine Polystyrene	Pluronic F127 Conditioned Polystyrene
C1s	98.40	89.60
O1s	1.60	10.40

Table 3. XPS atomic concentrations.

The results of the adhesion experiments of *Candida albicans* to pristine PS and Pluronic F127 treated PS are summarized in Figure 13. Figure 13 shows the number of cells adhering to a square millimeter area after exposure to a cell concentration of ~1×10^7 cells/ml at a rate of 1.5 ml/min for one hour. The number of cells that adhered to the Pluronic F127 conditioned PS (116 ± 31 cells/mm^2) was significantly less than that adhering to the untreated PS (16,241 ± 540 cells/mm^2).

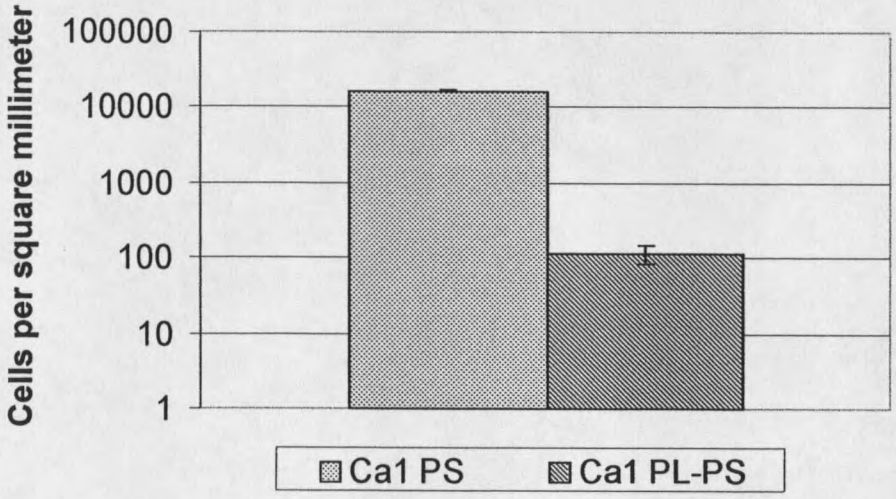


Figure 13. Adhesion of *Candida albicans* to treated (PL-PS) and untreated (PS) polystyrene after 60 minutes of exposure to a cell concentration of ~ 1×10^7 cells/ml at a rate of 1.5 ml/min. Values are expressed as mean number of cells per unit area. The error bar represents the standard deviation.

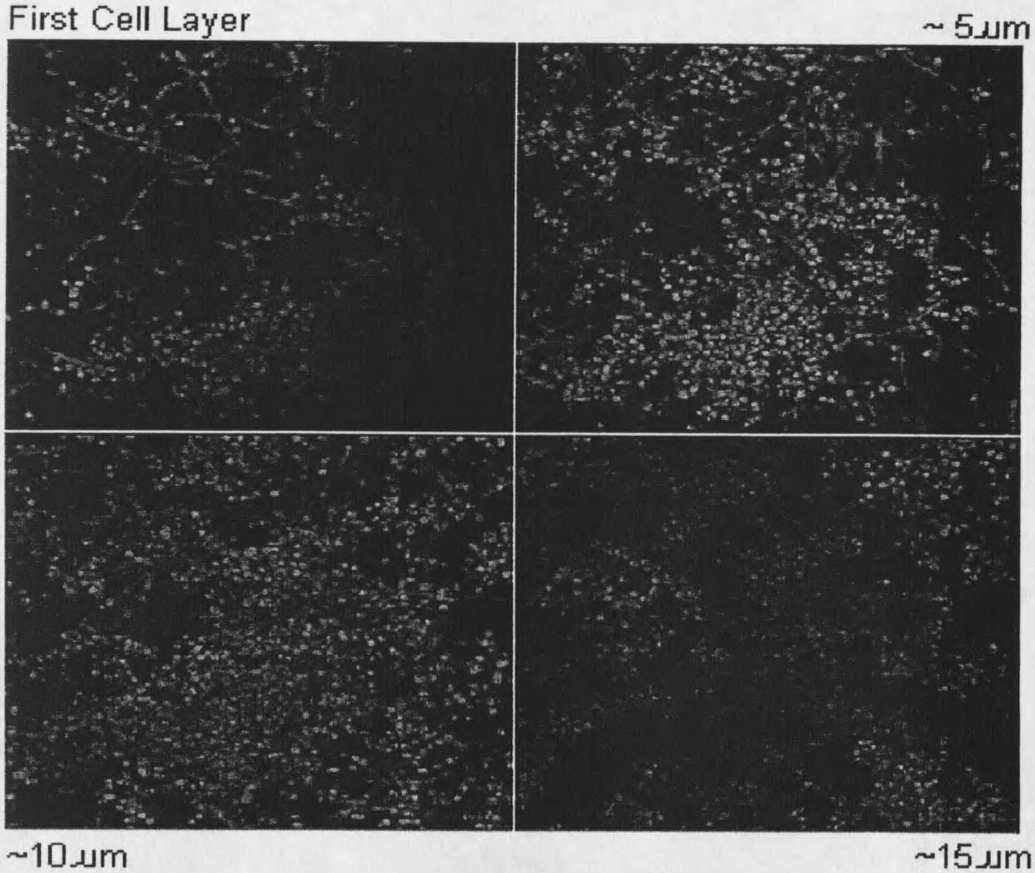


Figure 14. Biofilm formation on pristine polystyrene at 48 hours with the first cell layer originating at the surface and the consecutive layers within the biofilm given in ~ 5 μm increments.

The findings from the adhesion experiments prompted the question as to whether the influence of Pluronic F127 on initial cell adhesion persists during the formation of the biofilm. The biofilms that formed on the pristine PS and on the Pluronic F127 treated PS were strikingly different. Figure 14 displays CSLM images for a *C. albicans* biofilm formed on pristine polystyrene following 48 hours of growth at ~37°C. The CSLM images are of four layers of a biofilm beginning at the first layer of visible cells and extending towards the bulk fluid. The first layer of visible cells was determined by

scanning through the biofilm until no cells were observed and then scanning back up to the first layer of visible cells. Figures 15, 16, and 17 show the results for biofilm formation on pristine PS indicating the percent surface coverage as a function of distance above the first cell layer. Figure 15 represents a typical plot of surface coverage (%) as a function of height (μm), with the surface coverage reaching a maximum at approximately 5μm above the first cell layer and then tapering off as the biofilm extends into the bulk fluid. A height of zero represents the first visible layer of cells on the surface. Thus, for yeast cells with an average diameter of 5μm, this indicates that the greatest surface coverage occurs at the second cell layer beyond the substratum. However, it may be that the biofilm begins at the 5μm point and the reason we are seeing anything below this point may be due to an artifact of the surface. That is, the surface may not be completely level. Also, as we scan through the biofilm, some cells appear clearly at one level and faintly at levels above and below. The distorted appearance of cells may be a consequence of passing the laser through air/solid/liquid interfaces. These factors may then influence the biofilm thickness obtained from the confocal imaging.

Figure 16 presents the percent surface coverage as a function of distance from the first cell layer for eight regions of the biofilm for a single experiment. Again, the surface coverage attains a maximum at approximately 5μm above the first cell layer and then tapers off with increasing distance. This figure exhibits the heterogeneous nature of the biofilm with surface coverage ranging from ~10% to ~60% at its apex. The figure also reveals the heterogeneity in biofilm thickness.

The mean percent surface coverage versus distance from the first cell layer for three experiments and an average of ten regions of the biofilm for each experiment is shown in Figure 17. As in the previous figures, the percent surface coverage reaches a peak at a height of ~5μm from the surface layer of cells and tapers off into the bulk fluid. The bars on the graph represent the heterogeneity of the biofilm. The average maximum surface coverage for the three experiments is 21%.

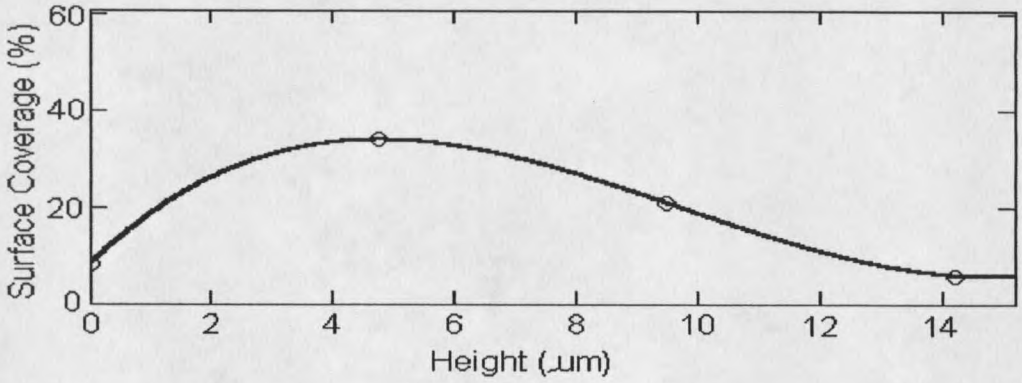


Figure 15. Percent surface coverage versus distance from the first cell layer for a single region of the biofilm formed on pristine polystyrene. Plus sign, data points; solid curved line, cubic spline interpolation.

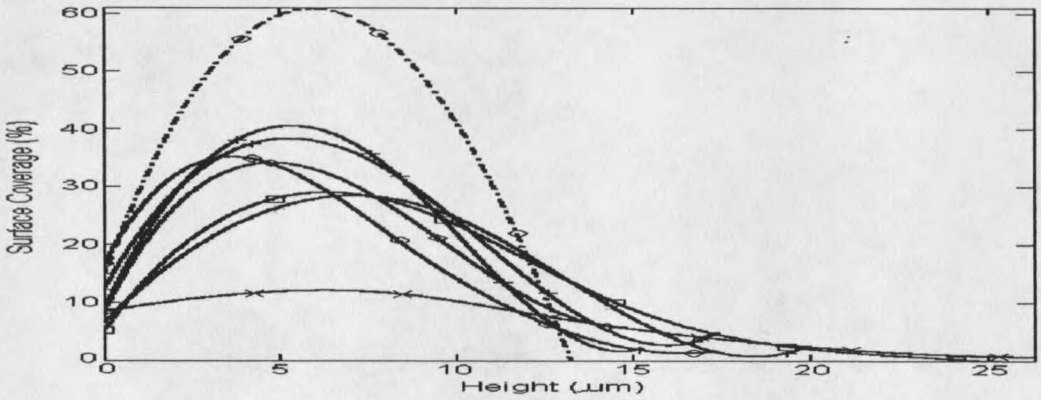


Figure 16. Percent surface coverage versus distance from the first cell layer for eight regions of the biofilm formed on pristine polystyrene. Symbols, data points; curved lines, cubic spline interpolation.

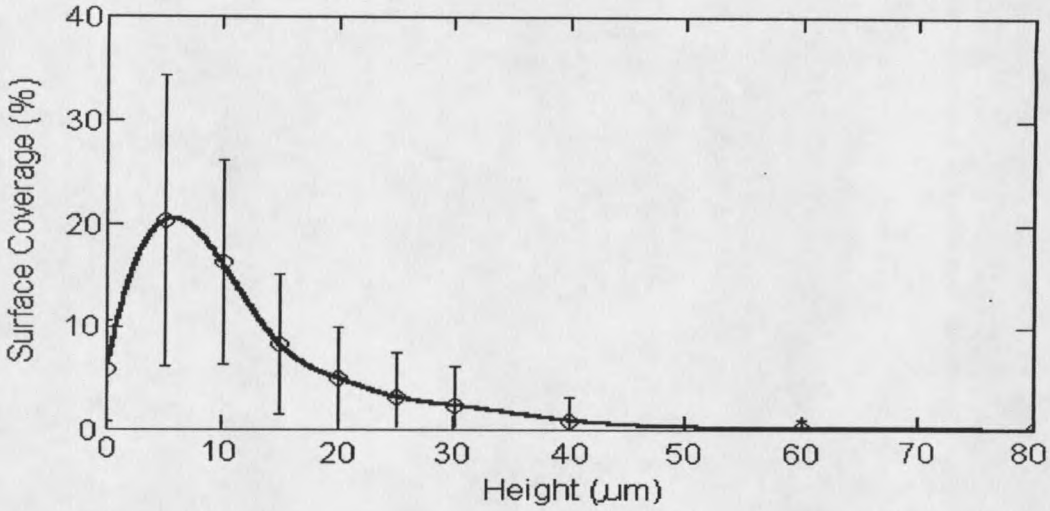


Figure 17. Mean percent surface coverage versus distance from the first cell layer for three experiments with an average of ten regions of the biofilm formed on pristine polystyrene per experiment. Open circles, data points; solid curved line, cubic spline interpolation; solid vertical lines, variation in surface coverage at a given height above the surface.

The various cluster groups within each layer of the biofilm were characterized into different cluster sizes. The cluster sizes that were considered, along with representative examples, are shown in Figure 18. The smallest cluster group consists of single cells and budding cells. The bar graphs displayed in Figure 19 indicate the cluster group fraction of the total cluster area in each layer of the biofilm. The graph in the top portion of the figure is for a single region of the biofilm for a single experiment. The graph in the bottom portion of the figure contains the mean values for eight regions for each of three experiments. Both graphs reveal that the largest of the total cluster groups (gray) represents the greatest fraction of the total cluster area in regions close to the surface, and the smallest cluster groups (backslash) occupy the greatest fraction of the total cluster area in regions distant from the surface, in general.

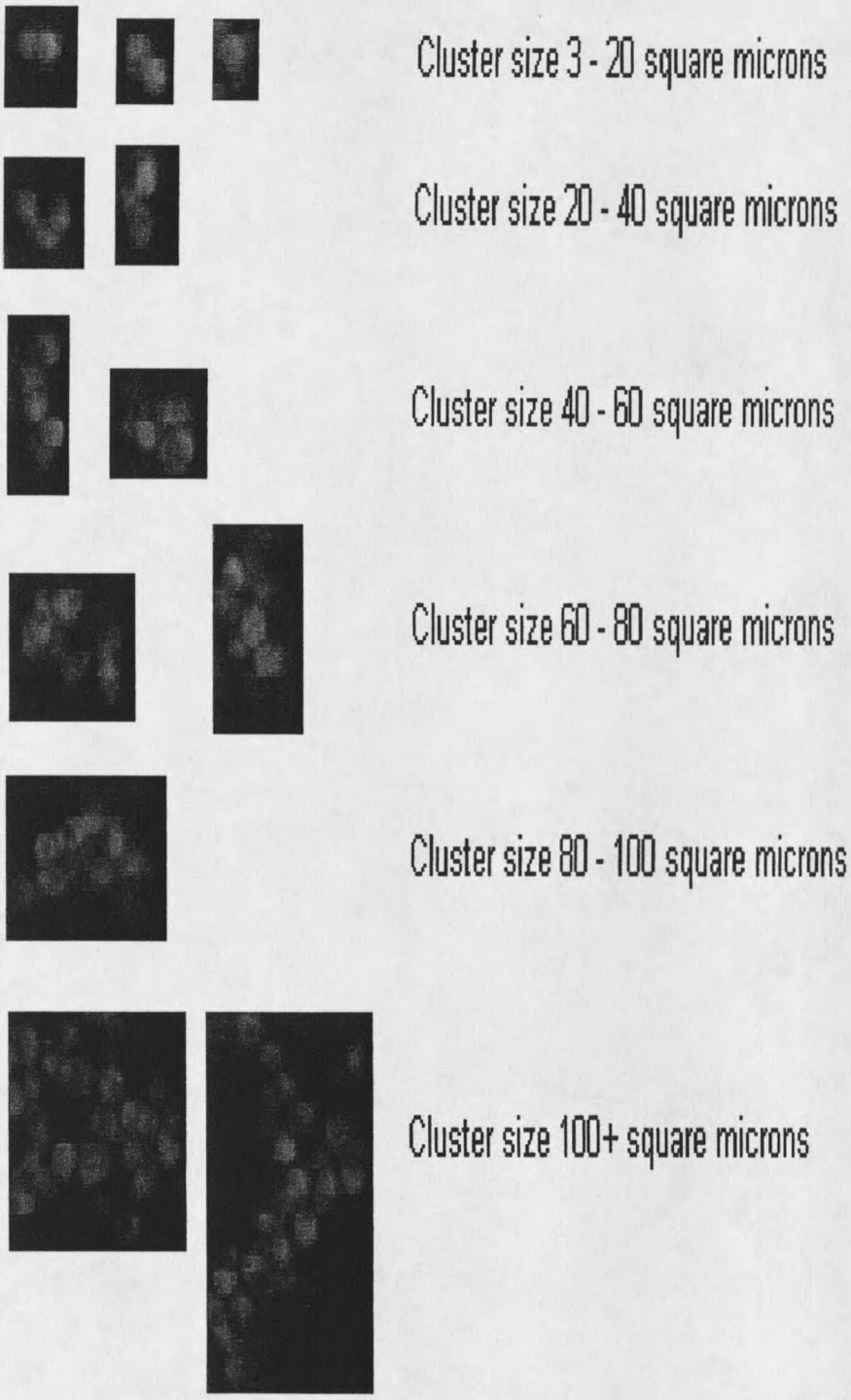
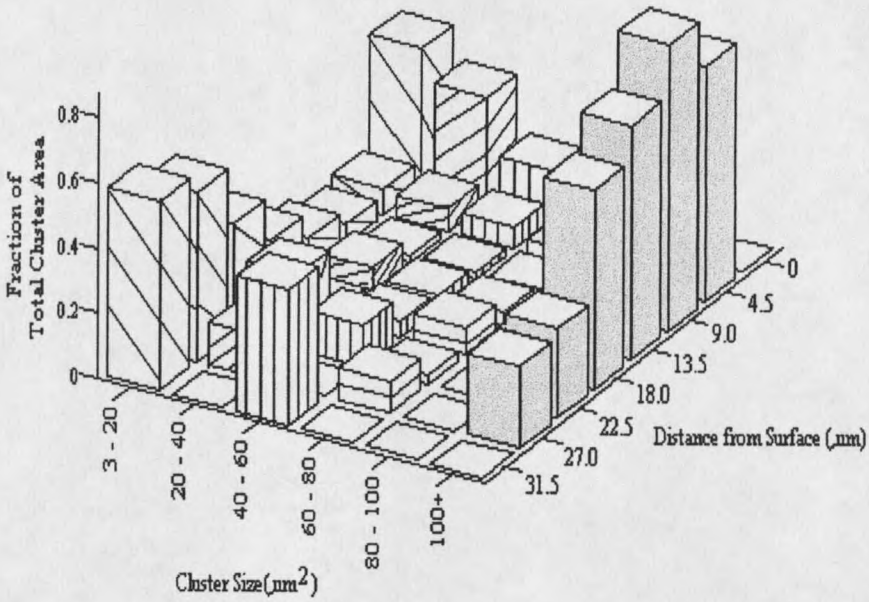


Figure 18. Examples of each cluster size used in quantifying the biofilm that formed on treated and untreated polystyrene.

A



B

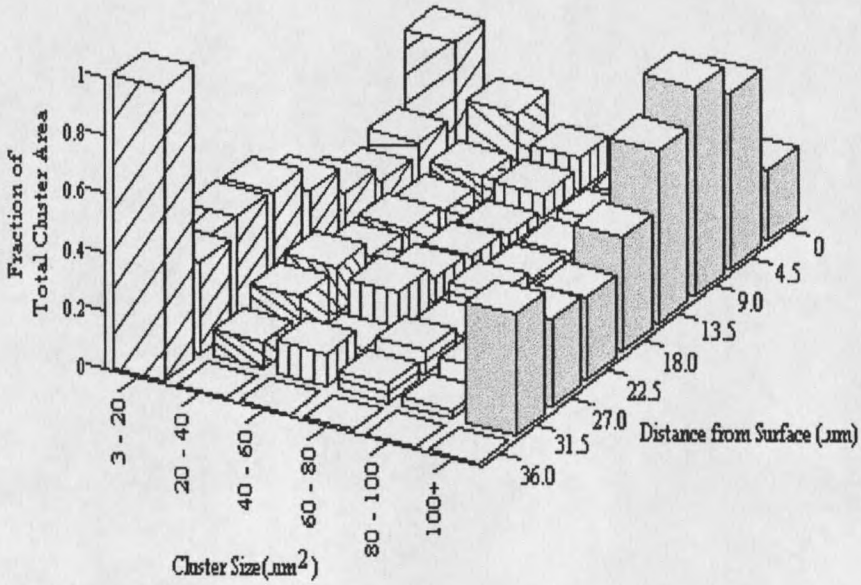


Figure 19. Cluster group fraction of the total cluster area in each layer of the biofilm formed on pristine polystyrene. (A) One region of the biofilm. (B) Average for eight regions of the biofilm.

The CSLM images for formation of *C. albicans* biofilm on Pluronic F127 conditioned polystyrene after 48 hours of growth at ~37°C are displayed in Figure 20. The CSLM images are of four layers of the biofilm starting at the first visible cell layer on the surface and projecting towards the bulk fluid at ~4 μm increments. As indicated earlier, as we scan through the biofilm, some cells appear clearly at one level and faintly at levels above and below. Thus, the biofilm shown here may actually be thinner than 12μm. The results for biofilm formation on Pluronic F127 conditioned PS are presented in Figure 21, indicating the mean percent surface coverage as a function of distance above the surface layer of cells. The findings are from an average of seven regions of the biofilm for each of four experiments. Again, the bars signify the heterogeneous character of the biofilm. As with the pristine PS, the greatest percent surface coverage arises at approximately 5μm above the first cell layer. However, in this instance, the average maximum percent surface coverage is 0.5% in comparison to 21% observed on the untreated PS (Figure 17).

The various cluster groups within each layer of the biofilm were characterized into the same cluster sizes as previously described for the untreated surface. The bar graphs displayed in Figure 22 indicate the cluster group fraction of the total cluster area in each layer of the biofilm. The graph in the top section of the figure is for a single region of the biofilm for a single experiment. The graph in the lower section of the figure contains the mean values for an average of seven regions for each of four experiments. These graphs reveal that the smaller cluster groups predominantly occupy a greater fraction of the total cluster area throughout the layers of the biofilm. The appearance of the larger cluster

groups has decreased dramatically in comparison to that associated with the biofilms that formed on the untreated PS (Figure 19).

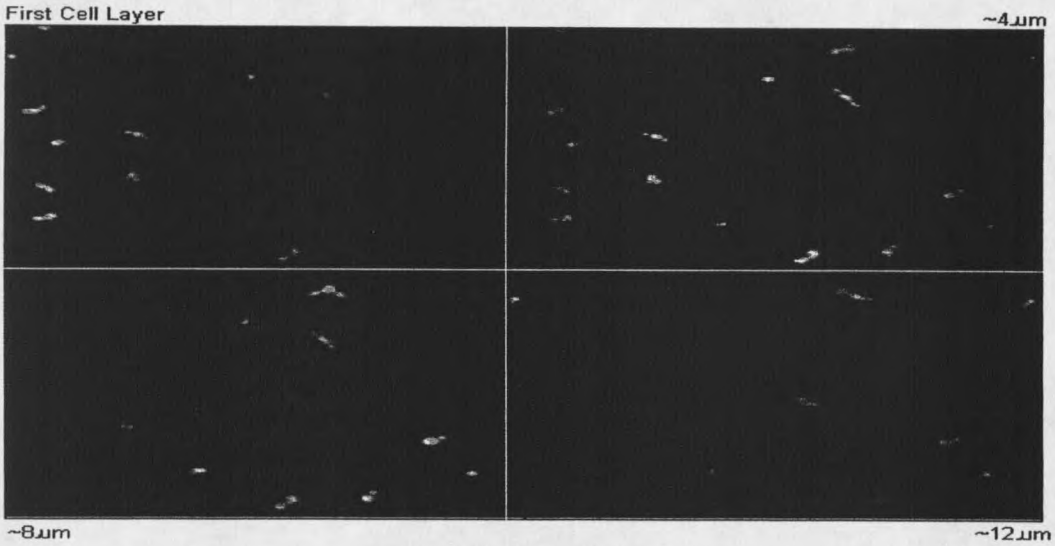


Figure 20. Biofilm formation on Pluronic F127 conditioned polystyrene with the first cell layer originating at the surface and consecutive layers within the biofilm given in ~ 4 μm increments.

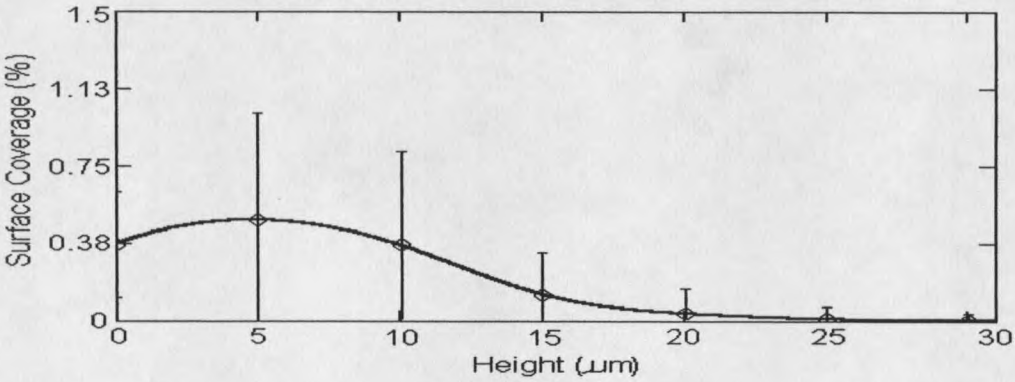


Figure 21. Mean percent surface coverage versus distance from the first cell layer for four experiments with an average of seven regions of the biofilm formed on Pluronic F127 conditioned polystyrene per experiment. Open circles, mean data points; solid curved line, cubic spline interpolation; solid vertical lines, variation in the surface coverage at a given height beyond the first cell layer.

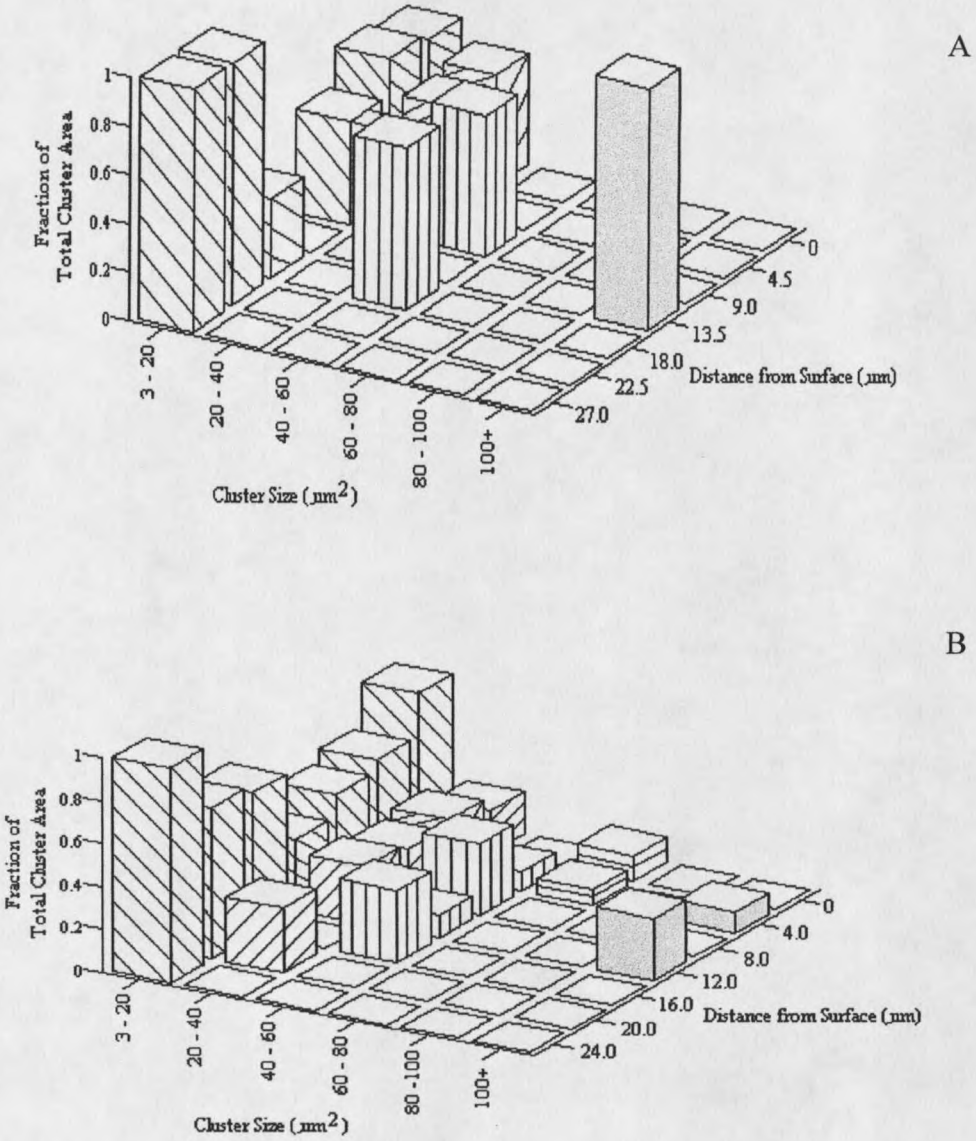
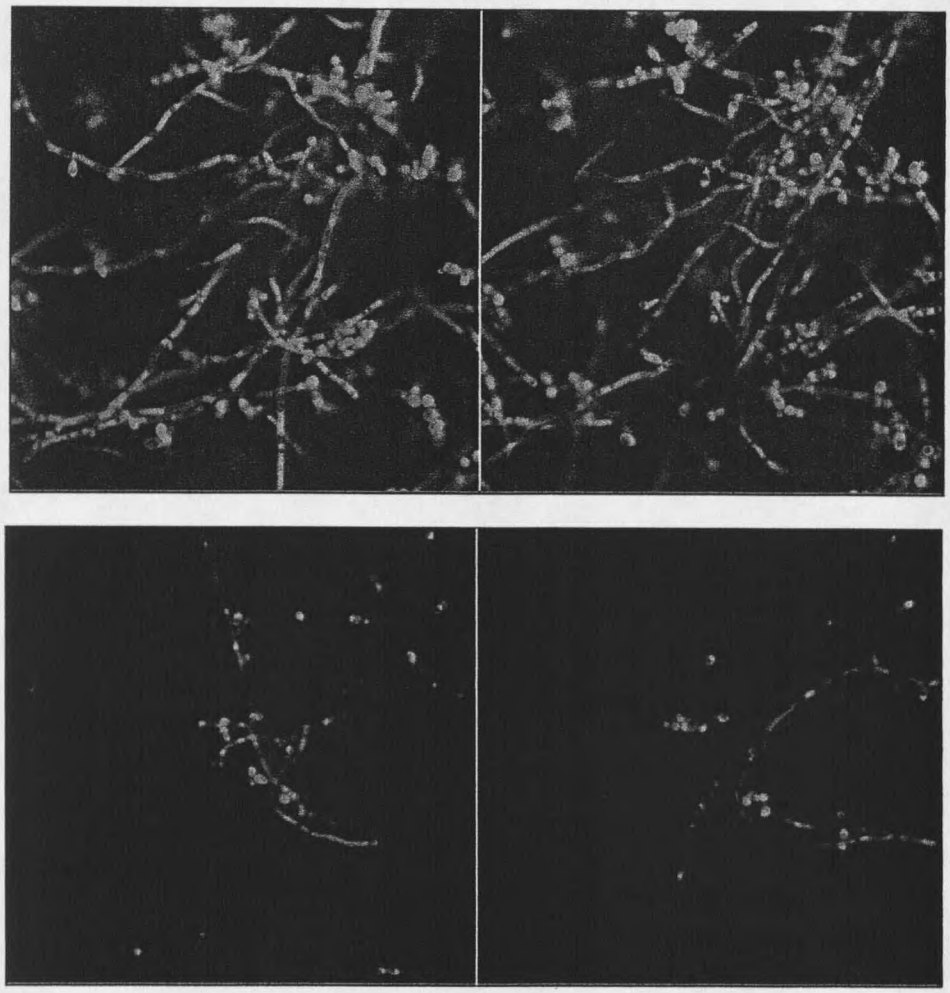


Figure 22. Cluster group fraction of total cluster area in each layer of the biofilm formed on Pluronic F127 conditioned polystyrene. (A) One region of the biofilm. (B) Average for seven regions of the biofilm.

The formation of hyphal elements within the biofilm was frequently observed on native PS. However, upon treatment with Pluronic F127 the expression of hyphal elements was scarce. The CSLM images displayed in Figure 23 reveal the typical presence of

hyphal elements within a biofilm formed on native PS (A) and the rare appearance of hyphal elements within a biofilm formed on Pluronic F127 conditioned PS (B).



A

B

Figure 23. Hyphal formation on pristine polystyrene (A) and on Pluronic F127 conditioned polystyrene (B) after 48 hours of biofilm growth.

Conclusions

Pluronic F127 reduced the adhesion of *Candida albicans* to polystyrene by more than 99% over untreated polystyrene. Pluronic F127 diminished the mean surface

coverage from 21% to 0.5% at ~5μm from the first visible cell layer on the surface. Again, we must consider that the maximum percent surface coverage may actually be at the surface and that anything observed below this point is due to an artifact of the surface. Pluronic F127 curtailed the formation of larger cluster areas with the smallest cluster group representing the greatest fraction of the total cluster area throughout the biofilm. Pluronic F127 also minimized the presence of hyphal elements. Thus, based on these studies, it appears that the initial adhesion events are reflected in the consequent formation of the biofilm. That is, the effect of the surface chemistry on the initial adhesion events persists during the development of the biofilm - limited adhesion results in limited biofilm growth.

ADHESION AND BIOFILM STRUCTURE OF TWO STRAINS OF CANDIDA ALBICANS OF DIFFERENT SEROTYPES ON SURFACES OF DIFFERING HYDROPHOBICITY

Introduction

Candida is a part of the normal human flora located on gastrointestinal and genitourinary tracts and mucosa.(36,43) Candidiasis is an opportunistic infection caused by species of *Candida* with *Candida albicans* being the major etiologic agent. The growth of *Candida* is normally kept in check by the body's immune defenses and normal flora bacteria, however, overgrowth can occur in immunocompromised individuals, mechanical trauma victims, and as a result of iatrogenic factors.(8,43)

Hyphal formation, adhesion properties, and the dynamic cell surface are proposed virulence factors of *C. albicans* which are related to the study at hand. *C. albicans* can take on one of three morphological states: blastoconidia, hyphae, or pseudohyphae. Environmental conditions such as pH, incubation temperature, inoculum size, and composition of the growth medium influence the form of the yeast.(8,43,49,81)

The transition from the yeast to the hyphal form is accompanied by chemical and structural changes in the cell wall components.(86) Upon contact with a surface, morphological changes may occur in response to modifications in gene regulation.(32) A shift from the yeast to hyphal form is accompanied by an increase in adhesion and cell surface hydrophobicity (CSH). The increase in CSH is the result of changes in the outer fibrillar layer mannoproteins which are thought to be the primary adhesins.(19,24,38)

The growth form and environmental conditions affect the hydrophobicity/ hydrophilicity of *C. albicans*. Both excessive and limited carbohydrate levels in the growth medium result in increased CSH. Yeast grown within a liquid display decreased CSH over those grown on solid or liquid cultures.(32,37) Stationary phase yeast cells grown at 28°C are generally more hydrophobic than cells grown at 37°C, although temperature alone does not dictate CSH. Actively growing yeast cells display modest levels of CSH, pseudohyphae display variable amounts of CSH, and hyphae are highly hydrophobic.(19,24,36,37)

C. albicans is capable of binding to a variety of biomedical implants including contact lenses, prosthetic devices, pacemakers, artificial joints, and catheters. Following adhesion to a surface, yeast can develop microcolonies sheathed within a polymeric matrix, forming a biofilm which acts as a protective barrier. A biofilm serves as a setting for yeast to proliferate and release cells into the surrounding fluid and tissues, facilitating acute disseminated infections. Although slow to develop, the infections are relentless as the microorganisms are unreachable by host defenses and antimicrobial agents. Thus, removal of the implant is typically required to suppress the infection.(32,92)

Approximately 10 million infections that occur in the United States each year are precipitated by biofilm formation on permanent medical implants. Upon exposure to body fluids, surfaces become coated with a conditioning film consisting of host proteins and other substances. Host proteins can serve as binding sites for bacteria and yeast.(19,68) The formation of a conditioning film is believed to be an initiating stage for infectious biofilm development.(68)

Highly hydrophilic surfaces which are non-thrombogenic can be developed by adsorption of hydrophilic polymers onto a polymer surface. The adsorption of amphiphilic poly(ethylene oxide) (PEO) block copolymers onto glass, polystyrene, and polyethylene limited adhesion of albumin, fibrinogen, and blood platelets as compared to the unmodified surfaces.(21) Poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) triblock copolymers are surfactants which are commercially available as Pluronics (produced by BASF) or Poloxamers (produced by ICI).(1)

The recalcitrance of bacterial biofilms is thought to be the result of a multicellular endeavor.(59,82) Both a transport based mechanism and a physiological based mechanism of resistance have been proposed for bacterial biofilms.(83) Communication between cells allows for a group virulence response. *P. aeruginosa* biofilms generate signal transduction proteins which relate information about the environment to chromosomal elements. Under sufficient population sizes, *P. aeruginosa* signaling molecules, homoserine lactones, attain concentration levels necessary for gene activation. This type of gene regulation is termed "quorum sensing and response".(10)

Our initial work involved characterizing biofilms of *C. albicans* strain 1 that formed on two surfaces: native polystyrene (PS) and Pluronic F127 modified PS. The work reported here examined the formation of *C. albicans* strain 662 biofilms to these same surfaces. *C. albicans* strain 1 was initially reported as being of serotype A. (17,27,28,45,70) Later reports indicated that strain 1 is of serotype B as determined by IATRON *Candida* Check.(17,28) Using the IATRON *Candida* Check method, we ascertained that *C. albicans* strain 662 was of serotype A. Therefore, assuming the test is

capable of distinguishing between two different serotypes, our initial and later studies were conducted on a strain of *C. albicans* of serotype B and of serotype A, respectively.

In the work presented here, we were interested in whether a surface of increased hydrophilicity generated by adsorbing Pluronic F127 onto PS could limit both the interaction of *C. albicans* strain 662 with the surface and the presence of hyphal phenotypes. Since the emergence of candidiasis is attributed in large part to the formation of a biofilm (66), we were also interested in learning whether the initial adhesion events can lead to diminished growth and thwart the appearance of a mature differentiated biofilm. The recalcitrance of bacterial biofilms is described as being a multicellular endeavor (59,82), studies indicate that this is also true for *Candida* biofilms (3,5,33). Biofilms in the early stages of development (24hrs) can usually be eradicated by antimicrobial agents.(68) Therefore, a surface which limits adhesion and obstructs the appearance of multicellular structures should increase the sensitivity of adhering yeast cells to antimicrobial agents.

Our previous findings revealed that adsorption of Pluronic F127 to PS reduced the adhesion of *C. albicans* strain 1 by more than 99% over the unmodified PS surface. The mean surface coverage was decreased from ~20% on unmodified PS surfaces to ~0.5% on Pluronic F127 modified surfaces. The presence of hyphal elements was also minimized by the adsorption of Pluronic F127.(88) Here we found similar results for *C. albicans* strain 662.

Materials and Methods

Cells and Culture Media

C. albicans strain 662 (CA662) and strain 1 (CA1) were maintained in a sub-zero freezer. A new subculture was generated every month as described previously for CA1.(88) Serotyping and confirmation of the identity of CA662 was carried out using four tests that are commercially available. These tests included the api 20 C AUX Yeast Identification System, IATRON *Candida* Check, CHROMagar *Candida,* and *C. albicans* screen. All the tests indicated CA662 as being *C. albicans* with the exception of the last test which was inconclusive. The first two tests listed also identified CA662 as being of serotype A. CA1 was originally given in the literature as being a serotype A strain as determined by Hasenclever's original antiserum.(17,27,28,45,70) However, more recent literature reports indicate that the IATRON Candida Check characterizes CA1 as being a serotype B strain.(17,28)

Stationary phase cultures of CA662 at 35°C were collected and rinsed with PBS to generate a solution with a concentration of ~ 10^7 cells per milliliter for adhesion and biofilm studies in a manner described previously for CA1.(88) The relative hydrophobicity/hydrophilicity of the cells used in adhesion and biofilm investigations was determined using the microsphere hydrophobicity assay (HMA) described previously by Hazen et al..(33,34,39) The mean percentage of hydrophobic cells in the CA662 and CA1 solutions were 5.735 and 3.068 with a standard deviation of 6.361 and 2.962, respectively. Glee et al.(2001) states that yeast cultures grown at 37°C are not completely hydrophilic,

but generally have cell surface hydrophobicity (CSH) levels of 8 percent or less.(25)

Adhesion and Biofilm Growth Experiments

The adhesion and biofilm growth studies were performed in a Kynar flow cell fitted with silanized glass coverslips spin-coated with polystyrene (PS). Some of the PS spin-coated coverslips were exposed to a 4% Pluronic F127 solution as described previously.(88) Confirmation of the presence of the desired chemical species was achieved through X-ray photoelectron spectroscopy (XPS) also described previously.(88) The adhesion and biofilm growth experiments were conducted for 60 minutes and 48 hours, respectively, in the manner described for CA1 (88) with the following exceptions. The flow rate was increased to 3.4 milliliters per minute in the adhesion assays to compensate for the design changes associated with the Kynar flow cell. The adhesion process was observed at a central location for an hour using phase contrast microscopy in the transmitted light mode and recorded using Imaging Program for Windows®. Following the 60 minute observation period, six other regions were viewed and recorded. Thus, through all the trials, the seven regions examined remained the same (Figure 24), but the location within these regions was randomly selected.

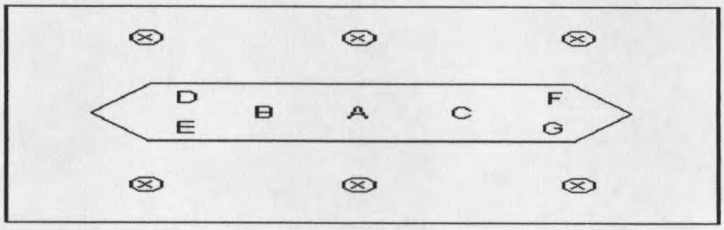


Figure 24. Seven regions examined during the adhesion and biofilm studies The direction of flow was from left to right.

Pluronic F127 Toxicity Analysis

Toxicity tests were conducted by generating growth curves for CA1 and CA662. The growth curves were produced for suspension cultures at 37°C in reduced GYEP medium (0.05% glucose, 0.03% yeast extract, and 0.1% bacto-peptone) with and without 0.04% (v/v) Pluronic F127. Growth curves were also generated for suspension cultures at 37°C in GYEP medium (5% glucose, 0.3% yeast extract, 1% bacto-peptone) with 0.0004% or 0.04% (v/v) Pluronic F127 or in the absence of Pluronic F127. Due to the difference in the effect of chemicals on planktonic cells versus their sessile counterparts, the impact of Pluronic F127 adsorbed to the surface on cell viability was examined through the use of propidium iodide (PI). Cells on or near the surface following the initial adhesion event (60 minutes) and extended biofilm growth (48 hours) were stained with 25 μg/ml of PI.

The reliability of PI in identifying non-viable cells of *C. albicans* was tested by staining "live" cells and "dead" cells. The "live" cells were obtained from a 35°C stationary phase suspension culture. The "dead" cells were obtained by autoclaving the "live" cells for 15 to 20 minutes. Both groups of cells were stained with 25 μg/ml of PI and loaded into a hemacytometer. The cells in one of the four corner blocks were counted and the percentage of compromised cells was established. Compromised cells fluoresced red under UV light as observed using an Olympus microscope fitted with a 20 x objective (Olympus DplanApo 20 UV) under x15 magnification. In staining heat killed cells with PI, all of the cells counted fluoresced red for both strains, whereas none of the CA662 "live" cells fluoresced red upon exposure to PI and only 2% of CA1 cells fluoresced red.

Based on these results, PI appeared to be a reliable measure of whether cells within the biofilm were compromised.

Biofilm Imaging

Stained biofilms were inspected with a Leica DMRXE confocal scanning laser microscope fitted with an argon ion laser (488 nm) reported previously.(88) Biofilms stained with acridine orange (excitation wavelength and emission wavelength of 550 nm and 526 nm, respectively, for acridine orange (AO) bound to DNA; excitation wavelength and emission wavelength of 460 nm and 650nm, respectively, for AO bound to RNA) provided a structural image of the biofilm. Biofilms stained with PI (excitation wavelength and emission wavelength of 535 nm and 637 nm, respectively, for PI bound to nucleic acids) were used for viability analysis as aforementioned. Images were collected as indicated for CA1.(88)

Statistical Analysis

In order to assess whether there was a significant difference in the number of cells adhering to Pluronic F127 treated PS (PL-PS) in comparison to PS at 60 minutes at location A, a randomization test was performed using Matlab®. A relationship between location on the surface and the number of cells per unit area on PS and PL-PS was established through regression analysis using Minitab®. The maximum percent surface coverage on PS versus PL-PS at each of the seven locations on the surface was analyzed by counting using combinations.

In order to determine whether there was an effect due to strain or Pluronic F127

on the log number of cells per unit volume in a suspension culture grown in reduced medium, the data was analyzed by balanced ANOVA using Minitab®.

In the case of adhesion to the PL-PS surface, the regression equation was expressed in terms of the log of cells per unit area in order to deal with problems of heteroschedasticity and non-normality. By performing the log transformation on the response, the problems associated with the assumption of normality and constant variance were abrogated (Anderson-Darling normality test, $p = 0.485$). The log transformation on the response for adhesion on the PS surface was given for comparison, but was not required to deal with constant variance or normality assumption problems (Anderson-Darling normality test, $p = 0.351$ without the transformation). However, transforming the response did not introduce any negative effects associated with the constant variance and normality assumptions.

Results and Discussion

Binding of CA662 to Native Polystyrene and Pluronic F127 Modified Polystyrene

We previously described the adhesion of CA1 to native PS and Pluronic F127 modified PS (PL-PS).(88) In the present study, we examined the ability of CA662 to bind to these same surfaces. The adhesion was observed by phase contrast microscopy during 60 minutes of exposure to a continuous flow of yeast cells suspended in PBS. As shown in Figure 25, CA662 and CA1 adhered readily to unmodified PS, but adhesion was limited on PL-PS.

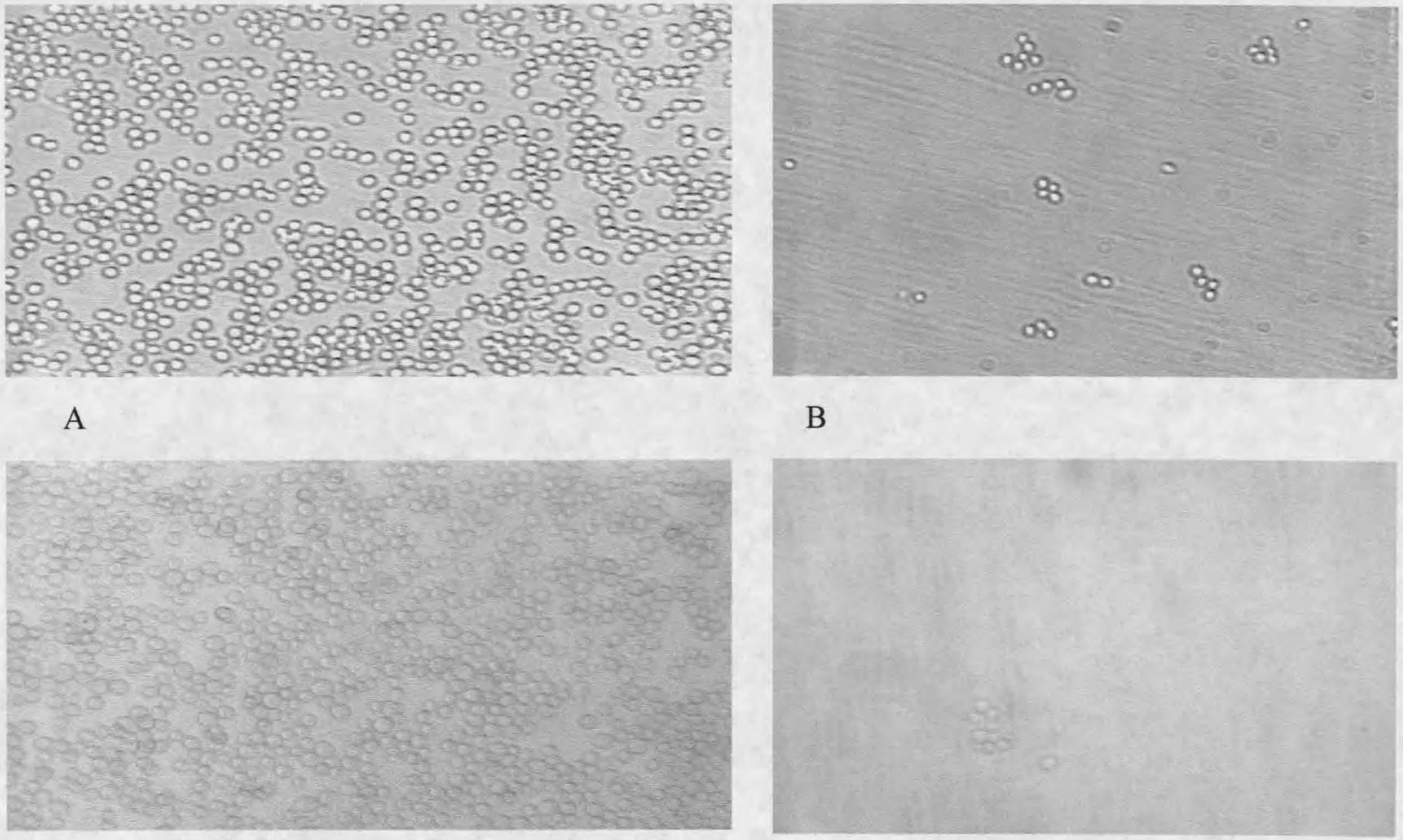


Figure 25. Adhesion of *C. albicans* to polystyrene (A - CA662, C - CA1) and Pluronic F127 modified polystyrene (B - CA662, D - CA1). Objective, 20x. Magnification, x15..

The studies were performed using stationary phase cultures grown at 37°C having a mean cell surface hydrophobicity (CSH) value of approximately 6% as determined by hydrophobic microsphere assay (HMA) described by Hazen et al.(34,35,40). Based on the HMA results, a cell solution of 1×10^7 cell/ml, a flow rate of 3.4 ml/min, and a surface area of 52.46 cm^2, the mean number of hydrophobic cells exposed to a unit area over a 60-minute period is 22,000 cells per square millimeter. This value is more than the number of cells observed adhering to a unit area of PS after 60 minutes. Thus, the number of hydrophobic cells is sufficient to explain the number of cells adhering to PS after one hour. Therefore, the yeast cells adhering from the hydrophilic cultures might be the hydrophobic cells existing in the culture.

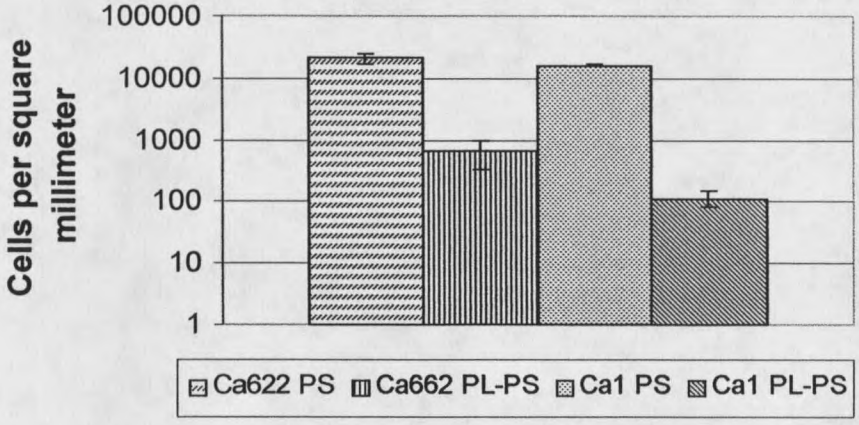


Figure 26. Adhesion of CA662 and CA1 to polystyrene (PS) and Pluronic F127 conditioned polystyrene (PL-PS) as determined by phase contrast microscopy. The bars represent mean ± standard deviation.

As shown in Figure 26, CA662 adhered strongly to PS (20,817 ± 3428), but binding to PL-PS was limited (657 ± 318). The adhesion results for CA662 on both PS

and PL-PS were slightly greater than those observed for CA1 (16,241 ± 540 for CA1 on PS; 116 ± 31 for CA1 on PL-PS). This difference in adhesion rates may in part be due to the use of two different flow cells. The shear rate was slightly lower for the CA662 binding studies than for the CA1 studies (i.e., 14 sec^{-1} versus 16 sec^{-1}). This variation in shear rate was a consequence of limiting factors in the experimental conditions. The results from the randomization test performed using Matlab® indicate that the adsorption of Pluronic F127 onto PS significantly reduced the adhesion in comparison to the unmodified PS surface. The probability of this difference occurring by chance is less than 0.05. A randomization test performed using Matlab® revealed that strains with different serotypes did not cause a significant difference ($p = 0.41$) in the mean number of cells adhering to PS. San et al. (1996) reported similar results for strains of *C. albicans* from two serotypes in binding to PS. They also reported that yeast phase cells grown at 25°C prevented adhesion to PS.(75) The adhesion studies described here were carried out using yeast phase cells grown at 37°C.

Regression analysis was used to establish a relationship between distance from the inlet and the number of cells adhering in a unit area. For both unmodified PS and PL-PS, the number of cells adhering per unit area increased linearly with increasing distance from the inlet (Figure 27). The regression equation expressing cells per unit area as a function of distance from the inlet is given by the following equations. The area is expressed in units of square millimeters and the distance is given in units of centimeters. The regression equation for binding to unmodified PS is given by

$$\text{Log(Cells Per Unit Area)} = 3.80 + 0.16 \text{ Distance}$$

The regression equation for binding to Pluronic F127 modified PS is given by

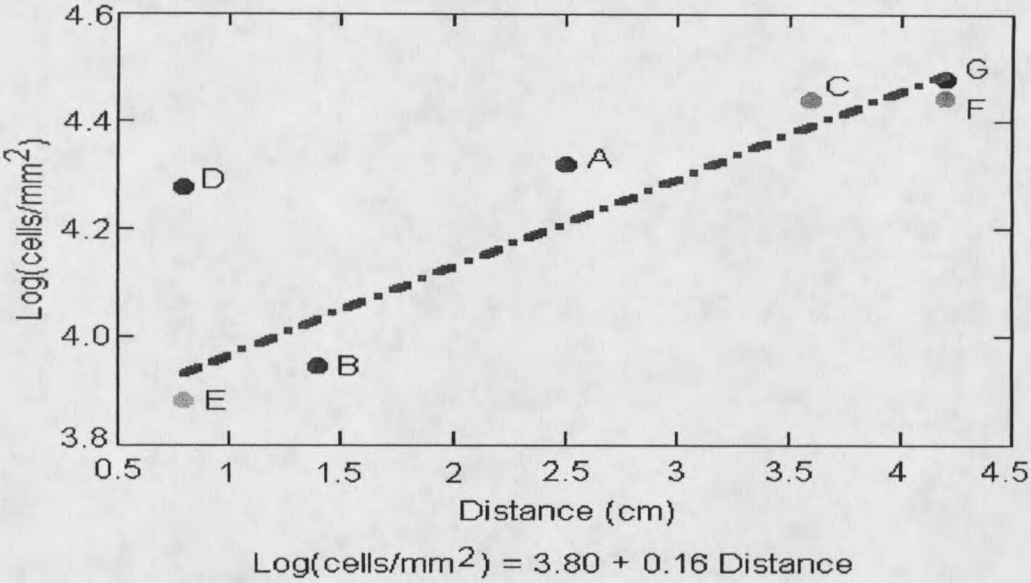
$$\text{Log(Cells Per Unit Area)} = 2.01 + 0.25 \text{ Distance.}$$

For adhesion of CA662 and CA1 to PS, distance is a significant predictor of the log number of cells adhering per unit area and explains approximately 74% and 64% of the variability in the log number of cell adhering per unit area, respectively.

The increase in adhesion with increasing distance from the inlet may be the result of sudden expansion of the cross section upon entering the flow cell and sudden contraction of the cross section upon leaving the flow cell. Sudden expansion upon entering the flow cell is due to the entrance of the flow cell being a circular region with a diameter of 0.16 cm that opens up into a rectangular region that is 1.0 cm by 0.16 cm. This sudden expansion of the cross section results in the presence of vortex motion in the entrance region between the expanding jet and the flow cell wall. It is thought that this vortex motion may hinder the binding of yeast cells in the entrance region of the flow cell. At the exit region of the flow cell, the sudden contraction of the cross section is due to the fluid leaving a rectangular region of 1.0 cm by 0.16 cm through a circular region with a diameter of 0.16 cm. This sudden contraction of the cross section is thought to generate stagnant regions within the exit region of the flow cell that may allow for the increased adhesion of yeast cells. The discrepancy in the adhesion levels observed between locations D and E may be a consequence of the machining of the flow cell.

Adhesion to the top surface was observed in the Kynar flow cell due to difficulties in focusing on the lower surface. Both the top and bottom surfaces were made of either PS or Pluronic F127 modified PS. Qi et al. (2002) indicated that when a particles density

A



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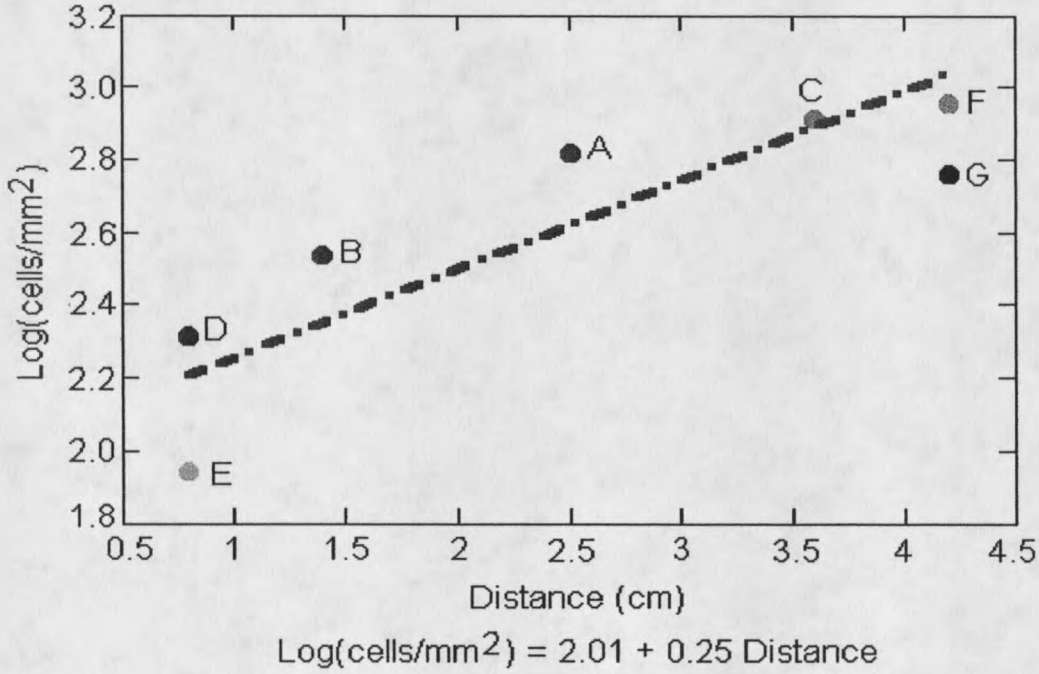

Figure 27. The log number of adhering cells per square millimeter as a function of distance from the inlet for adhesion of *C. albicans* strain 662 to PS (A) and for adhesion to PL-PS (B). The letters (A-G) refer to the locations in Figure 24.

is slightly greater than that of the fluid, the particle will migrate to regions close to the wall as a result of inertial lift forces associated with the curvature of the velocity profile.(65) Others have described these forces as playing a role in the lateral migration of particles in fluid-flow.(16) The density of a yeast cell is 1.001 g/cm^3, and the density of water at 25°C is 0.997 g/cm^3. Thus, the inertial lift forces may allow for the propinquity to the surface and this along with particle diffusion may explain the ability of yeast cells to attach to the top surface.

CA662 Biofilm Formation on Native Polystyrene
and Pluronic F127 Modified Polystyrene

The biofilm formation of CA1 on native PS and PL-PS was characterized previously.(88) Here we describe the formation of CA662 biofilms on the same surfaces. After allowing the biofilm to develop over a 48-hour period, it was stained with acridine orange and then examined using confocal scanning laser microscopy (CSLM). The biofilms were formed within a flow cell to simulate an *in vivo* environment and viewed using CSLM in order to preserve the undisturbed biofilm structure. Figure 28, on the following page, shows a single layer of CA662 and CA1 biofilms that developed on PS and the entire biofilm of these strains on PL-PS. The cells frequently appear individually on the PL-PS surface, although small groups of cells can be found as shown in image D.

Stationary phase yeast cultures grown at 37°C, as described for the adhesion studies, were used to seed the flow cell. Biofilms of *C. albicans* are structurally heterogeneous in nature as has been described by others.(3,5,32) The maximum percent surface coverage can vary with location across the surface and with respect to the distance

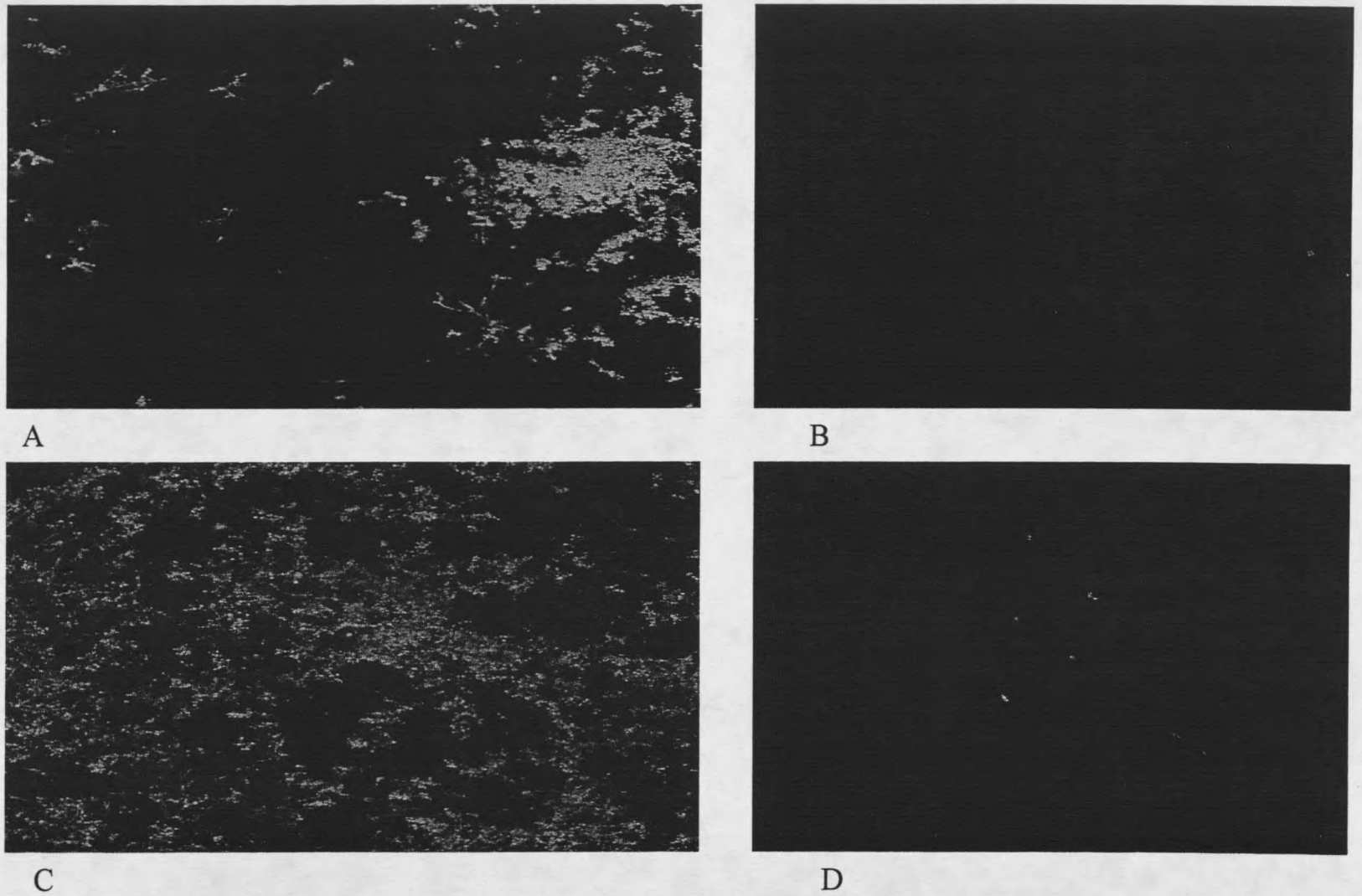

Figure 28. Biofilms of *C. albicans* formed on polystyrene (A – CA662, C – CA1) and Pluronic F127 modified polystyrene (B – CA662, D – CA1). Objective, 20x. Magnification, x10.

above the surface as shown in Figure 29 for PS and in Figure 30 for PL-PS. For biofilms that form on PS, the maximum percent surface coverage occurs at approximately 12 μm above the surface layer of cells. However, it may be that the biofilm actually begins at a point closer to the surface. The diameter of a yeast cell is approximately 5 μm and thus, a distance of 12 μm represents two cell layers. The reason we may be seeing anything below the actual surface layer of cells may be due to an artifact of the surface. That is, the surface may not be completely level. Also, as we scan through the biofilm, some cells appear clearly at one level and faintly at levels above and below. The distorted appearance of cells may be a consequence of passing the laser through air/solid/liquid interfaces. These factors may then influence the biofilm thickness obtained from the confocal imaging. Similarly, for biofilms that form on PL-PS, the maximum percent surface coverage occurs at approximately 8 μm above the surface layer of cells. It may be that the biofilm actually begins at this point for the reasons discussed above.

The average maximum percent surface coverage was significantly reduced ($p < 0.05$) on PL-PS surfaces in contrast to biofilms formed on PS, regardless of the location on the surface as shown in Figure 31. That is, the probability of finding a difference in the average maximum percent surface coverage between PS and PL-PS as extreme or more extreme than that observed purely by chance is less than 0.05.

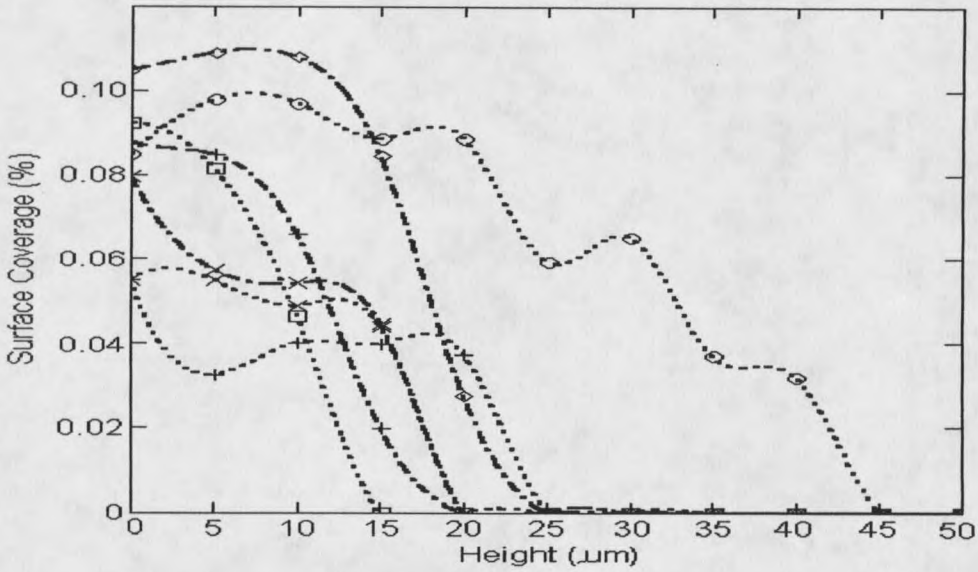

Figure 29. Percent surface coverage versus height from the first cell layer for seven different regions on the polystyrene surface averaged over seven experiments. Symbols, data points; curved lines, cubic spline interpolation.

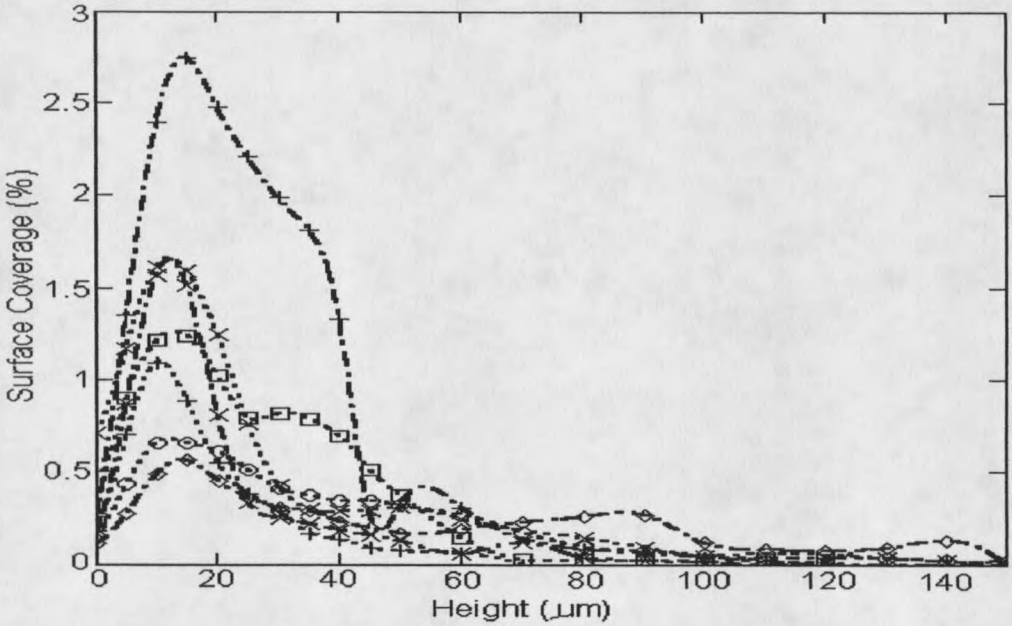

Figure 30. Percent surface coverage versus height from the first cell layer for seven different regions on the Pluronic F127 treated polystyrene surface averaged over four experiments. Symbols, data points; curved lines, cubic spline interpolation.

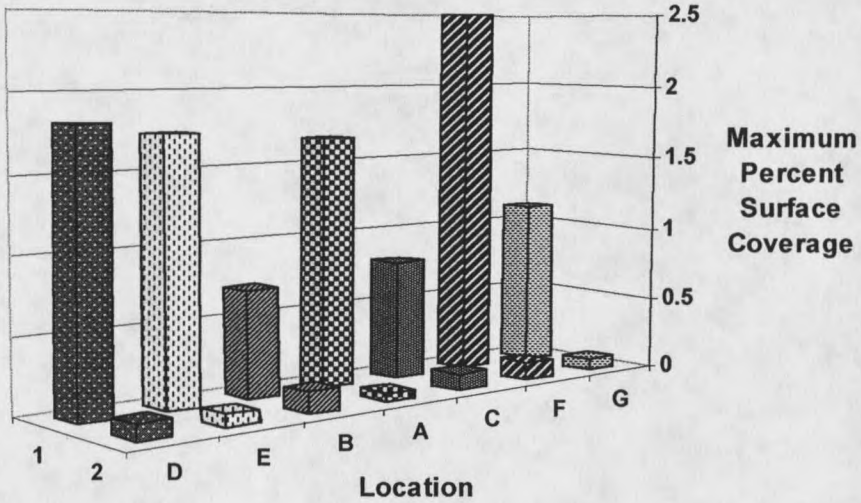

Figure 31. Average maximum percent surface coverage as a function of location (A - G) for biofilms formed on polystyrene (1) and Pluronic F127 modified polystyrene (2).

Growth Curves for CA662 and CA1 in
Reduced Concentration Medium

The average maximum percent surface coverage for CA662 was less than that observed for CA1 on PS and PL-PS (Figure 32). As shown in Figure 29, the maximum percent surface coverage can vary with location, a characteristic of the heterogeneous nature of biofilms. CA1 biofilms that form on PS surfaces can display a maximum percent surface coverage of seven percent, whereas CA662 can develop biofilms that attain a maximum percent surface coverage of eleven percent.

Similarly, the thickness varies throughout the biofilm. Thickness of the biofilm is defined as the distance from the surface layer of cells to the last layer of cells observed in the bulk fluid. On average CA662 biofilms were thicker than CA1 biofilm formed on PS (Figure 33). Biofilms ranged in thickness from one to thirty-three cell layers for CA662

on PS with an average of eleven cell layers and from one to twenty-one cell layers for CA1 on PS with an average of seven cell layers. In contrast, less variability between strains was observed for biofilms that were generated on PL-PS, with thicknesses ranging from a single layer to eight cell layers with an average of three cell layers for CA662 and from a single layer to seven cell layers with an average of three cell layers for CA1. The variability in biofilm formation on PS between the two strains could be the result of an enhanced growth rate and a greater detachment rate for CA662.

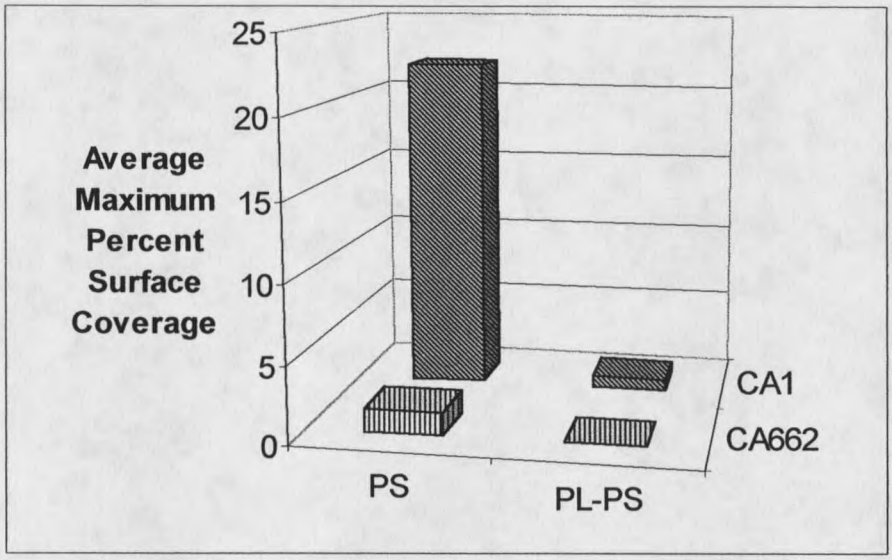

Figure 32. Average maximum percent surface coverage for CA1 and CA662 biofilms formed on polystyrene and Pluronic F127 modified polystyrene. The average maximum percent surface coverage for CA662 was obtained by averaging over the seven regions.

The difference in biofilm thickness between the two strains was found to be statistically significant ($p < 0.03$). The greater average thickness observed in CA662 biofilms may be due to a greater growth rate of CA662 in comparison to CA1. To

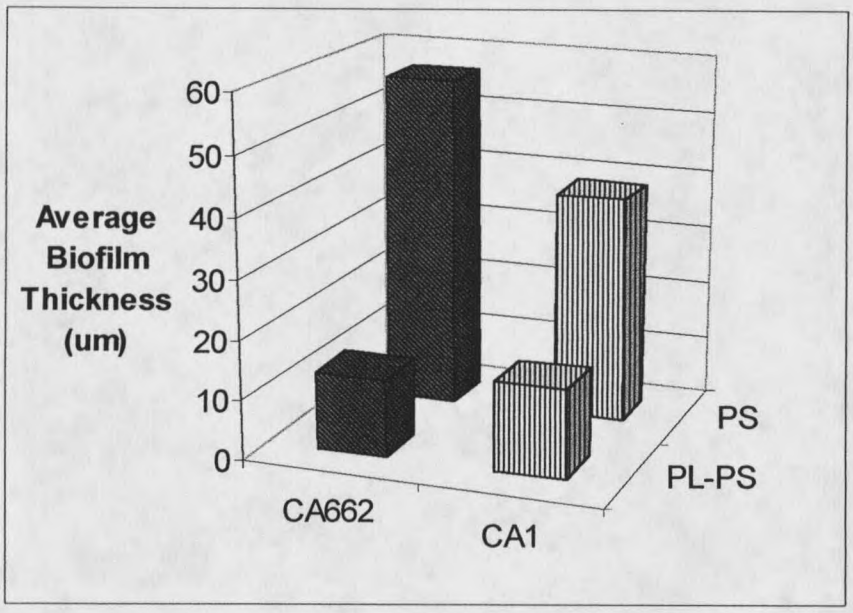

Figure 33. Average biofilm thickness (μm) for CA662 and CA1 formed on polystyrene and Pluronic F127 modified polystyrene.

ascertain whether the dissimilarity in the ability of these strains to develop biofilms is a consequence of differences in growth rates, growth profiles for the two strains were compared. The two strains were grown in suspension cultures containing reduced medium. The results of the statistical analysis (ANOVA) indicate that there is a significant interaction between strain and time ($p = 0.001$). Therefore, the significant interaction "masks" the significance of the main effects. In looking at the main effects plot (Figure 34) and the interaction plot (Figure 35), the log of cell concentration is greater for CA662 than for CA1 during the exponential phase of CA662. However, CA662 plateaus at a lower concentration than CA1. The main effects plot does not reveal a significant effect of strain on the log of cell concentration. The specific growth rate for CA1 and CA662 during exponential phase was determined to be 0.68 h^{-1} and 0.67 h^{-1}, respectively. Thus,

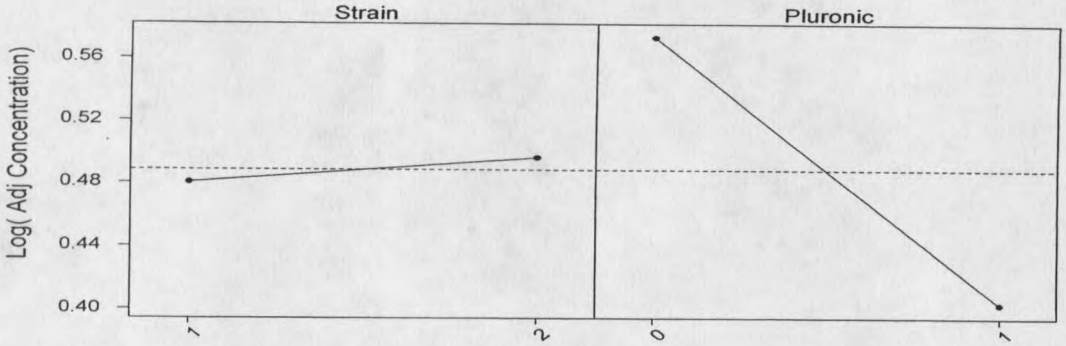

Figure 34. Main effects plot, data means for log(concentration), from the statistical analysis of the growth profiles for CA1 and CA662 in reduced medium with and without the addition of Pluronic F127 (0.04% (v/v)). Strain 1, CA1; strain 2, CA662. Pluronic 0, Pluronic F127 absent; Pluronic 1, Pluronic F127 present.

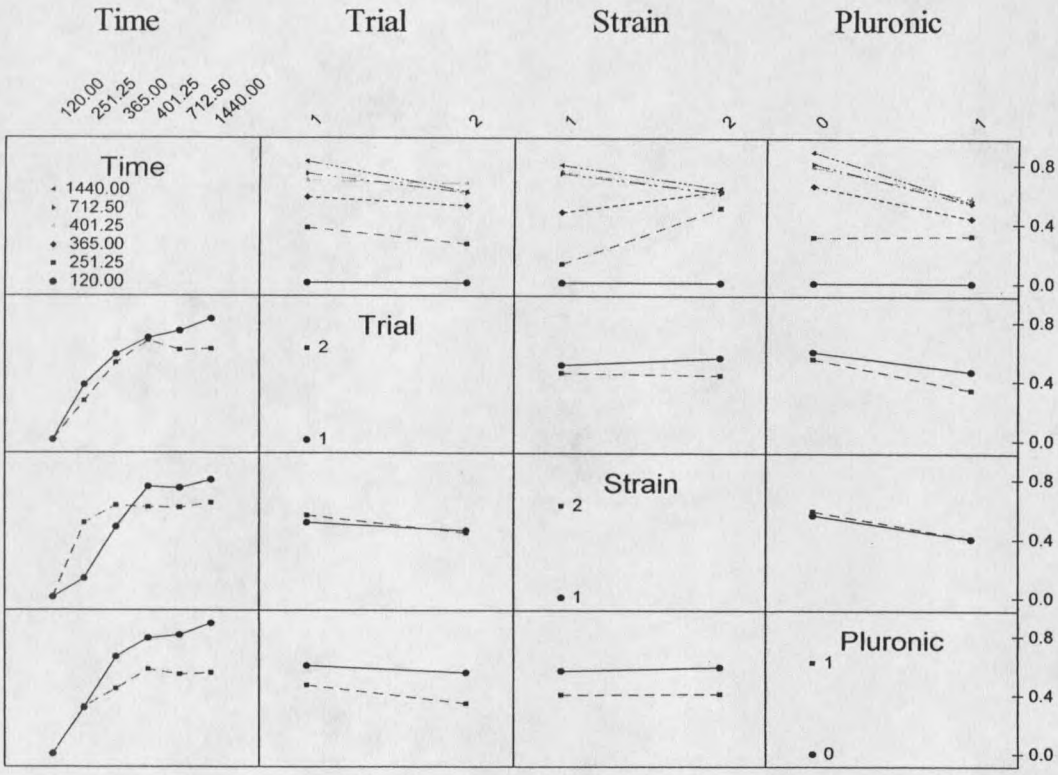

Figure 35. Interaction plot, data means for log(concentration), from the statistical analysis of the growth profiles for CA1 and CA662 in reduced medium with and without the addition of Pluronic F127 (0.04% (v/v)). Strain 1, CA1; strain 2, CA662. Pluronic 0, Pluronic F127 absent; Pluronic 1, Pluronic F127 present.

based on these findings, there does not appear to be a large enough difference in growth rate to explain the observed variation in the quantity of biofilm produced by these two strains. However, these growth rates were found using planktonic cultures and may not reflect the growth of *C. albicans* in a biofilm. Thus, it is unclear whether a difference in growth rate significantly affects the appearance of the biofilm. The rate of detachment appears to be the major contributing factor in the difference observed between the two biofilms.

Toxicity Analysis of Pluronic F127

In an attempt to determine whether Pluronic F127 is toxic to *C. albicans*, the surfactant (0.04% (v/v)) was added to the growth medium of a suspension culture as described in the materials and methods section of this dissertation. The growth profile for *C. albicans* grown in medium containing Pluronic F127 was compared to those generated for cells grown in the absence of the surfactant. The results from statistical analysis (ANOVA) indicate that Pluronic F127 (0.04% (v/v)) has a significant effect on the log concentration of cells ($p < 0.0001$), independent of strain. For both strains, the presence of Pluronic F127 (0.04% (v/v)) caused a significant reduction in log concentration of cells. The presence of Pluronic F127 (0.04% (v/v)) decreased the plateau level by approximately one-third and one-half for CA1 and CA662, respectively. Thus, in a twenty-four hour period Pluronic F127 (0.04% (v/v)) limited the growth of planktonic cultures of *C. albicans*. However, due to the differences in chemical sensitivity observed between sessile cells and their planktonic counterparts (5,33), the potential effect of Pluronic F127 on cells

within a biofilm, formed on a surface modified by adsorption of Pluronic F127, was investigated.

Observation of PI stained biofilms using CSLM revealed that CA1 biofilms formed on PS had regions that fluoresced red (i.e., were stained with PI and therefore presumably compromised) in most of the areas of the biofilm examined. However, the regions stained by PI were small in comparison to the total area of the biofilm. In contrast, regions of CA1 biofilms generated on PL-PS that were stained by PI constituted a greater portion of the total cellular area. However, less than half of the regions examined were stained. Those cellular components which fluoresced red were hyphal elements, but not all hyphal elements observed were stained. Similar findings were obtained for biofilms of CA662 formed on PS and PL-PS, except that the majority of the regions observed were not stained with PI. Also, the stained regions were a small proportion of the total biofilm, regardless of the culture surface. Again, for CA662 biofilms formed on PL-PS, the cellular components that fluoresced red were hyphal elements. On average less than one-quarter of the regions observed for biofilms of *C. albicans* that form on PL-PS were stained with PI. One should also consider the findings of others that Pluronic alters the permeability of cells.(52) Therefore, the cells that are stained may be stained as a result of increased cell permeability and not as a consequence of cell death. Based on these results, it is not evident that Pluronic F127 is toxic to the yeast cells on the surface.

Conclusions

Others have shown that Pluronic inhibits the adhesion of bacteria (4,64) and proteins (1,21,30) to polymer surfaces. Here we have shown that this is true for two strains of *C. albicans* of different serotypes. Pluronic F127 conditioned PS surfaces reduced the adhesion of CA662 (serotype A) and CA1 (serotype B) by ~ 97% and ~99%, respectively, in comparison to the unmodified PS surface. This limitation in the adhesion of *C. albicans* to the substratum resulted in the formation of "biofilms" after 48 hours that was devoid of thick, expansive microcolonies. The surface of the Pluronic F127 modified surface contained widely dispersed, thin, small groups of cells and the presence of a single cell in a region was frequently observed. Adsorption of Pluronic F127 on the PS surface significantly reduced the mean maximum percent surface coverage of CA662 and CA1 by ~93% and ~97%, respectively, in relationship to the native surface. The presence of Pluronic F127 also significantly diminished the mean biofilm thickness by ~84% and ~70% for CA662 and CA1, respectively, compared to the unmodified surface. The mean maximum percent surface coverage for CA1 biofilms was larger than that observed for CA662. This observed difference is thought to be due to a greater detachment rate for CA662 over CA1.

Planktonic cultures of *C. albicans* grown in reduced medium in the presence of Pluronic F127 (0.04% (v/v)) displayed reduced stationary phase concentrations in contrast to cultures grown in the absence of Pluronic F127. The log number of cells per unit volume was decreased by 31% and 34% for CA1 and CA662 planktonic cultures grown in

the presence of Pluronic F127, respectively, from that observed for cultures grown in the absence of the surfactant. The anti-adhesive quality of Pluronic F127 towards *C. albicans* is probably due to steric stabilization of the surface as has been indicated for bacteria and proteins, rather than a product of toxicity.(1,4,21)

Thus, based on these findings, the limited adhesion event was reflected in the ensuing biofilm. However, to use the term biofilm is somewhat of a misnomer since the cellular components observed on the modified surface did not resemble the mature differentiated biofilm that formed on the unmodified surfaces. The effect of the surface chemistry on the initial adhesion was displayed previously for CA1 (88) and was manifested again using a second strain of *C. albicans* (CA662) of a different serotype. Therefore, what happens at the adhesion level appears to indicate what will be observed at the biofilm level, regardless of serotype.

CONCLUSIONS

The adhesion of *C. albicans* to inert (18,43,71,73,84) and biological (2,14,16,18,24,25,35,44,71,74) surfaces has been described previously by others. In our studies, we examined the adhesion of two strains of *C. albicans* of differing serotypes to polystyrene (PS) and Pluronic F127 conditioned PS. The adsorption of Pluronic F127 to the PS surface significantly reduced the adhesion of *C. albicans* in comparison to the unmodified PS surface, regardless of serotype.

The existence of candidiasis is largely associated with the development of biofilms. Therefore, to stave off the development of an infection, the formation of a biofilm must be prevented or at least hindered. Others have studied biofilms of *C. albicans* that form on non-biological surfaces, characterizing their development, structure, and antifungal resistance.(3,5,15,30,31,32) Here we examined the formation of two strains of *C. albicans* of differing serotypes on the same surfaces as described for the adhesion studies. The Pluronic F127 modified PS surfaces showed significantly diminished biofilm formation over the untreated PS surface. The "biofilms" that formed on Pluronic F127 conditioned PS were significantly thinner and the mean maximum percent surface coverage was significantly reduced.

The application of Pluronic F127 as an anti-fouling substance appears hopeful, since it has also been shown to limit the adhesion of proteins (1,20,29) and bacteria (4,63). The formation of a conditioning film on a surface following exposure to human fluids has been implicated as an initiating stage in the development of infectious biofilms.(66) A

disadvantage of adsorbing Pluronic F127 to PS is that over time the Pluronic F127 may be removed from the surface. This factor may therefore limit the utility of a surface generated by adsorption of Pluronic F127. To increase the life of a Pluronic F127 treated PS surface, the PEO-containing triblock copolymer should be incorporated into the PS matrix. Others have been able to successfully incorporate PEO-triblock copolymers into polyurethane surfaces.(20,53)

The biofilm studies described in this thesis were conducted for 48 hours due to space limitations imposed by performing the studies at 37°C in an incubator. Thus, the life of a Pluronic F127 treated PS surface beyond 48 hours is not known. This point becomes important when considering application of these surfaces in medical settings. Another point to consider with regard to *in vivo* applications is that under "real life" situations, the surface will be exposed repeatedly to human fluids and microorganisms. In the studies describe in this thesis, the surface was exposed to yeast cells over a 30-minute seeding period after which time exposure to new cells was discontinued. Repeated exposure of the surface to new yeast cells might allow one to determine whether the cells that remain on the surface after a period of biofilm growth are altering the character of the surface and thereby making it friendlier for newly exposed yeast cells.

Based on our studies, the ability of Pluronic F127 to limit the adhesion of *C. albicans* seems to be the result of steric stabilization of the surface rather than a consequence of toxicity. Others have described steric stabilization as being the mechanism by which Pluronic impedes the binding of bacteria and proteins.(1,4,20)

Thus, based on our findings, the effect of the surface chemistry on the initial adhesion events persists during the development of the biofilm, That is, limited adhesion of *C. albicans* translates into limited biofilm formation, independent of serotype. The "biofilm" that developed on the Pluronic F127 conditioned surface more closely resembled the surface during the initial adhesion events than it did the mature differentiated biofilm that formed on the unmodified surface.

LITERATURE CITED

LITERATURE CITED

1. Alexandridis Paschalis and T. Alan Hatton. Poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) block copolymer surfactants in aqueous solutions and at interfaces: thermodynamics, structure, dynamics, and modeling. *Colloids and Surfaces A: Physiochemical and Engineering Aspects* 1995;96,1-46.

2. Bailey Andrea, Elsa Wadsworth, and Richard Calderone. Adherence of *Candida albicans* to Human Buccal Epithelial Cells: Host-Induced Protein Synthesis and Signaling Events. *Infection and Immunity* 1995;63(2),569-572.

3. Baillie George S. and L. Julia Douglas. Matrix polymers of *Candida* biofilms and their possible role in biofilm resistance to antifungal agents. *Journal of Antimicrobial Chemotherapy* 2000;46,397-403.

4. Bridgett MJ., MC. Davies, and SP. Denyer. Control of staphylococcal adhesion to polystyrene surfaces by polymer surface modification with surfactants. *Biomaterials* 1992;13(7),411-416.

5. Chandra Jyotsna, Duncan M. Kuhn, Pranab K. Mukherjee, Lois L. Hoyer, Thomas McCormick, and Mahmoud A. Ghannoum. Biofilm formation by the fungal pathogen *Candida albicans*: Development, Architecture, and Drug Resistance. *Journal of Bacteriology* 2001;183(18),5385-5394.

6. Costerton JW. and PS. Stewart. Battling biofilms. *Scientific American*; New York; July 2001.

7. Crithley IA. and LJ. Douglas. Isolation and partial characterization of an adhesin from *Candida albicans*. *Journal of General Microbiology* 1987;133(3),629-636.

8. Cutler JE. Microbiology 440 Lecture Notes 1999.

9. Cutler Jim E., Diane L. Brawner, Kevin C. Hazen, and Mark A. Jutila. Characteristics of *Candida albicans* Adherence to Mouse Tissues. *Infection and Immunity* 1990; 58(6),1902-1908.

10. Davies David G., Matthew R. Parsek, James P. Pearson, Barbara H. Iglewski, JW Costerton, and EP Greenberg. The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* 1998;280,295-298.

LITERATURE CITED - CONTINUED

11. de Nys R., PD. Steinberg, P. Willemsen, SA Dworjanyn, CL. Gabelish, and RJ. King. Broad spectrum effects of secondary metabolites from the red alga *Delisea pulchra* in antifouling assays. *Biofouling* 1995;84(4),259-271.

12. Dewez JL., JB. Lhoest, E. Detrait, V. Berger, CC. Dupont-Gillain, LM. Vincent, YJ. Schneider, P. Bertrand, PG. Rouxhet. Adhesion of mammalian cells to polymer surfaces: from physical chemistry of surfaces to selective adhesion on defined patterns. *Biomaterials* 1998;19,1441-1445.

13. Dewez Jean-Luc, Aurora Doren, Yves-Jacques Schneider, Paul G. Rouxhet. Competitive adsorption of proteins: Key of the relationship between substratum surface properties and adhesion of epithelial cells. *Biomaterials* 1999;20, 547-559.

14. Douglas LJ. Adhesion of *Candida* species to epithelial surfaces. *Critical Review of Microbiology* 1987;15(1),27-43.

15. Douglas LJ. *Candida* biofilms and their susceptibility to antifungal agents. Society for General Microbiology - 147th Ordinary Meeting - 12-15 September 2000 - University of Exeter, Exeter, UK.

16. Dubini G., R. Pietrabissa, and FM. Montevecchi. Fluid-structure interaction problems in bio-fluid mechanics: a numerical study of the motion of an isolated particle freely suspended in channel flow. *Medical Engineering and Physics* 1995;17(8), 609-617.

17. Edens Heather A., Charles A. Parkos, Tony W. Liang, Algirdas J. Jesaitis, Jim E. Cutler, and Heini M. Miettinen. Non-serum-dependent chemotactic factors produced by *Candida albicans* stimulate chemotaxis by binding to the formyl peptide receptor on neutrophils and to an unknown receptor on macrophages. *Infection and Immunity* 1999;67(3),1063-1071.

18. Edwards John E., Jr., M.D. Research Unit Coordinator *Candida* Adherence Mycology Research Unit.

19. El-Azizi Mohamed and Nancy Khardori. Factors influencing adherence of *Candida* spp. to host tissues and plastic surfaces. *Indian Journal of Experimental Biology* 1999;37,941-951.

20. Elibol Murat. Mass transfer characteristics of yeast fermentation broth in the presence of Pluronic F68. *Process Biochemistry* 1999; 34, 557-561.

LITERATURE CITED - CONTINUED

21. Freij-Larsson C., P. Jannasch., and B. Wessle'n. Polyurethane surfaces modified by amphiphilic polymers: effects on protein adsorption. *Biomaterials* 2000;21,307-315.

22. Gimeno CJ., and GR. Fink. Induction of pseudohyphal growth by over expression of PHD1, a *Saccharomyces cerevisiae* gene related to transcriptional regulators of fungal development. *Molecular and Cell Biology* 1994;14(3),2100-2112.

23. Givskov Michael, Rocky de Nys, Michael Manefield, Lone Gram, Ria Maximilien, Leo Eberl, Soren Molin, Peter D. Steinberg, and Staffan Kjelleberg. Eukaryotic interference with homoserine lactone-mediated prokaryotic signaling. *Journal of Bacteriology* 1996;178(22), 6618-6622.

24. Glee PM, Sundstrom P, Hazen KC. Expression of surface hydrophobic proteins by *Candida albicans* in vivo. *Infection and Immunity* 1995;63,1373-1379.

25. Glee Pati M., Jim E. Cutler, Evelyn E. Benson, Robert F. Bargatze, and Kevin C. Hazen. Inhibition of hydrophobic protein-mediated *Candida albicans* attachment to endothelial cells during physiological shear flow. *Infection and Immunity* 2001; 69(5),2815-2820.

26. Gow NAR., S. Bates, AJP. Brown, ET. Buurman, LM. Thomson, and C. Westwater. *Candida* cell wall mannosylation: importance in host-fungus interaction and potential as a target for the development of antifungal drugs. *Biochemical Society Transactions* 1999; 27(4),512-515.

27. Han Yongmoon and Jim E. Cutler. Antibody response that protects against disseminated candidiasis. *Infection and Immunity* 1995;63(7),2714-2719.

28. Han Yongmoon, Richard P. Morrison, and Jim E. Cutler. A vaccine and monoclonal antibodies that enhance mouse resistance to *Candida albicans* vaginal infection. *Infection and Immunity* 1998;66(12),5771-5776.

29. Han Yongmoon, Toshio Kanbe, Robert Cherniak, and Jim E. Cutler. Biochemical characterization of *Candida albicans* epitopes that can elicit protective and nonprotective antibodies. *Infection and Immunity* 1997;65(10),4100-4107.

30. Harper GR. , MC. Davies, SS. Davis, ThF. Tadros, DC. Taylor, MP. Irving, and JA. Waters. Steric stabilization of microspheres with grafted polyethylene oxide reduces phagocytosis by rat Kupffer cells *in vitro*. *Biomaterials* 1991; 12(7), 695-700.

LITERATURE CITED - CONTINUED

31. Hawser SP., GS. Baillie, and LJ. Douglas. Production of extracellular matrix by *Candida albicans* biofilms. *Infection and Immunity* 1998;47(3),253-256.

32. Hawser SP. and LJ. Douglas. Biofilm formation of *Candida* species on the surface of catheter materials in vitro. *Infection and Immunity* 1994;62,915-921.

33. Hawser Stephen P. and L. Julia Douglas. Resistance of *Candida albicans* biofilms to antifungal agents in vitro. *Antimicrobial Agents and Chemotherapy* 1995; 39(9), 2128-2131.

34. Hazen BW. and KC. Hazen. Modification and application of a simple, surface hydrophobicity detection method to immune cells. *Journal of Immunological Methods* 1987;107(2),157-163.

35. Hazen BW., RE. Liebert, and KC. Hazen. Relationship of cell surface hydrophobicity to morphology of monomorphic and dimorphic fungi. *Mycologia* 1988;80(3),348-355.

36. Hazen Kevin C., Diane L. Brawner, Marcia H. Riesselman, Mark A. Jutila, and Jim E. Cutler. Differential adherence of hydrophobic and hydrophilic *Candida albicans* yeast cells to mouse tissues. *Infection and Immunity* 1991;59(3),907-912.

37. Hazen KC. and PM. Glee. Cell surface hydrophobicity and medically important fungi. *Current Topics in Medical Mycology* 1995;6,1-31.

38. Hazen KC. and BW. Hazen. Hydrophobic surface protein masking by the opportunistic fungal pathogen *Candida albicans. Infection and Immunity* 1992; 60(4),1499-1508.

39. Hazen KC. and BW. Hazen. Surface hydrophobic and hydrophilic protein alterations in *Candida albicans. FEMS Microbiology Letters* 1993;107,83-88.

40. Hazen KC. and BW. Hazen. A polystyrene microsphere assay for detecting surface hydrophobicity variations within *Candida albicans* populations. *Journal of Microbiology Methods* 1987;6,289-299.

LITERATURE CITED - CONTINUED

41. Hellung-Larsen Per, Fahed Assaad, Stanislava Pankratova, Birgitte L. Saietz, and Lene T. Skovgaard. Effects of Pluronic F-68 on *Tetrahymena* cells: protection against chemical and physical stress and prolongation of survival under toxic conditions. *Journal of Biotechnology* 2000; 76,185-195.

42. Jenkinson HF. and Hilary M. Lappin-Scott. Bioflms Adhere to Stay. *Trends in Microbiology* 2001;9(1),9-10.

43. Kaiser GE. *Biology 141 Lab Manual* 1997.

44. Kaita H.. Adherence of *Candida albicans* to acrylic surfaces. *Fukuoka Shika Daigaku Gakkai Zasshi* 1989;16(3),407-422.

45. Kanbe Toshio, Yongmoon Han, Bryce Redgrave, Marcia H. Riesselman, and Jim E. Cutler. Evidence that mannans of *Candida albicans* are responsible for adherence of yeast forms to spleen and lymph node tissue. *Infection and Immunity* 1993;61(6), 2578-2584.

46. Kapteyn JC., RC. Montijn, GJP. Dijkgraaf, H. Van den ende, and FM. Klis. Covalent association of β-1,3-glucan with β-1,6-glucosylated mannoproteins in cell walls of *Candida albicans*. *Journal of Bacteriology* 1995;177,3788-3792.

47. Kayes, JB. and DA. Rawlins. Steric stabilization of suspensions, the adsorbed polymer layer thickness. *Colloid and Polymer Science* 1979;257,622.

48. Kirkpatrick R., JL. Lopez-Ribot, RK. Mcatee, and TF. Patterson. Growth competition between *Candida dubliniensis* and *Candida albicans* under broth and biofilm growing conditions. *Journal of Clinical Microbiology* 2000;38(2), 902-904.

49. Kobayashi SD. and JE. Cutler. *Candida albicans* hyphal formation and virulence: is there a clearly defined role? *Elsevier Science Ltd.* 1998.

50. Kohler Julia R. and Gerald R. Fink. *Candida albicans* strains heterozygous and homozygous for mutations in mitogen-activated protein kinase signaling components have defects in hyphal development. *Proceedings of the National Academy of Science of the United States of America* 1996;93(23),13223-13228.

51. J. Kuby, *Immunology*, WH. Freeman and Company, New York, New York, 1994.

LITERATURE CITED - CONTINUED

52. Laouar, L., KC. Lowe, and BJ. Mulligan. Yeast responses to nonionic surfactants. *Enzyme and Microbiology Technology* 1996;18(6),433-438.

53. Leberer Ekkehard, Doreen Harcus, Ian D. Broadbent, Karen L. Clark, Daniel Dignard, Karl Ziegalbauer, Axel Schmidt, Neil A.R. Gow, Alistair J.P. Brown, and David Y. Thomas. Signal transduction through homologs of the Ste20p and Ste7p protein kinases can trigger hyphal formation in the pathogenic fungus *Candida albicans*. *Proceedings of the National Academy of Science of the United States of America* 1996;93(23),13217-13222.

54. Lee JH., YM. Ju, WK. Lee, KD. Park, and YH. Kim. Platelet adhesion onto segmented polyurethane surfaces modified by PEO- and sulfonated PEO-containing block copolymer additives. *Journal of Biomedical Materials Research* 1998; 40(2),314-323.

55. Liu H., J. Kohler, and GR. Fink. Suppression of hyphal formation in *Candida albicans* by mutations of a STE12 homolog. *Science* 1994;266(5191),1723-1726.

56. Macura AB. and E. Tondyra. Influence of some carbohydrates and concanavalin A on the adherence of *Candida albicans* in vitro to buccal epithelial cells. *Zentralbl Bakteriol* 1989; 272(2),196-201.

57. Mah, Thien-Fah C. and George A. O'Toole. Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology* 2001;9(1),34-39.

58. McCourtie J. and LJ. Douglas. Extracellular polymer of *Candida albicans*: isolation, analysis and role in adhesion. *Journal of General Microbiology* 1985;131(3),495-503.

59. Netting Jessa. Sticky Situations. *Science News Online* 2001; 160(2).

60. Nikawa H., H. Nishimura, S. Makihira, T. Hamada, S. Sadamori, and LP. Samaranayake. Effect of serum concentration on *Candida* biofilm formation on acrylic surfaces. Mycoses 2000;43,139-143.

61. Odds FC. *Candida* and candidosis. Baltimore: University Park Press;1979, pp.16-28.

LITERATURE CITED - CONTINUED

62. Parent SA., JB. Nielsen, N. Morin, G. Chrebet, N. Ramadan, AM. Dahl, MJ. Hsu, KA. Bostian, and F. Foor. Calcineurin-dependent growth of an FK506- and CsA-hypersensitive mutant of *Saccharomyces cerevisiae*. *Journal of General Microbiology* 1993;139(12),2973-2984.

63. Pickering SAW. Implant infections - the orthopaedic perspective. Society for General Microbiology - 147th Ordinary Meeting - 12-15 September 2000 - University of Exeter, Exeter, UK.

64. Portoles Marta, Miguel F. Refojo, and Fee-Lai Leong. Poloxamer 407 as a bacterial abhesive for hydrogel contact lenses. *Journal of Biomedical Materials Research* 1994;28(3),303-309.

65. Qi Dewei, Lishi Luo, Raja Aravamuthan, and William Strieder. Lateral Migration and Orientation of Elliptical Particles in Poiseuille Flows. *Journal of Statistical Physics* 2002;107(112), 101-120.

66. Ramage Gordon, Kacy Vande Walle, Brian L. Wickes, and Jose′ L. Lopez-Ribot. Standardized Methods for In Vitro Antifungal Susceptibility Testing for *Candida albicans* Biofilms. *Antimicrobial Agents and Chemotherapy* 2001; 45(9),2475-2479.

67. Ramirez, OT. and R. Mutharasan. The role of the plasma membrane fluidity on the shear sensitivity of hybridomas grown under hydrodynamic stress. *Biotechnology and Bioengineering* 1990;36,911-920.

68. Reid G. Biofilms in infectious disease and on medical devices. *International Journal of Antimicrobial Agents* 1999;11,223-226.

69. Reinhart H., G. Muller, and JD. Sobel. Specificity and mechanism of in vitro adherence of *Candida albicans*. *Annuals of Clinical Laboratory Science* 1985; 15(5),406-413.

70. Ren-Kai Li and JE. Cutler. Chemical definition of an epitope/adhesin molecule on *Candida albicans*. *The Journal of Biological Chemistry* 1993;268(24),18293-18299.

71. Reynolds TB. and Gerald R. Fink. Baker's Yeast, a Model for Fungal Biofilm Formation. *Science* 2001; 291(5505), 878-881.

LITERATURE CITED - CONTINUED

72. Riesselman Marcia H., Toshio Kanbe, and Jim E. Cutler. Improvements and Important Considerations of an Ex Vivo Assay to Study *Candida albicans*-Splenic Tissue Interactions. *Journal of Immunological Methods* 1991;145,153-160.

73. Rotrosen D., RA. Calderone, and JE. Edwards Jr. Adherence of *Candida* species to host tissues and plastic surfaces. *Review of Infectious Diseases* 1986;8,73-85.

74. Ryan DR., AK. Russell, WD. Leukes, PD. Rose, and SG. Burton. Suitability of a modified capillary membrane for growth of fungal biofilms. *Desalination* 1998; 115,303-306.

75. San Millan R., PA. Ezkurra, G. Quindos, R. Robert, JM. Senet, and J. Ponton. Effect of monoclonal antibodies directed against *Candida albicans* cell wall antigens on the adhesion of the fungus to polystyrene. *Microbiology* 1996;142(8),2271-2277.

76. Sandin, R.L., A.L. Rogers, R.J. Patterson, and E.S. Beneke. Evidence for mannose-mediated adherence of *Candida albicans* to human buccal cells in vitro. *Infection and Immunity* 1982; 35(1),79-85.

77. Schwartz Dennis S., Howard H. Larsh, and Peter A. Bartels. Enumerative fluorescent vital staining of live and dead pathogenic yeast cells. *Stain Technology* 1977;52(4),203-210.

78. Shepherd MG. Cell envelope of *Candida albicans. CRC Critical Reviews in Microbiology* 1987; 15,7-25.

79. Silyn-Roberts G. and G. Lewis. A technique in confocal laser microscopy for establishing biofilm coverage and thickness. Water Science Technology 1997;36(10), 117-124.

80. Smith William F. Principles of materials science and engineering. 2nd Edition, McGraw-Hill Publishing Company 1990, p. 358.

81. Soll DR. The regulation of cellular differentiation in the dimorphic yeast *Candida albicans*. Bioessays 1986;5(1):5-11.

82. Stewart Philip S. Multicellular resistance: Biofilms. *Trends in Microbiology* 2001; 9(5), 204.

LITERATURE CITED - CONTINUED

83. Stewart Philip S. Theoretical aspects of antibiotic diffusion into microbial biofilms. *Antimicrobial agents and chemotherapy* 1996;40(11),2517-2522.

84. Stoldt Volker R., Anja Sonneborn, Christoph E. Leuker, and Joachim F. Ernst. Efg1p, and essential regulator of morphogenesis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. *The European Molecular Biology Organization Journal* 1997;16(8),1982-1991.

85. Tronchin G., JP. Bouchara, RR. Annaix, and JM. Senet. Fungal cell adhesion molecules in *Candida albicans*. *European Journal of Epidemiology* 1991;7,23-33.

86. Tronchin G., JP. Bouchara, R. Robert, and JM. Senet. Adherence of *Candida albicans* germ tubes to plastic: ultrastructural and molecular studies of fibrillar adhesins. *Infection and Immunity* 1988;56,1987-1993.

87. Tyler BJ., KJ. Siedlecki, H. Shi, and DL. Brawner. The influence of hydrodynamic shear on the adhesion of *Candida albicans* to two polymer surfaces. Submitted to the *Journal of Biomaterials Science, Polymer Edition.*

88. Wesenberg KE. and BJ. Tyler. Unpublished data. - Influence of surface chemistry on the adhesion and biofilm structure of *Candida albicans.*

89. Xie Hua, Guy S. Cook, J. William Costerton, Greg Bruce, Timothy M Rose, and Richard J. Lamont. Intergeneric communication in dental plaque biofilms. *Journal of Bacteriology* 2000;182(24), 7067-7069.

90. Yao Zhengbin, Guisheng Zhou, Xuhong Sunny Wang, Amy Brown, Katrina Diener, Hong Gan, and Tse Hua Tan. A novel human STE20-related protein kinase, HGK, that specifically activates the c-Jun N-terminal kinase signaling pathway. *Journal of Biological Chemistry* 1999; 274(4), 2118-2125.

91. Yu L., KK. Lee, K. Ens, PC. Doig, MR. Carpenter, W. Staddon, RS. Hodges, W. Paranchych, and RT. Irvin. Partial characterization of a *Candida albicans* fimbrial adhesion. *Infection and Immunity* 1994;62,2834-2842.

92. Zhang X., R. Whitbourne, and RD. Richmond. Antiinfective coatings for indwelling medical devices. *Medical Plastics and Biomaterials Magazine* 1997.

APPENDICES

APPENDIX A

BACKGROUND

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Candida albicans Virulence, Morphology, and Surface Characteristics

Epidemiology and Ecology

Candida is a normal commensal of the gastrointestinal and genitourinary tracts and mucosa.(36,43) The body's immune defenses and normal flora bacteria usually keep *Candida* under control. However, *Candida* is an opportunistic pathogen and can become problematic if the host becomes immunosuppressed or if the normal flora bacteria are quashed by use of broad spectrum antibiotics.(43) Most life-threatening fungal diseases are attributed to species of *Candida*. *Candida* species are the fourth leading cause of all bloodstream and urinary tract nosocomial (hospital-acquired) infections in the United States.(49)

Candidiasis is an infection caused by species of *Candida* of which *Candida albicans* is the major etiologic agent. Oral mucocutaneous candidiasis (thrush), vaginitis, onychomycosis (nail infections), and dermatitis (moist skin infections, e.g. diaper rash) are the most prevalent forms of candidiasis. Conditions favorable for severe opportunistic candidal infections of the skin, lungs, heart, and other organs can be precipitated by antibiotic therapy, as well as cytotoxic and immunosuppressive diseases such as diabetes, leukemia, and AIDS. In fact, currently 10% of the cases of septicemia are due to *Candida*. Also, the existence of AIDS can be indicated by the existence of candidiasis of

the esophagus, trachea, bronchi, or lungs in concurrence with a positive HIV antibody test.(43)

An estimated three million central venous catheters (CVCs) are used in the United States each year and contribute to an increase in the frequency of septicemia. Approximately 120,000 cases of CVC-related septicemia are reported each year. The common etiological agents associated with nosocomial sepsis and nosocomial septicemia are *Streptococcus epidermidis, S. aureus,* and *C. albicans*. *C. albicans* is the most prevalent of the pathogenic *Candida* species. *C. albicans* is accredited with 78% of all nosocomial fungal infections and 76% of all *Candida* infections followed by *Candida tropicalis*.(19,43,81)

Morphogenesis

C. albicans can undergo three different morphogenetic processes. This organism generally appears as an oval, nonencapsulated blastospore (~ 4 μm in diameter) and multiplies by formation of buds on the blastospores. *Candida* can also grow as pseudohyphae and true hyphae. Pseudohyphae are strands of lengthened buds (blastoconidia, 4-6 μm in diameter) with constrictions at the point of contact between the cells. Hyphae develop by germination of blastocondia with parallel walls at the location of germ tube formation and have true cross-walls or septa.(43,49,81) Chlamydospores, thick-walled survival spores, may develop at the ends or as part of the pseudohyphae. The growth phenotype depends on the environmental conditions such as CO_2 levels, pH, composition of the growth medium, serum factors, incubation temperature, and inoculum

size.(43,49,81) Studies of *C. albicans* biofilm growth in high galactose medium on disks of PVC catheter material revealed the presence of both yeast forms and hyphae. However, only the yeast forms were found when *C. albicans* was grown on an agar surface or within a liquid culture of the same medium. Regulation of morphogenesis by *C. albicans* is poorly understood, but studies indicate that it may involve gene expression in response to surface contact.(32)

Saccharomyces cerevisiae has been proposed as a model system for finding *C. albicans* genes that may play a role in the hyphal development. Due to *C. albicans* diploid genome and perceived absence of a sexual state, it is difficult to produce hyphal mutants. Mutants that are unable to form hyphae *in vitro* in some growth environments were generated through double-allelic knockout of the *S. cerevisiae* mating gene homologs in *C. albicans*. However, both the wild-type and gene-disrupted mutants of *C. albicans* generated hyphae in the presence of serum. Neither the wild-type nor gene-disrupted mutants of *S. cerevisiae* produced hyphae when serum was present. Thus, it is apparent that there are dissimilarities between *S. cerevisiae* and *C. albicans* in the genes required for regulation and control of filament production.(49)

Pseudohyphae production in *S. cerevisiae* is controlled by the PHD1 (pseudohyphal determinant) gene.(22,49) The gene homolog in *C. albicans* is EFG1 (enhanced filamentous growth).(49,84) Removal of the EFG1 gene or limitation of expression through an inducible promoter results in the production of pseudohyphae, but not hyphae in the presence of serum. The EFG1/efg1 heterozygote generate hyphae *in vitro* in the media containing 20% serum. The EFG1/efg1 heterozygotes are less virulent

than the EFG1/EFG1 homozygotes. The formation of defective hyphae and yeast is also observed when the expression of EFG1 is limited. Therefore, it is thought that the EFG1/efg1 hyphae and yeast forms are defective. Thus, EFG1 appears to be required for virulence, but may not be necessary for the yeast to hyphal transition. It is not known whether the EFG1/efg1 heterozygote produces normal hyphae *in vivo*.(49)

The disruption of the *S. cerevisiae* mitogen-activated protein kinase (MAPK) gene homolog in *C. albicans* (CPH1, cytosolic cyclophilin) along with EFG1 results in the generation of a mutant (HLC54) that does not generate hyphae in several environments such as medium with serum.(49,55,62) This double mutant is less virulent in murine models than the wild-type and the single disruption mutant. This double mutant may provide information as to the mechanism required for morphogenesis.(49)

The transition from the yeast to filamentous form has been indicated as being necessary for virulence. However, the preceding findings for the EFG1/efg1 heterozygote, along with those for HST7 and CST20 deletion mutants, do not support this hypothesis. The HST7 and CST20 *Candida* homologs of *S. cerevisiae* genes STE7 (mitogen-activated protein kinase kinase) and STE20 (serine/threonine kinase), deletion mutants form hyphae *in vivo*, but are not as virulent as the wild-type.(49,50,53,90) The *S. cerevisiae* gene homologs in *C. albicans* affect the morphogenesis, but fail to provide insight into the relationship between hyphal formation and virulence.(49) Adhesion studies of *C. albicans* have shown the yeast-mycelial transition to be important. This phenomenon of multi-morphogenetic states involves rearrangement, or chemical changes in the cell wall components. During germination, the density and distribution of cell wall

surface mannoproteins have been shown to change along with reorganization of the cell wall. Intense labeling of the surface mannoproteins was observed in resting blastoconidia. However, upon germ tube formation, the surface labeling of the mother cell decreased, whereas that of the hyphal surface was strongly labeled. This seemed to indicate that upon germination, reorganization of the cell wall was accompanied by enzymatic degradation of the surface mannoproteins on the mother cell and by generation of new components on the hyphal surface. This activity which accompanies morphological changes along with multifunctional potential of some of the surface mannoproteins appears to be important in the expression of the pathogenicity of *C. albicans.*(86)

Structure:
Environmental Influences and Adhesion

F.C. Odds (1988) describes the cell wall of *C. albicans* blastospores in terms of five different layers, which are described as being electron dense (the two outermost layers), weakly electron dense, strongly electron dense, or electron transparent. The electron transparent layer containing mostly glucans is located just outside the innermost layer of the cell wall which is the strongly electron dense layer containing chitin and glucan with some mannan.(61,78)

The cell wall of *C. albicans* contains a greater amount of glucan polymers than mannan polymers. Beta-glucans serve as the major structural elements of the cell wall. Three types of glucans have been identified in intact yeast, hyphal, and germ tube forming cells: an alkali-soluble polymer which is primarily low molecular weight, a branched acid-soluble glucan consisting of mainly beta-1,6-linked residues, and an insoluble highly

branched complex containing variable amounts of beta-1,3- and beta-1,6-linkages. A portion of the insoluble glucan fraction is linked to chitin. None of the glucans analyzed contained mixed intrachain beta-1,3-/beta-1,6-linkages. Yeast and hypha have approximately the same ratios of beta-1,3- to beta-1,6-linkages in the insoluble glucan. However, in germ tube forming cells, 67% of the insoluble glucan had beta-1,3-linkages, whereas in yeast cells, 32% of the insoluble glucan contained beta-1,3-linkages. The beta-1,6-glucan epitopes and probably the beta-1,3-glucan epitopes are believed to be attached to a glycosyl phosphatidyl inositol (GPI) anchor which is phosphodiester linked to a C-terminal amino acid of a mature protein.(46,61,78)

Beta-1,6-glucans and chitin are found in the innermost layer of the cell wall. The total glucan and mannoprotein content of the cell wall remains fairly constant independent of the stage of growth of germ tube formation. However, the percent of alkali-soluble and insoluble glucans, as well as chitin change during germ tube formation, increased from 0.6% to 2.7%. It has been shown that the mycelial cells contain three times as much chitin as in yeast cells. Chitin is a linear polysaccharide made up of N-acetyl-D-glucosamine (GlcNAc) units connected through beta-1,4-linkages. The largest amount of chitin is located in the bud scars with the next largest amount located in the layer close to the plasma membrane (mentioned previously) and a smaller amount of chitin is distributed throughout the cell wall. The cell wall chitin is associated with glucan.(61,78)

Lipids are located throughout the cell wall of *C. albicans*. Lipids comprise 1 to 5% of the cell wall. The portion of lipids in the cell wall increases during the yeast-

mycelial transformation. The lipids are believed to be derived from the plasma membrane. The major lipids in the cell wall are triglycerides, phospholipids, and sterol esters.(61,78)

The plasmalemma of *C. albicans* is a typical eukaryotic phospholipid bilayer. The plasma membrane is the primary site of action of many antifungal agents such as polyenes and azoles. Polyenes attack the plasma membrane directly. Azoles affect the final composition of the plasma membrane. The plasma membrane is composed of ~ 9.5% (w/w) protein, ~3.7% carbohydrate, and 80.9% lipid. Solutes are transported across the plasma membrane by either simple diffusion (nonspecific, nonsaturable) or specific protein carriers. Alcohols, undissociated organic acids, fatty acids, and the majority of drugs are transported by simple diffusion. The majority of ions and other solutes are transported by a specific protein carrier. Various enzymes involved in cell wall biosynthesis have been found in the cell wall including glucan synthase, chitin synthase, and mannosyl transferase.(61,78)

An electron dense layer consisting of mostly mannans is located just inside of the electron dense outermost fibrillar layer composed of mostly mannoproteins. Mannan polysaccharides are also found distributed throughout the rest of the cell wall. Mannan is composed of a polymer of alpha-1,6-linked mannose residues with alpha-1,2- and alpha-1,3-linked oligosaccharide side chains consisting of mannose residues. The alpha-1,3-linked side chains make up a smaller proportion of the side chains. A central protein core supports the mannan backbone and the oligosaccharide side chains. The mannose groups are covalently bound through either an o-glycosidic linkage to serine or threonine or via GlcNAc to an asparagine residue.(61,73,78)

Germination is accompanied by a two- to fifty-fold increase in adhesion of *C. albicans* over yeast phase cells.(19,73) This increase in adhesion could be due to qualitative changes in the mannoproteins, germ-tube specific mannoproteins, exposure of mannoproteins, or an increase in the number of exposed mannoproteins due to an increase in surface area.(73) The transition from yeast-to-hypha is accompanied by a change from surface hydrophilicity to hydrophobicity. Germ tubes and young hyphae are highly hydrophobic. Germ tubes are thought to be involved in invasion of host tissues. Variations in cell surface hydrophobicity (CSH) are associated with changes in the length and concentration of fibrils on the outermost layer of the cell wall.(19,24,38)

An outer fibrillar layer has been observed on blastoconidia and germ tubes. The presence of an additional outermost fibrogranular layer containing mannoproteins was revealed on germ tubes adhering to plastics that was not evident on the parent blastocondidium. It is believed that the glycoproteins of the cell wall coat regroup upon contact with the plastic surface, forming the outer fibrillar layer.(78,85,86) Studies indicate that these surface mannoproteins serve as the primary adhesins in *C. albicans*.

From fungal fibrils contained within the adhesion plaques located at the contact site of germ tubes with the substratum, four cell wall proteins with molecular weights (MWs) of 60, 68, 200, and greater than 200 kDa were obtained. The 60 and 68 kDa proteins also behave as receptors for host proteins such as laminin, fibrinogen, and complement. Glycosylated proteins with MWs of 62 and 70 kDa have been associated with pseudohyphae, but not with yeast forms of *C. albicans*.(14,73,85) Others have identified glycoproteins with MWs of 20 to 68 kDa that are believed to be responsible for

the surface hydrophobicity of hyphae. Four mannoproteins, two of which are larger than 200 kDa, have been identified as attachment factors for adhesion of *C. albicans* to plastic. These four proteins are thought to be hydrophobic surface molecules that play a role in the hydrophobic interactions with the plastic surface. It is not known whether some of these proteins appear as a consequence of contact with the plastic.(37) Others, however, have reported the appearance of new *Candida* proteins, accompanied by the presence of signal proteins, upon binding to human buccal epithelial cells (BECs).(2)

Cutler and colleagues (1990) found that *C. albicans* cells grown to stationary phase (24 and 48 hours) bind to tissue sections in greater numbers than logarithmic phase cells (6 hours). The stationary phase yeast cells differed in their ability to adhere to tissue sections, in particular to splenic tissue, with some strains binding to a greater extent than others. However, regardless of the strain, stationary phase yeast cells always adhered to tissue sections in greater numbers than the log-phase cells.(9,19) In general, humans are subject to endogenous stationary phase yeast cells which are more likely to undergo germination, an associated virulence factor.(9)

Both *ex vivo* and *in vivo* methods of observing binding to tissue sections were utilized by Cutler and colleagues (1990). Tissue section tests were generally carried out at 4°C, although the results at 22°and 24°C were found to be comparable. One noted difference between the two methods was the absence of cellular adhesion to the tunica media of arterial walls of kidneys in the *in vivo* method. This indicates that the receptors on the walls are not accessible thereby not allowing for interactions between the host and *C. albicans*. In spite of this discrepancy between the two methods, the *ex vivo* method

still serves as a valid test for identifying specific host cell receptors and fungal cell antigens which permit the localization of cells to host tissue. The surface antigenic makeup of *C. albicans* depends on the strain and also on the cell cycle and environmental growth conditions. The *ex vivo* method serves as a suitable means of determining the pathogenesis of candidiasis based on adherence to tissue sections.(9)

S. cerevisiae displays a similar tissue distribution following clearance from the bloodstream as does *C. albicans* following a 30 to 60 minute inoculation period. Viable cells are found in the kidney, heart, liver, spleen, and lungs with the numbers being comparable for *S. cerevisiae* and *C. albicans*. Over time, the cell numbers decrease, except for the case of *C. albicans* in the kidneys which is the only organ that regularly supports growth, leading to disease. Since *S. cerevisiae* is non-pathogenic, adhesion to host tissue alone does not account for the onset of disease.(9,19)

Ex vivo methods revealed that *C. albicans* binds to lymph nodes and spleens in regions rich with macrophages, but not in regions of the spleen containing monocytes. At concentrations of *C. albicans* between 1 - 2 $\times 10^8$ cells/ml under stationary incubation, the binding sites in the marginal zones of mouse splenic tissue were saturated. At concentrations of 3×10^8 cells/ml or greater, adhesion to spleen marginal zones was diminished. This may be due to shedding of cell surface adhesins which then vie for the same tissue binding sites as the yeast cells. (9,72)

Under stationary incubation conditions, the binding to spleen marginal zones was 20 times that observed under rotational incubation. The centrifugal field under rotation was approximately $0.1 \times g$ which allowed the yeast cells to alight onto the tissue sections,

but it may not permit intimate contact where weak attractive forces are at play. By varying the rotational force, it may be possible to study different kinds of binding interactions.(72)

C. albicans also adheres to the endothelial aspects of kidney convoluted tubules and glomeruli. In otherwise immunologically normal mice, *C. albicans* binds to tissue rich in macrophages which may exhibit candidacidal or candidastatic activity, but the yeast grows in the kidney until the onset of inflammatory changes. Deep-seated candidiasis, in which organs other than the kidney are affected, may be attributed to abnormal macrophage function.(9)

Interactions of yeast cells with tissue macrophages were revealed through rat monoclonal antibodies, SER-r and MONTS-4, which specifically stain macrophages. Immunoperoxidase-stained tissue sections allow for the same observation to be made using *ex vivo* methods. Adherence of the *C. albicans* strain CaOs to spleen, lymph node, and kidney tissue was prevented by chelators (EDTA and EGTA) of divalent cations. Removal of the chelators along with the addition of Dulbecco modified Eagle medium (DMEM) restored the ability of the yeast to adhere to the tissues.(9)

The binding of yeast cells of varying CSH to tissues revealed that hydrophobic yeast cells (high CSH levels) adhered in greater numbers than hydrophilic yeast cells (low CSH levels), independent of tissue type or isolate tested. Wild-type isolates tested had CSH levels that are less than 10% at 37°C and greater than 70% at 24°C. *Ex vivo* adherence assays of hydrophobic yeast cells reveal that these cells are more widely distributed and in greater numbers than hydrophilic yeast cells. Hydrophilic yeast cells

(37°C) adhere to regions of lymph node and spleen tissue that are rich in macrophages. However, growth is not observed in these areas due to the antifungal nature of phagocytes. In contrast, hydrophobic yeast cells adhere to all tissues and in areas where macrophages are absent or nearly so. This was observed in the adhesion of hydrophobic yeast cells to the red and white pulp in splenic tissue and not just in marginal zones rich in macrophages. However, strains with the same level of CSH (>70%) may not display the same degree of tissue adhesion.(36)

On the other hand, hydrophilic yeast cells bind to particular areas of each tissue and primarily in areas that are macrophage rich via specific adhesin-receptor interactions. These tissue binding characteristics for hydrophobic and hydrophilic yeast cells were observed in all four isolates tested. If the same adhesion patterns are observed *in vivo*, then the greater number of hydrophobic cells than hydrophilic cells adhering to areas that are lacking macrophages may provide an advantage to hydrophobic cells in colonizing host tissues after removal from the bloodstream. This notion is in line with the findings of increased virulence in mice associated with hydrophobic cells over hydrophilic cells.(19,36)

The characteristics associated with increased virulence that are influenced by growth temperature include the synthesis of coenzymes, specific adhesin expression, and tolerance to toxic substances. However, growth temperature does not affect the adhesion pattern of hydrophobic and hydrophilic yeast cells to tissues. At 37°C, the mutant strain A9V10, which is hydrophobic, adheres to tissues in significantly greater numbers than the hydrophilic wild-type strain A9.(36)

A relationship between CSH levels and adhesion to epithelial cells at 24°C must be determined on a strain by strain basis. There is a positive correlation between CSH levels and adhesion to epithelial cells for a single strain. The limitations in comparing different strains is due to variations in the expression and concentration of surface factors such as adhesins, fibrils, and integrin receptors which are involved in adhesion to host tissues. In addition to a positive correlation between CSH and adhesion to epithelial cells for the same strain, there may also exist a positive correlation for the mutant strain A9V10 and the wild-type strain A9. In adhesion experiments for these two strains with splenic tissue, increased levels of CSH were associated with increased adhesion.(36)

In *ex vivo* adherence assays, the presence of EDTA prevented the binding of hydrophilic yeast cells to mouse tissue. The presence of divalent cations are necessary for cell-cell adherence, and electrostatic forces play a role in adhesin-receptor interactions. In contrast, the adherence of hydrophobic cells was not affected by EDTA. The long range (up to 10 nm) hydrophobic interactions allow for short range (< 2 nm) specific adhesin-receptor interactions by bringing the two surfaces in closer proximity than could be obtained otherwise.(36)

Hydrophobic cells form germ tubes one hour after intravenous inoculation in mice, whereas hydrophilic cells exist in the yeast form. Hydrophobic cells seed kidney tissues, germinate at a faster rate than hydrophilic cells, display decreased killing by phagocytoses, are thought to express specific adhesins, and adhere to host tissues during dissemination. These findings indicate that hydrophobic cells may play an important role in the genesis of invasive disease.(36)

The cell wall engages in other pathogenic events aside from adhesion to host or plastic surfaces. It is also involved in obtaining nutrients from its surroundings, resisting the affects of drugs, activating the immune response, and tolerating the immune response products. CSH has been shown to be fundamental in each of these functions. CSH may facilitate the adsorption of nutrients by acting as a surfactant.(37) The demise of hydrophobic cells by polymorphonuclear neutrophils is less than that observed for hydrophilic cells.(19)

The hydrophobicity/hydrophilicity of the cell surface is determined by both the growth form and the environmental conditions. The environmental conditions that influence the composition of the cell wall include nutrient availability, growth medium, temperature, hydration, and environmental stresses and toxins.(37) CSH can be affected by the carbohydrate composition of the medium. If carbohydrates serve as the only carbon source, the CSH levels will increase. CSH levels are increased by an excessive concentration of carbohydrates and may also be increased by limited carbohydrate concentrations.(32,37) Short-term starvation of yeast cells signals sporulation. Spores of many fungi are hydrophobic. Since morphological changes result in reorganization of the cell wall, it seems plausible that starvation could induce elevated CSH levels.(37)

The form of the growth medium has a critical bearing on the apparent cell surface hydrophobicity. That is, cells may be less hydrophobic if they are grown within a liquid medium in comparison to cells grown on a solid or liquid culture. This is especially true if the growth on the solid or liquid culture is preceded or accompanied by sporulation.(37)

Temperature is another parameter that can affect the level of CSH. Stationary-phase yeast cells grown in complex media at 28°C are more hydrophobic than the same cells grown at 37°C.(19,24,36,37) Temperature alone, however, does not dictate the degree of CSH. It is also affected by physiologic and morphologic factors along with other environmental conditions. When incubated at 37°C, yeast cells that are actively growing show modest levels of CSH, pseudohyphae show variable amounts of CSH, and yeast cells grown on solid media exhibit a range of CSH. Yeast cells appear hydrophobic prior to germination, and young hyphae are highly hydrophobic.(24,37)

Temperature and pH influence the phenotype (budding or hyphal form) of yeast grown in defined media. Generally, at low temperatures or low pH, the budding phenotype is expressed whereas at high temperature and pH, the hyphal phenotype is expressed. However, at low temperature or low pH, some strains will express the hyphal phenotype. Stationary phase cells will form buds and grow solely in this form when placed in media at 37°C with a pH of 4.5. If stationary phase cells are placed in media at 37°C with a pH of 6.7, the cells will grow solely as hyphae as long as the pH remains above 6.0. If stationary phase cells are placed in media with a pH between 5.5 and 6.5, initially the daughter cells will be elongated, but will revert to the budding form and intermediate phenotypes.(81)

It is unknown whether *C. albicans'* ability to change its surface phenotype from hydrophilic to hydrophobic contributes to its parasitism. Establishing a relation between CSH and the pathogenicity is difficult because expression of surface hydrophobicity (or hydrophilicity) is a dynamic event. *C. albicans* can change from hydrophilic to

hydrophobic within 60 minutes after inoculation in tissue culture medium or fresh growth medium.(36,37) Also, cells suspended in buffer at low concentrations (10^8 cells/ml) on ice may change from hydrophilic cells to hydrophobic cells. CSH status can change within 15 minutes and also may accompany morphologic conversion. Cell surface hydrophilicity can be maintained for up to four hours, however, if the cells on ice are pelleted. Thus, relating surface hydrophobicity to adhesion of *C. albicans* to plastic is difficult because within the time frame of most assays (one hour), the surface hydrophobicity status of the yeast could change. Thus, the adhesive ability of the yeast may be altered. Also, during the extended incubation period, the yeast may secrete substances which coat the surface and thereby increase the affinity of the cells for the surface. CSH is believed to contribute to the adhesion of *C. albicans* to hydrophobic plastic surfaces.(37)

As described previously, the cell wall of *C. albicans* is a complex structure composed of proteins, polysaccharides (chitin, glucan, and mannans), and lipids. The cell wall can be described with regard to layers or more precisely as "zones of enrichment". The number of cell wall layers is between five and eight layers. The number and composition of the layers depends on the age, growth environment, growth form (yeast or hyphal), and the cytochemical techniques utilized. The cell wall has been shown to thicken with the disappearance of the layers following prolonged starvation. The cell wall accounts for 30% of the cell dry weight. The composition of the cell wall of yeast and germ tubes is ~ 3 to 6% (w/w) proteins, ~ 77 to 85% carbohydrates, and ~ 2% lipids. Other authors have offered slightly different compositions.(14,61,78)

The multilayered cell wall of *C. albicans* consists of three regions, an endomural region with a thin layer of glucan and chitin, a mesomural region containing glycoproteins, glucans, mannans, and chitin in various proportions, and an ectomural fibrillar region containing mannoproteins. The ectomural layer differs between hydrophilic and hydrophobic cells. The length of the fibrils of hydrophilic cells is 2.3 times that of hydrophobic cells, 0.198 μm compared to 0.085 μm. In hydrophobic cells, the fibrils are blunt and aggregated unlike the fibrils in hydrophilic cells which are distinct, thin, and tightly packed. The long fibrils associated with the hydrophilic cells are composed of high molecular mass mannoproteins. The ectomural region is responsible for surface hydrophobicity as determined by hydrophobic microsphere assay.(38)

The hydrophobic microsphere assay (HMA) determines the percentage of cells (at least 100 cells are counted in each sample) which have three or more microspheres attached. This percentage indicates the portion of hydrophobic cells in the population and the remaining difference from 100% is the portion of hydrophilic cells.(36)

Hydrophobic and hydrophilic cells have similar hydrophobic proteins. However, in the hydrophilic cells, the hydrophobic proteins are not readily exposed. Thus, differentiation between surface hydrophobicity and hydrophilicity depends not on the qualitative difference of hydrophobic proteins, but rather on the presence of hydrophilic proteins. The attachment of hydrophobic microspheres to hydrophilic cells is inhibited by an increase in the ectomural mannoproteinaceous fibrillar layer. The hydrophilic proteins mask the hydrophobic proteins. The hydrophobic proteins are exposed when the large

mannoproteins are altered. Hydrophobic surface proteins can be exposed using tunicamycin, a protein glycosylation inhibitor, or by treatment with dithiothreitol.(36,38)

Germination of hydrophilic stationary-phase yeast cells results in exposure of the hydrophobic surface proteins. A reduced ectomural fibrillar region is observed on young buds and the apices of growing hyphal cells, both of which are hydrophobic, unlike their hydrophilic parent. A decrease in the "water-interactive" state of *C. albicans* could ensue from a stimulus that initiates minor modifications of the ectomural layer such as decreased protein glycosylation or shedding of the fibrils. Subsequent exposure of the hydrophobic proteins is associated with increased virulence of *C. albicans* due to enhanced adhesion and increased resistance to phagocytic killing as expressed earlier.(38,39)

The cell wall proteins of *C. albicans* are either loosely associated with the wall matrix or covalently bound to mannans via phosphatidyl bonds. The cell surface hydrophobic proteins are tightly associated with the cell wall whereas the large hydrophilic cell wall mannoproteins are loosely associated and are synthesized and shed throughout cell growth.(38,39) Hydrophobic surface proteins are small (34-40 kDa) and poorly glycosylated (less than 5% polysaccharide). They are present throughout the various stages of growth regardless of the temperature (23° or 37°C) or growth medium.(37)

Exposure of the hydrophobic surface proteins is dependent on the growth phase, growth form, and temperature. Hydrophilic proteins are high molecular mass fibrillar proteins usually containing less than 50% polysaccharide.(37) Lei Yu et al. (1994) reported a fibril subunit consisting of ~ 15% protein by weight and ~ 85% carbohydrate and a molecular weight of 66 kDa. The protein component consists of 50% hydrophobic

amino acid residues and 12.5% basic amino acid residues with a molecular weight of 8,644 Da. The carbohydrate portion consists primarily of D-mannose.(91)

Adhesion of *C. albicans* to inert, low surface charge substrata entails nonspecific hydrophobic interactions, unlike attachment to a host surface which requires specific hydrophobic adhesins. As expected, adhesion of hydrophobic *C. albicans* to a hydrophobic inert surface utilizes hydrophobic interactions. The hydrophobicity of both the plastic and fungal surface regulate the degree of attachment. If the substrata has hydrophobic and hydrophilic microareas (e.g., polystyrene with low parking area), then the adhesion of *C. albicans* to the surface involves other interactions such as electrostatic and polar-polar interactions along with hydrophobic interactions.(37)

There are a number of factors which influence the adhesion of *Candida* spp. to biological and non-biological surfaces such as environmental factors (e.g., sugars, pH, temperature, ions), host proteins, characteristics of the fungi, and interactions with other microorganisms. Techniques used to quantify the attachment of *Candida* spp. to biological and non-biological surfaces include direct observation with microscopes, radiolabelled cells, tetrazolium salt, XTT, fluorescence assays that employ fluorescent dyes such as calcofluor white which binds to chitin and glucan, and immunofluorescence assays.(19)

Some *Candida* spp. utilize carbon sources to synthesize extracellular polymeric material (EP). The EP is formed from the cell surface components and is thought to play a role in adhesion. The EP is characteristically mannoprotein, containing 85-90% carbohydrates of which 82-87% is mannose, 7-9% protein, 1.5% glucosamine, and 0.5%

phosphate. McCourtie and Douglas (1985) found that EP from the culture supernatant of *C. albicans* grown in media containing 50mM glucose, 500mM galactose, or 500mM of sucrose had a similar chemical makeup. They also found that galactose more than sucrose was associated with increased adherence and EP synthesis, and both galactose and sucrose displayed increased adherence and EP synthesis over glucose. The EP from five *C. albicans* strains contained adhesins with specificities for glycoproteins of epithelial cells consisting of L-fucose, N–acetyl-D-glucosamine, and D-mannose.(19,58)

The attachment of *Candida* spp. to host tissues and plastic surfaces is influenced by the type and quantity of sugars in the growth medium and in the medium under which attachment is assessed. The sugars are either necessary for the development of extracellular materials that play a role in adhesion or can compete with the yeast for adhesion sites.(19)

Attachment of *C. albicans* GDH2346 and *C. albicans* GDH2023 to buccal and epithelial cells was hindered by L-fucose and N-acetyl-D-glucosamine and the attachment of the latter strain was also inhibited by D-glucosamine. Reinhart et al. (1985) reported that adhesion of *C. albicans* to vaginal epithelial cells was diminished by amino sugars such as glucosamine, galactosamine, and mannosamine and augmented by dextrose, galactose, mannose, α-methyl-mannoside, N–acetyl-glucosamine, N–acetyl-galactosamine, and N-acetyl-mannosamine This adhesion was also enhanced at low pH.(19,69) Macura and Tondyra (1989) determined that the attachment of *C. albicans* to epithelial cells was enhanced by growth in D-glucose, D-galactose, D-mannose, and sucrose while growth in D-xylose, D-ribose, D-fructose, maltose, lactose, and raffinose reduced attachment.

Others have reported that amino sugars and mannose prevent adhesion of *C. albicans* to epithelial cells.(19,56)

The amino sugars blocked attachment to polystyrene (PS). Glucose concentrations in the growth media of 50mM augmented *C. albicans* attachment to PS, but concentrations greater than this prevented attachment. The attachment of *C. albicans* CA51 to PS was also enhanced by galactose, glucose, and divalent ions such as Ca^{2+} and Mg^{2+} and hindered by Fe^{3+}. The binding of *C. albicans* CA51 to PS was greater at 37°C than at 20° and 25°C and attained a maximum at 40°C.(19)

Exposure of duodenal discs to mannose and N-acetylglucosamine prior to exposure or in conjugation with *C. albicans* exposure prevented attachment to the gastrointestinal tract by taking up sites of attachment used by the yeast. Crithley and Douglas (1987) found that adhesion of yeast to BECs was mediated by the protein portion of the mannoprotein as indicated by the ability of EP and EP treated with sodium periodate or α-mannosidase, to block attachment of yeast to BECs by 60%. EP treated with dithiothreitol or proteolytic enzymes did little if anything to prevent the interaction.(7,19)

Kaita (1989) described an adhesive substance (AS) procured from the surface of *C. albicans* IFO1385 that was involved in the attachment of the yeast to acrylic. The composition of the AS was 62-68% carbohydrates in the form of glucose and 23-26% protein. The attachment to acrylic was augmented by growth in YNB medium containing 500mM galactose over that grown in YNB medium containing 500mM glucose.(19,44) McCourtie and Douglas (1985) determined that the binding to acrylic surfaces increases

with increasing amounts of sugars in the growth medium.(19,58) Adhesion of *C. albicans* to dental acrylics was shown to be unaffected by the presence of lactose or xylitol, but increased linearly upon incubation in sucrose, galactose, glucose, maltose, and fructose.(73) The attachment of *Candida* spp. to biological and non-biological surfaces is augmented by growth in galactose to a greater extent than by other mono or disaccharides. This is thought to be the result of increased EP synthesis, spheroplast development, and cell surface hydrophobicity (CSH).(19)

The attachment of *Candida* spp. to biological and non-biological surfaces is also enhanced by divalent ions including Ca^{2+} and Mg^{2+}, more than monovalent ions, by minimizing the electrostatic repulsion between the yeast and the negatively charged surface.(19)

C. albicans possesses cell surface receptors for host proteins, such as fibrinogen, fibronectin, laminin, and type I and type IV collagen, which allow for adhesion to the conditioning films that form on implanted surfaces and subsequent infections. Germ tubes and hyphae have receptors for fibrinogen, laminin, and complement (a group of serum proteins), while yeast cells do not. Fibrin deposition on catheters, in addition to decreased phagocytic activity, is thought to contribute to the persistent colonization by *C. albicans* of these implants.(19,73) The binding of *C. albicans* to extracellular matrix (ECM) proteins such as type I collagen and fibronectin requires the presence of calcium. Hyphae adhere to fibrinogen to a greater extent than germ tubes. This adhesion can be minimized by 2-mercaptoethanol and/or pronase or α-mannosidase or trypsin. Fibronectin is a peripheral glycoprotein that is expressed on normal cell surfaces and serves as a site for

microorganisms to bind to host cells and to biomaterials. Laminin is a large glycoprotein that is located primarily in the basal membrane, but can also be found in plasma.(19)

ECM proteins have regions for the attachment of heparin. Thus, glycosaminoglycans (GAGs) such as heparin, heparin sulfate, and dextran sulfate can prevent binding of *C. albicans* to ECM proteins by concealing molecules recognized by the yeast cell surface receptor. Hemoglobin also expresses receptors for ECM proteins and thereby can compete with *C. albicans* for binding sites.(19)

Increased adherence of yeast cells to epithelial tissue is attributed to CSH. The relationship between CSH and adhesion is contingent on the species involved. There is a positive relationship between CSH and the binding of *C. krusei* to HeLa cells (He(nrietta) La(cks), from whose cervical cancer such cells were obtained in 1951) and a negative relationship between CSH and the binding of *C. krusei* to acrylic. The attachment of *C. albicans* to HeLa cells in citrate and phosphate buffers was augmented at a pH of 7 and to a greater extent at a pH of 3. Growth phase, medium, and temperature all have an affect on the hydrophobicity of *T. glabrata* and *C. albicans.* The binding of *C. albicans* to epithelial cells is mediated by specific adhesin-receptor interactions which are influenced by CSH.(19)

There is an increased likelihood for attachment when the surface free energy of yeast cells and the substratum is nearly the same. The binding of *C. albicans* to plastic surfaces is mediated by hydrophobic interactions or attractive London van der Waals forces. All living cells, including yeast, possess a net negative surface charge. Thus, in

order for microorganisms to bind to a biological surface, they must overcome repulsive forces.(19)

The outer cell wall of *C. albicans* interacts with the host as a point for adhesion to epithelia and endothelia and factor in colonization. The outer cell wall mannoproteins play a role in the pathogenesis and biology of the yeast. These mannoproteins serve as major antigens, as a defensive mechanism against host enzymes, and as a means of controlling and averting detection by the immune system. The constitution of the surface mannoproteins is active and changing. The regulation of mannoprotein gene expression to manipulate antigenic variation provides a means by which *C. albicans* can circumvent the immune system. The known hyphal-specific genes encode mannoproteins on the cell surface.(26)

Interactions of *C. albicans,* particularly in the hyphal form, with host tissue is due in part to integrin-like surface molecules. Surface molecules, termed adhesins, are responsible for the adherence properties of *Candida albicans*. Such adhesins include cell wall mannoproteins, cell wall lipids, and chitin which has been implicated in adhesion to vaginal epithelium.(45)

The protein portion of mannoproteins is involved in adhesion of the yeast to buccal epithelial cells (BECs), endothelial cells, and host proteins consisting of arginine-glycine-aspartic acid (RGD). The hosts tolerance for *C. albicans* is affected by RGD-peptides. Interactions between the cell wall proteins of the yeast and the endothelial cells and extracellular matrix proteins of the host determine the pathogenesis of disseminated

candidiasis. The protein portion also contributes to the hydrophobic nature of the cell.(45)

The mannan portion serves as a fibrinogen receptor and sites within this portion may also play a role in adhesion to buccal epithelium. The mannans facilitate adhesion to mouse spleen and lymph node tissue. Yeast cells grown at 37°C adhere preferentially to spleen marginal zone macrophages of mice. The method of adhesion is similar to that observed in adhesion to the subcapsular and medullary sinuses of peripheral lymph node tissue. The binding system displayed by spleen marginal zone macrophages of mice is unlike that for macrophages in other portions of the spleen and in other organs like the thymus.(45)

Mannans procured from yeast cell walls displayed the ability to bind to specific locations in spleen and lymph node tissue in *ex vivo* and *in vivo* experiments. Mannan adhesins were extracted from fungal cell walls using 2-mercaptoethanol (2-ME). The carbohydrate and protein composition of the 2-ME extract was 850 and 75 micrograms per milligram (dry weight basis), respectively.(45)

Tissues from the subcapsular and medullary sinuses of the lymph node and spleen pretreated with different quantities of the extract prohibited the adhesion of yeast cells in a dose-dependent manner. The level of inhibition for the lymph node and spleen tissue were comparable. *In vivo* experiments performed on splenic tissue showed similar results. A dose-response reduction in adhesion occurred one hour after injection of the 2-ME extract. Three hours after the intravenous injection of 500μg of the extract, the level of

inhibition for binding to spleen marginal zone macrophages was greater than 80% and was still greater than 50% twenty-four hours after injection.(45)

The results for the mannan adhesins obtained by 2-ME extraction did not appear to be influenced by strain or serotype. The 2-ME extract of strain A9 (serotype B) prevented adhesion of serotype A strain 1 and serotype B strain 222. Similarly, the 2-ME extract of serotype A strain 1 prevented adhesion of serotype B strain A9.(45)

The 2-ME extract was fractionated into five fractions with the corresponding carbohydrate and protein composition, respectively, in micrograms per milligram (dry weight basis): Fraction I 220, 680; Fraction II 920, 38; Fraction IIa 970, 35; Fraction IIb 975, 33; and Fraction IIc 960, 25. The adhesion ability of Fraction IIa and Fraction IIb was markedly greater than for the other fractions.(45)

Fraction IIa had slightly greater adhesion activity than the 2-ME extract and prevented binding of yeast cells to spleen marginal zone macrophages. Subcapsular and medullary sinuses of the lymph node tissue pretreated with different quantities of Fraction IIa prohibited the adhesion of yeast cells in a dose-dependent manner. Binding was notably curtailed to the lymph node tissue even when pretreated with only 0.1 μg/ml.(45)

Heat and proteinase K digestion did not alter the ability of Fraction IIa to bind to tissue. Prior to proteinase K digestion, the carbohydrate and protein composition was 750 and 35 micrograms per milligram, respectively. Following proteinase K digestion, the carbohydrate and protein composition was 750 and 3.5, respectively. Therefore, proteinase K digestion produced an approximate 10-fold reduction in the protein content. Exposure of Fraction IIa to 20mM of sodium periodate or 5 U of α-mannosidase all but

prohibited adhesion to tissue. Similar findings for *in vivo* experiments of Fraction IIa exposed to sodium periodate or digested by proteinase K were obtained. That is, the effect of proteinase digestion on adhesion activity was insignificant while periodate oxidation prohibited adhesion activity.(45)

Mannose was found to be the primary constituent of Fraction IIa as determined by analysis of trimethylsilyl (TMS) derivatives (98% mannose) and analysis of alditol derivatives (~99% mannose). The adhesion of hydrophilic yeast cells to tissues such as the spleen and lymph node is due in part to the mannose portion of mannoproteins, while in hydrophobic yeast cells, the cell wall proteins are important for adhesion to tissues. These results indicate that the pathogenesis of disseminated candidiasis is not solely due to interactions between the cell wall proteins and endothelial cells and extracellular matrix proteins.(45)

Antigenic Determinants

An antigen (10G Ag) located in the cell wall surface and plasma membrane (plasmalemma) was discovered through its reaction with a monoclonal antibody (mAb 10G). The 10G Ag is solubilized from the cell surface using β-mercaptoethanol and zymolase. The extract was exposed to periodate oxidation, proteolytic enzymes, and heat. The former destroyed the antigenic activity of the extract whereas the latter two had no effect. The mannoproteins in the extract were separated into fractions using hexadecyltrimethyl ammonium bromide. The 10G Ag fraction was then broken down through mild acid hydrolysis and passed through a P-2 size exclusion column. A

tetrahexose was eluted from the column and determined to be the 10G Ag epitope (10G epitope, antigenic determinant). Gas-liquid chromatography, mass spectroscopy, and H-1 proton NMR analysis techniques were used to determine that the 10G epitope is a linear β-1,2-linked tetramannose.(70)

The 10G Ag and the 10G epitope conjugated to latex beads attached to the marginal zone macrophages of mouse spleen. The adherence of *C. albicans* was inhibited in *ex vivo* binding assays by the 10G Ag and the 10G epitope. The percentage of hydrophilic cells used in *ex vivo* binding assays had to be greater than 95%.(70)

As aforementioned, the cell walls of *C. albicans* are composed in large part of mannoproteins thought to be the adhesins that promote binding to host tissue and thereby may play a part in the pathogenesis of *C. albicans*. The β-1,2-linked tetramannose portion is one of the adhesin sites on the mannoprotein which might contribute to the pathogenesis of disseminated candidiasis. The β-1,2-linked tetramannosyl oligosaccharide is believed to be part of the acid-labile region of the mannan complex.(70)

Phosphate-bound oligomannosyl residues of various sizes, consisting of only β-1,2-linkages, can be released from the C. *albicans* mannans using mild acid. In contrast to native mannoproteins, acid-modified mannoproteins display poor agglutination which could be explained by the 10G epitope being part of the acid-labile phosphomannosyl side chains of the mannan. Agglutination of the 10G Ag to mAb 10G coupled to latex beads was not influenced by the presences of nonspecific proteases. The 10G epitope is part of the antigenic factor 5 determinant. Antigenic factor 5 is a portion of the phosphate-bound, acid-labile β-1,2-linked oligomannosyl residues.(70)

Either the protein or carbohydrate portion of the cell wall mannoproteins can be responsible for binding to host tissues, depending on the tissue type. β-1,2-linked oligomannoses preferentially bind to marginal zone macrophages. Mouse marginal zone macrophages have a novel adhesion system which allows for the observed interaction between yeast cells and splenic tissue. Binding to mouse spleen marginal zone macrophages is precipitated through yeast cell wall polysaccharides.(70)

One report indicates that the protein portion is responsible for binding to BECs. Upon exposure to heat, dithiothreitol, and proteolytic enzymes, the ability of cell wall mannoproteins to limit adhesion of *C. albicans* to BECs was significantly reduced or eliminated. Exposure to sodium periodate and α-mannosidase had very little, if any, influence on the ability of the mannoproteins to block adhesion of yeast cells to BECs. These results indicate that the protein portion is necessary for adhesion to BECs. Another report claims that the carbohydrate portion of factors 5 and 6 in the phosphomannan complex contributes to the binding of yeast cells to BECs. Sandin et al. (1982) showed that use of α-D-mannopyranside limited the binding of *C. albicans* to BECs.(70,76)

C. albicans monoclonal antibodies (MAb) B6.1 and B6 are of the immunoglobin M isotype or class (IgM), with the former conferring increased resistance against experimentally disseminated candidiasis in inbred mice, outbred mice, SCID (severe immunodeficiency disease) mice, and mice with newly acquired and advanced neutropenia, while the latter antibody did not. MAb B6.1 and MAb B6 are specific for yeast cell wall mannan.(29)

The MAb B6.1 epitope and MAb B6 epitope are nearly absent from the surface of hyphae as revealed through epiflourescent observation of hyphae stained with one of the monoclonal antibodies. The B6.1 and B6 epitopes are part of the cell wall phosphomannan (PM) complex. Confocal images indicate that the B6.1 epitope is apportioned evenly across the surface of stationary phase yeast cells, whereas the B6 epitope is unevenly expressed. Thus, the MAb B6.1 and MAb B6 show specificity for different epitopes.(29)

The composition of the PM complex differs for serotype A and serotype B. In serotype B, the PM consists of an α-(1-6)-linked mannan backbone with α-(1-2)-linked oligomannosyl side chains of different sizes. The oligomannosyl side chains contain principally α-(1-2)-linkages although α-(1-3)- and α-(1-6)-linkages are also present. Under certain growth environments, the oligomannosyl side chains can have phosphate linked oligomannosyl side chains which contain only β-(1-2)-linkages. The phosphodiester bonds can be hydrolyzed and thereby the β-(1-2)-linked side chains liberated by boiling in 10mM HCl. Thus, this portion of the PM is referred to as the acid-labile portion (PM-AL). The backbone and α-(1-2)-linked side chains are stable under mild acid treatment and therefore are termed the acid-stable portion of the PM (PM-AS). The PM of serotype A is nearly the same as that of serotype B, except the PM-AS portion contains some non-phosphodiester-linked β-(1-2)-oligomannosyl side chains.(29)

Thus, the PM-AS and PM-AL portions can be separated by acid hydrolysis and size exclusion chromatography. The PM-AL portion elutes according to mannan chain length and the PM-AS portion appears in the void volume. The B6 epitope was shown to

be part of the PM-AS portion through dot blot analysis performed on nitrocellulose (NC). The interaction of MAb B6 with PM blotted on NC was prevented by PM-AS portion and PM. The PM also inhibited the interaction of MAb B6.1 with the PM blotted on NC, whereas the PM-AS portion did not. Thus, the B6.1 epitope is not part of the PM-AS portion. These findings were also observed in agglutination reactions in which the PM-AS portion agglutinated to MAb B6 coupled to latex beads, but not to those coupled to MAb B6.1. The mannan character of the B6 epitope was displayed by the inability of the PM-AS portion treated with α-mannosidase to interact with MAb B6 coupled to latex beads. Treating the PM-AS portion with protease had no effect on its interaction with MAb B6 coupled to latex beads.(29)

The B6.1 epitope is part of fractions III and IV of the PM-AL portion, whereas the B6 epitope is not associated with the PM-AL portion. The B6.1 epitope is mostly in fraction III as indicated by the increased ability of fraction III over fraction IV to prevent interaction between MAb B6.1 and PM. The presence of both fractions did not increase the blocking ability. Therefore, fraction IV either has an epitope with less of a propensity for the monoclonal antibody or else it contains the epitope in addition to other molecular groups.(29)

Fraction IV is composed of two molecular groups, one a mannotriose and another a mannotetraose. Fraction III is composed primarily of isomers of mannotriose which are in equilibrium with the preponderance being in the α-configuration at the reducing terminus. The B6.1 epitope is a β-(1-2)-mannotriose.(29)

Adhesion of Fungi and Bacteria and Subsequent Biofilm Formation

Fungal Adhesion and Biofilms

C. albicans can adhere to a variety of biomedical implants, including contact lenses, prosthetic devices, pacemakers, artificial joints, and urinary, central venous, or peripheral catheters. Microorganisms can attach to these biomaterials, creating microcolonies through cellular division that are sheathed within an extracellular polymeric matrix. The mono- or multilayers of cells together with the extracellular material forms a protective barrier, a biofilm. The biofilm provides a setting where the yeast can proliferate and can subsequently be released into the surrounding fluids and tissues. Release of these microorganisms may promote acute disseminated infections. Often the implants must be removed in order to eliminate the associated infections. These infections are relentless because the microorganisms within the biofilm are unreachable by both host defenses and antibiotics.(32,92)

In nature, microorganisms do not exist as pure planktonic cultures but rather are a conglomeration of multiple species that are associated with surfaces. Multiple species biofilms display interspecies cooperation and form complex structures. *C. albicans* and *Candida dubliniensis* have been procured simultaneously from the mouths of immunocompromised individuals. Increased adhesion of *Candida* species in an oral environment is related to sucrose- or glucose-rich diets, acidic pH, cell surface hydrophobicity, and cell surface mannoproteins.(48)

Growth competition between *C. albicans* and *C. dubliniensis* was examined under both sessile and planktonic growth conditions. Sabouraud liquid broth modified antibiotic medium 13 (SDB) was added to culture tubes. Some of the culture tubes also contained a piece of PVC which is used in the fabrication of feeding tubes and urethral catheters. *C. albicans* existed at greater levels than *C. dubliniensis* under planktonic growth conditions in SDB , independent of the minimum inhibitory concentration (MIC), with *C. dubliniensis* being imperceptible in 2 out of 11 sets of tubes and comprising 8 to 35% for each of the other 9 sets of tubes. After 96 hours of growth, only 2 of the 11 sets of tubes still contained *C. dubliniensis*.(48)

A monoculture may emerge in planktonic cultures due to competitive forces which result in the diminished presence of competitors. If the microorganisms are exposed to consistent conditions of pH, temperature, nutrient levels, and waste removal, then the phenotypes or organisms with enhanced growth rates tend to predominate with time. Under planktonic growth conditions in SDB, the log-phase cell doubling rate was greater for *C. albicans* than for *C. dubliniensis* in 10 out of 11 sets of assays with one exception, Ca1649 at 96 hours.(48)

After 96 hours of growth, *C. albicans* subsisted at greater levels than *C. dubliniensis* under sessile growth conditions in SDB, independent of MIC, with *C. dubliniensis* being imperceptible in 6 out of 11 sets of tubes and comprising 1 to 52% for each of the other 5 sets of tubes. In biofilms, population differences that result from variations in growth rates are not as noticeable as in suspension cultures.(48)

With an equivalent number of colony forming units (CFU), *C. albicans* has a greater competitive advantage over *C. dubliniensis* in suspension culture as compared to growth under biofilm conditions. In biofilms, the cells are spatially organized. The cells near the nutrient source are generally of a different phenotype than those that are located deeper within the biofilm close to the substratum. The heterogeneity of biofilms allows yeast to subsist together, thereby increasing biofilm survival. Whereas under planktonic growth conditions, the yeast succumb to competitive forces.(48)

Candidiasis can be chronic with the preponderance of manifestations being associated with biofilms which can form on either inert or biological surfaces and appear in both mucosal and systemic sites. The sessile cells of biofilms are phenotypically distinct from their free-floating planktonic counterparts and are frequently resistant to antimicrobial agents. The National Committee for Clinical Laboratory Standards (NCCLS) investigation of *in vitro* susceptibility specifies the utilization of free-floating planktonic cells. This method of testing doesn't allow for reliable *in vitro - in vivo* correlation in infections attributed to biofilms due to the phenotypic disparity of sessile and planktonic cells and the increased antimicrobial resistance of sessile cells. Therefore, an alternate testing strategy was derived by Gordon Ramage et al.(2001). This antifungal susceptibility test is a microtiter-based colorimetric assay which measures the metabolic activities of sessile cells through the reduction of formazan salt. There are other means of quantifying biofilms, and thereby measuring the antimicrobial susceptibility, which include microscopic methods, plate counting, metabolically active dyes, radiochemistry, and luminometry.(66)

There are many redundant copies of proposed virulence genes in the *Candida* species which makes elucidation of the virulence function by mutational analysis difficult. Reynolds and Fink (2001) believe that since *S. cerevisiae* is capable of undergoing the initial stages of biofilm formation, it may prove a valuable tool in discerning the role of cell surface proteins in pathogenesis. They also conclude that *S. cerevisiae* might be beneficial in evaluating compounds for their ability to block fungal adhesion.(71)

The adhesion of *S. cerevisiae* to plastic surfaces increases with a decreasing concentration of glucose, from 2% to 0.1% glucose. However, in the absence of glucose, the adhesion is reduced. FLO11 is a gene encoding a cell surface glycoprotein (Flo11p) which is necessary for adhesion to agar. Glucose is thought to restrict FLO11 transcription. FLO8 is a gene encoding a regulatory protein needed for the expression of FLO11. At low glucose concentrations, genetically uniform strains, missing either the FLO11 gene (flo11Δ) or the FLO8 gene (flo8Δ), demonstrated diminished adhesion to polystyrene. FLO11 is important for adhesion to plastics, the multicellular morphology displayed on 0.3% agar plates, and invasive growth.(71)

The Flo11p cell surface protein is necessary for cell-cell adhesion as observed in filamentous growth and cell-surface adhesion. Flo11p must have distinct attributes from other cell surface proteins that allow for the initiation of biofilm formation. A related cell surface protein, Flo1p, also plays a role in cell-cell adhesion, but does not allow for cell-surface adhesion. The function of Flo11p in the adhesion of *S. cerevisiae* to plastics may be similar to that observed for the cell surface glycopeptidolipids (GPLs) of the nonflagellated bacterium *Mycobacterium smegmatis*. The GPLs are considered to be

necessary for biofilm formation and the sliding function in this bacterium. These phenomenon are attributed to the increased hydrophobicity of the cell surface by the GPLs.(71)

The hydrophobicity of FLO11 and flo11Δ strains were examined through partitioning between water and octane phases. Twelve percent of the FLO11 strain partitioned into the aqueous phase, while 91% of the flo11Δ strain partitioned into the aqueous phase. Therefore, the FLO11 strain displays increased hydrophobicity. This hydrophobic property is thought to be responsible for the adhesion of the yeast to plastic surfaces. The hydrophobic property is also believed to be the driving force for the mat formation observed on 0.3% agar plates by decreasing the interaction between the yeast and the aqueous surface. The reduced adhesion to the surface allows the yeast to move across the surface more freely. The pattern observed in the mat formation is thought to be due to frictional forces and cell-cell interactions.(71)

Orthologs of Flo11p exist in the opportunistic pathogens *C. albicans* and *Candida glabrata*. Both form mats and the former also produces biofilms, as aforementioned. Reynolds and Fink (2001) hypothesize that these orthologs of Flo11p are virulence factors, since expression in *S. cerevisiae* led to adhesion to mammalian cells.(71)

Not all biofilms are destructive, some provide beneficial services such as in bioremediation. The fungi *Trametes veriscolor* (*coriolus*) and *Neurospora crassa* have potential applications in bioremediation in the removal of organic pollutants such as phenols from water.(74)

Bacterial Adhesion and Biofilms

Biofilms are observed in a number of different locales including ship hulls, drilling pipes of oil wells, food fermentors, and dental plaque of humans. In humans, biofilms are also associated with diseases such as upper respiratory infections, kidney stones, prostate infections, urogenital infections, periodontal disease, Legionnaire's disease, peritonitis, and infections of the middle ear. Biofilms that form on permanent medical implants are responsible for approximately 10 million infections each year in the United States. Although these infections form slowly, they are also very difficult to eliminate.(6,68) Implanted medical devices such as artificial veins, joints, hearts, heart valves, catheters, and stents are susceptible to biofilm formation and disease, because they serve as binding sites for microorganisms and host defenses do not provide adequate protection to these locations.(68)

The formation of biofilms by pathogens such as *Staphylococcus epidermidis* and *Staphylococcus aureus* on enclosed implants pose a life-threatening condition. In North America, more than 100 million urethral catheters and urinary stents are used each year. In the absence of antibiotic treatment, the infection rate can be as great as 28% with ureteral stents and 100% with catheters and can result in death. Biofilms form on surfaces coated with host proteins or other substances, constituting a conditioning film. The presence of a conditioning film on a medical device is considered an initiation stage for infectious biofilm formation. The conditioning film coats the device upon exposure to body fluids, including saliva, blood, and urine. The conditioning film consists of

macromolecular components, several of which are proteinaceous such as serum albumin, fibrinogen, collagen, and fibronectin. Some of these proteinaceous components have been found to influence bacterial adhesion. Due to the significance of the conditioning film and the initial adhesion events, three regions of the biofilm should be considered. The regions include the "linking film, base film, and surface film".(68)

The linking film is the conditioning film composed of organic material (e.g., carbohydrates, proteins, glycoproteins, electrolytes) from the bulk phase that diffuses to the surface within 15 minutes upon exposure. The linking film imposes a net negative surface charge which neutralizes or changes surface properties such as the surface free energy. Bacterial fimbriae, stretching across the free energy barrier, interact with the linking film through short-range, reversible electrostatic, covalent, or hydrogen bonds.(68)

At small distances ($\leq$1-10 nm), attractive and repulsive forces (Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory forces: London van der Waals forces) influence the adhesion of microorganisms to the surface. The DLVO theory provides for two minima, the primary and secondary minimum. The primary minimum refers to short distances ($\leq$1-10 nm) and the secondary minimum corresponds to greater distances (5-10 nm) between the surface and the assailing microorganism. The DLVO theory indicates that the repulsive electrostatic energy barrier decreases with decreasing bacterial radius. For instance, the gram-positive coccal has a small radius and displays a natural tendency to adhere to medical devices.(68)

The surface film allows for the transport of nutrients to the biofilm but hinders the movement of antimicrobial agents. The viability of microbes within a biofilm is affected by

diffusion which influences mass transport and growth. The biofilm thickness is affected by the transport of nutrients such as oxygen, fluid frictional resistance, and the efficiency of heat transfer. The solute flux within a biofilm is a constant proportional to the length of migration. The host conditioning film provides a foundation for the development of a mature biofilm. If the link between the conditioning film and the biofilm can be compromised or eliminated through means such as shear force fluctuation or surfactants, then it may be possible to eliminate an infection by sloughing off the biofilm or hindering its formation.(68)

The complex biofilm structures allow organisms to communicate and share information with one another and with their environment. It seems plausible that organisms within a biofilm could share genetic information similar to that observed in plasmid transfer between *E. coli* and gram-positive bacteria. Organisms within a biofilm communicate with their environment through signal transduction proteins which gather information and pass it on to chromosomal elements. *E. coli* may have more than 50 pairs of regulatory proteins which respond to specific stimuli. Communication allows pathogens to orchestrate a group virulence response.(68)

Antimicrobial agents such as silver and ciprofloxacin (fluoroquinolone) have been shown to reduce pathogen colonization of medical devices and subsequent infections. It has been hypothesized that oral antibiotics reduce biofilm formation and infection by adsorbing to the surface of medical devices. The outcome from experiments on 21 devices support the validity of the hypothesis. Device-related infections can be combated

with antimicrobial agents if the biofilm is new (i.e., ≤24hr), but longstanding biofilms currently require device removal to eliminate the infection.(68)

Mah and O'Toole (2001) have proposed that several mechanisms of resistance operate through a synergetic approach.(57,82) Stewart (2001) presents two conceivable mechanisms by which bacterial biofilms remain recalcitrant. Bacteria within biofilms do not display antibiotic resistance mechanisms employed by their free-floating counterparts. Free-floating bacteria evade antibiotics through enzymes that modify the antibiotic or through mutations of the targeted region. In biofilms, however, even strains that are sensitive to antibiotics, lacking a genetic disposition for resistance, are safeguarded. Therefore, understanding biofilm resistance requires looking for different genes and methods of protection.(82)

The bacteria within a biofilm mount resistance towards antibiotics through a multicellular endeavor. This multicellular endeavor requires a group approach and the concerted effect in which a consortium of bacteria activate specialized resistant genes that function together. An example is the inability of hydrogen peroxide to completely penetrate a biofilm due to degradation by catalase produced by bacteria. A single bacterium would be unable to tolerate an equivalent amount of hydrogen peroxide, but acting as a group allows for increased protection of the biofilm. The catalase activity is not determined by the viability of the bacteria. Therefore, bacteria within the surface layers are capable of breaking down the oxidant even after they have expired thereby protecting their neighbors deeper within the biofilm.(59,82)

Another example of the communal effort used to thwart the impact of antibiotics is based on aerobic conditions within the biofilm. The interior of the biofilm offers protection from antibiotics due to a lack of oxygen which is required for the function of some antibiotics. Bacteria in the outer layers of the biofilm, the aerobic zone, guard their deeper neighbors by consuming the oxygen. Even damaged bacteria can continue to deplete oxygen from the environment. This is a group effort since an individual bacterium is not capable of consuming enough oxygen to inhibit the activity of the antibiotics.(59,82)

An additional means by which biofilm bacteria confer resistance to antibiotics is through differentiation. Organisms of the same species within the biofilm may take on a static, spore-like state or may grow and reproduce. In the spore-like state, the bacteria are protected from many chemical and physical assaults while those that are growing are sensitive to antibiotics. Having bacteria within both states allows the biofilm to recover from aggressive actions. This protective strategy is necessarily a multicellular approach to antimicrobial resistance since it is impossible for one cell to occupy both states at once. Thus, although the majority of the bacteria are growing and subject to the antimicrobial agents, the minority which occupy the spore-like state are unresponsive to the antibodies and will persist to reseed the biofilm. This explains why antibiotics can suppress the effects of bacterial biofilms for a short period before they reappear.(59,82)

Aside from a physiologically based mechanism described above as a consequence of differences between the sessile and planktonic microorganisms, the recalcitrance of microbial biofilms to antimicrobial chemotherapy could be due to a transport-based mechanism whereby the biofilm hinders antibiotic diffusion. The diffusion limitations of a

non-interacting solute which does not sorb or react in the biofilm are not sufficient to explain the resistance to antimicrobial agents. The diffusion (Fickian diffusion) barrier for a reverse sorbing, nonreacting solute is not large enough to justify the increased tolerance to antibiotics. The inability of antibiotics to penetrate biofilms cannot be explained by reversible sorption. The penetration of irreversibly sorbing, nonreacting solutes is significantly hindered and this level of antibiotic retardation could explain the biofilm recalcitrance. In the case of an irreversibly sorbing, nonreacting antibiotic, the antibiotic and the biofilm binding site are "permanently sequestered". Thus, any inquiry into the binding of antibiotics to cells or other biofilm constituents should consider whether the sorption is reversible or irreversible since the degree of retardation depends upon this characteristic. However, there is no indication that there is extensive irreversible sorption of antibiotics to biofilm constituents.(83)

The diffusion of a stoichiometrically reacting solute is the same as that for irreversible sorption. The difference is that the antimicrobial agent reacts in a stoichiometric manner with a biofilm reaction site, thereby neutralizing the effect of both the antibiotic and the reaction site. There is no clear indication that diffusion of a stoichiometrically reacting solute can justify the reduced susceptibility of biofilms to industrial biocides such as chlorine.(83)

The catalytically reacting, nonsorbing solute can explain the recalcitrance of biofilms to antimicrobial agents if the reaction is fast enough. Antibiotics such as beta lactams react sufficiently fast to reduce penetration. However, the vast majority of antibiotics do not react rapidly enough to account for the increased tolerance. To

determine the potential effect of a reaction on solute transport, one must assertain whether the reaction is catalytic or stoichiometric, and the extent and rate of the reaction. Similarly, to understand the impact of sorption on solute transport, one must determine whether the sorption is irreversible or reversible, and the extent and rate of sorption.(83)

A theoretical investigation of the diffusion of antimicrobial agents in a biofilm reveals that transport limitations can account for the biofilm recalcitrance if the antibiotic reaction is rapid or sorption is irreversible. Transport limitation may play a role in the recalcitrance of biofilms to antimicrobial agents, but there are probably other mechanisms involved. A physiology-based mechanism as previously discussed, in which phenotypically altered and slow growing microorganisms exist, could serve as another mechanism. The transport models offered here fail to take into account two characteristics of real biofilms: external mass transfer resistance and structural heterogeneity. The external mass transfer resistance hinders transport, and structural heterogeneity tends to increase diffusion in most cases.(83)

In nature, bacteria form biofilms, sessile communities, which are physiologically and morphologically distinct from their planktonic counterparts. Biofilms form thick layers on solid surfaces which are exposed to a continuous source of nutrients. The biofilms differentiate into "mushroom- and pillar-like" formations that are demarcated by channels of water. The structures consist of an extracellular polysaccharide (EPS) matrix or glycocalyx within which bacteria are enveloped. Some bacteria such as the fruiting bacteria *Myxococcus* create macroscopic structures through communication. Common bacteria display similar activity in the proper environments.(10)

Cell-to-cell signaling plays a role in the biofilm differentiation of *Pseudomonas aeruginosa*. Differentiation of *P. aeruginosa* involve at least two extracellular signals which play a role in cell-to-cell communication and in the production of virulence components that depend on the size of the population. Two known cell-to-cell signaling processes in *P. aeruginosa* are the *las R - las I* and the *rhl R - rhl I*. The gene product of the former is necessary for the production of the diffusible extracellular signal N-(3-oxododecanoyl)-L-homoserine lactone ($3OC_{12}$-HSL), and the latter is responsible for the creation of the extracellular signal N-buytryl-L-homoserine lactone. Concentrations of the signaling molecules attain levels necessary for gene activation with suitable population size. This type of gene regulation is termed "quorum sensing and response".(10)

The wild-type (PAO1) biofilms consist of microcolonies which are cell clusters distinguished by water filled voids. Both the wild-type and the *las I - rhl I* double mutant adhere and grow on a glass surface, attaining steady-state within 2 weeks. Thus, the initial events of biofilm formation proceed as expected. The *las I - rhl I* double mutant produces continuous, densely packed, thin biofilms with a thickness which is approximately 20% that of the wild-type and doesn't generate quorum sensing signals. The biofilms produced by the *rhl I* mutant are similar to those formed by the wild-type with regard to thickness and cell packing. The *las I* mutant, on the other hand, creates biofilms whose thickness and cell packing are like those of the double mutant. The *P. aeruginosa* signal mutant, *las I*, forms flat, undifferentiated biofilms which in contrast to the wild-type (PAO1) are susceptible to the biocide sodium dodecyl-sulfate (SDS). The mutant biofilm appears normal, however, in the presence of synthetic signal molecules. Thus, the quorum sensing

signal $3OC_{12}$-HSL is necessary for normal biofilm differentiation. In the absence of adequate levels of this signaling molecule, the initial phases of biofilm formation, adhesion, and growth are normal, but the mature differentiated biofilm observed with the wild-type does not develop.(10)

The amount of EPS matrix in wild-type and *las I* mutants was determined by measuring the level of uronic acids (component of EPS alginate) and total carbohydrates. Both the wild-type and the *las I* mutant displayed similar levels of both uronic acids and total carbohydrates. The wild-type biofilms have similar amounts of EPS matrix as do their planktonic counterparts. The apportionment of glycocalyx differs between planktonic and sessile cells. The glycocalyx associated with planktonic cells is "compressed and incomplete". The glycocalyx of mutant biofilms is similar to that observed with planktonic cells and may explain the close packing manifested by mutant biofilms.(10)

Exposure of *P. aeruginosa* biofilms to 0.2% SDS had no effect on the wild-type strain, but within 5 minutes, the vast majority, if not all, of the *las I* mutant biofilm bacteria were released from the surface. However, in the presence of the synthetic signal molecule $3OC_{12}$-HSL, up to 24 hours after exposure to SDS no effect on the mutant biofilm was observed with the thickness of the biofilm being similar to that observed with the wild-type. The thickness of the mutant biofilm was 93 ± 21 μm initially and 24 hours after exposure, whereas the wild-type biofilm thickness was 102 ± 21 μm initially and 24 hours after exposure. Based on these findings, preventing cell-to-cell signaling necessary for normal biofilm differentiation may be useful in biofilm prevention.(10)

Biofilms consist of many microcolonies secured by extracellular matrix and bounded by water channels that carry nutrients and waste to and away from the microcolonies. Within a microcolony, the exterior cells receive adequate nutrients and waste removal, whereas those amenities are diminished for the interior cells. The interior cells are surrounded by numerous other cells and organic matrix which minimize the transport of water in this region. Therefore, the interior cells are limited to those substances that can diffuse into the microcolony. However, since the extracellular matrix is composed primarily of water, many small molecules move with ease, although the movement of molecules can be limited if they react with the biofilm cells or with the extracellular matrix. These chemical reactions can create small-scale variations in the surroundings.(6)

The oxygen levels can differ widely between two locations that are in close proximity, as near as five hundredths of a millimeter. The oxygen level can serve as a physiological indicator. For instance, biofilms of *P. aeruginosa* (associated with cystic fibrosis pneumonia) require oxygen for cellular activity and growth. Thus, those cells in the outer two to three hundredths of a millimeter of each microcolony are active and growing, whereas those within the interior are in an inactive state. This variation in metabolic states is generally not observed in suspension cultures which normally display a single metabolic state. Due to the chemical variations in the surroundings, two cells, which may be the same genetically, can display dissimilar appearances and behaviors. Thus, some cells may produce deleterious effects on the host through the development of toxins and other elements that induce disease while other cells may pose little threat.(6)

Chemicals used in medicine and industry are effective against planktonic bacteria, but fail to combat biofilm bacteria that have enhanced resistance. Biofilm bacteria also elude immune system molecules. The increased resistance of biofilm bacteria to antibiotics and antiseptics, as indicated earlier, may be due in part to an inability to penetrate the biofilm. The limited penetration of penicillin into biofilms is the result of break down by beta-lactamase enzymes at a rate greater than that of diffusion. Thus, penicillin is prevented from reaching the deeper layers of the biofilm. Penicillin targets the replicating cells of several bacteria species. Thus, the interior bacteria that have limited nutrient exposure, and are in an inactive, nonreplicating state, escape the damaging effects of penicillin whereas the exterior, replicating cells are destroyed. The destroyed cells serve as nourishment for those that are living and are able to reconstruct the biofilm within a few hours.(6)

Bleach, a reactive oxidant, has difficulty with eliminating biofilms. However, given enough time and bleach, it can eventually work its way through the biofilm. Antimicrobial agents that diffuse readily in the biofilm may destroy planktonic cells, but not those associated with the biofilm due to the presence of various metabolic states and types of bacteria. Thus, information obtained from studies of planktonic cells treated with antimicrobial agents does not provide much insight into how to tackle biofilms.(6)

Once bacteria adhere to a surface and form biofilms, they begin to produce large numbers of proteins, some of which are involved in movement across the surface prior to assuming a fixed position. These proteins are not generated by planktonic cells. Within 15 minutes after adhering to a surface, *P. aeruginosa* expresses several genes. One such

gene is algC whose expression is necessary for the production of alginate, which is a principal component of the extracellular matrix.(6)

When bacteria begin to generate biofilms, certain genes are expressed as a result of communication between microorganisms. As described previously, *P. aeruginosa* and related bacteria generate acylated homoserine lactones, signal molecules, in small amounts. When a sufficient number of bacteria are together, the amount of signal molecules increases to a level which alters the activity of several genes needed to produce a biofilm. As we saw earlier, this type of gene regulation is termed "quorum sensing". Biofilms that develop on urinary catheters and other surfaces employ signal molecules.(6,10)

In order to limit biofilm formation, drugs that are directed toward the novel attributes of biofilm bacteria are needed. In order to prevent the formation of the extracellular matrix, surfaces could be treated with chemicals that block these genes. Suppression of the *Staphylococcus epidermidis* genes necessary for the production of extracellular matrix prevented biofilm formation in both test tubes and in the tissue of laboratory animals. To prevent microorganisms from adhering to a surface and consequently biofilm formation, one could introduce molecules that would bind to the sites used by the microorganisms for attachment. By interfering with the signaling molecules, one could control the ability of the bacteria to form biofilms, generate toxins, and other detrimental agents.(6)

A group of individuals in Denmark and Australia have begun to develop chemical compounds to target bacterial communication. The inspiration for developing such chemical compounds came about as a result of observing the marine alga *Delisea*

pulchra.(59) In 1995, Staffan Kjelleberg and Peter Steinberg in Australia observed that biofilms did not develop in general on the fronds of the red alga *Delisea pulchra*. This lack of biofilm formation was attributed to substituted furanones. These substituted furanones occupy locations on bacteria that are normally taken up by signal molecules and thereby prevent the signal molecules from cuing the development of biofilms. The substituted furanones have been shown to not only inhibit biofilm formation, but promote the dispersion of biofilms that are already present. The use of substituted furanones in medical applications looks promising since it is not toxic and fairly stable in humans. Also, after exposure to furanones for millions of years, bacteria present in the oceans have not developed a tolerance.(6,11)

Dental plaque biofilms are composed of organisms that constitute more than 500 species within more than 30 genera. The formation of the biofilm is well orchestrated, beginning with initial adhesion by gram-positive bacteria, primarily streptococci. Later stages of plaque formation entail adhesion of gram-negative anaerobic bacteria including *Porphyromonas gingivalis*. Attachment of such bacteria indicates a turning point in the status of the biofilm from that of a commensal entity to one that is pathogenic. *P. gingivalis* is associated with acute adult periodontitis.(89)

P. gingivalis adheres to oral surfaces through fimbria which permits subsequent colonization. The *fimA* gene expression allows for production of a primary fimbriae protein subunit (FimA). The expression of the *fimA* gene is controlled through environmental factors and signaling molecules produced by plaque bacteria. *Streptococcus cristatus* produces a signal specific for modification of *fimA* gene

expression which prevents *P. gingivalis* biofilm formation. The signaling method taking place within the biofilm was studied using *P. gingivalis* strain UPF which contains a *fimA* promoter and lacZ reporter gene through chromosomal fusion. The level of *fimA* gene expression was used to determine the signaling activity of five bacteria: *Actinomyces naeslundii* NC-3, *Streptococcus cristatus* CC5A, *Streptococcus mutans* KPSK2, *Streptococcus sanguis* 10556, and *Streptococcus gordonii* G9B and M5. The protein concentration of the surface extract supernatant from each bacteria tested was measured. The level of *fimA* gene expression was measured after exposure of *P. gingivalis* UPF (10^5 cells) to the surface extract. The results indicate that *S. cristatus* CC5A significantly decreased *fimA* expression while the other biofilm bacteria tested did not.(89)

Exposure of *P. gingivalis* to *S. cristatus* CC5A extract reduced *fimA* gene expression in a dose-dependent manner. That is, increasing the extract concentration decreased *fimA* gene expression by 12 fold relative to controls. By reducing *fimA* gene expression and thereby fimbria-mediated adhesion, *S. cristatus* inhibited colonization by *P. gingivalis* which was prone to removal by saliva due to diminished ability to adhere to the oral surface. In contrast, *S. gordonii* provides for *P. gingivalis* adhesion to oral surfaces through specific adhesin-receptor interactions such as those between FimA and molecules on the surface of *S. gordonii.*(89)

Fungal and Bacterial Biofilms

The scope of biofilm description is changing from that of a collective characterization of structure and function to one detailing the activities and properties of

single cells, clusters, and microcolonies. Biofilms are dynamic bodies whose structural development is influenced by the physical interactions between organisms, nutrient supply, hydrodynamic flow, and the movement of cells which facilitates dissemination and diversification. Physical, biological, and environmental factors influence the biofilm during its various stages of life. Initial adhesion is a random event that depends on the surface free energy and the proximity of the organism to the surface. The initial adhesion event is followed by cell division and colonization of the surface. These latter events allow for additional cell adhesion and accumulation, resulting in the formation of a "linking film" followed by a multilayered community. Biofilms are heterogeneous with organisms of increased resistance due in part to diminished metabolic rates. Simon Pickering (2000) showed that the antibiotic sensitivity of *Staphylococci* can be enhanced electromagnetically.(42,63) Julia Douglas (2000) discovered that coadhering oral *S. gordonii* reduced the susceptibility of *C. albicans* biofilms to antifungal agents. Extracellular polymeric substance (EPS) may impart mechanical strength to biofilms and aid in the arranging of cellular populations, but it is not necessarily a diffusion barrier to inhibitory compounds.(15,42)

Both *S. epidermidis* and *P. aeruginosa* bind to biofilms of *C. albicans* that form on vascular catheters. *C. albicans* and *C. tropicalis* have been shown to adhere to oral biofilms of *S. gordonii* while *C. krusei* and *C. kefyr* do not. *C. albicans* expresses a receptor for a complex cell wall polysaccharide of *S. gordonii*. The adhesion of *C. albicans* to *Streptococcal* spp. is increased ten fold under conditions of glucose starvation. The binding of *C. albicans* and *C. krusei* to denture acrylic surfaces is

hindered by high quantities of bacteria such as *Porphyromonas gingivalis*, *S. sanguis*, and *S. salivarius*, but not by *E. coli*. When *C. albicans* is grown in sucrose together with *S. mutans*, there is an increase in binding of *C. albicans* to acrylic over that observed in the absence of *S. mutans*. Adhesion of *C. albicans* to epithelial cells is enhanced by the presence of *E. coli* (piliated bacteria) over that of *Klebsiella pneumonia* (non-piliated bacteria). The introduction of *C. tropicalis* prior to or in conjunction with that of *C. albicans* hinders the binding of *C. albicans* to PS. Indigenous microflora of Syrian hamsters form a thick layer in the mucus gel, hindering the adhesion of *C. albicans* by taking up binding sites and synthesizing inhibitory substances such as volatile fatty acids and bile acids.(19)

Poly(ethylene oxide) and Pluronic

Highly hydrophilic surfaces are non-thrombogenic because the low blood-material interfacial tension creates a low driving force for adsorption of blood proteins. Grafting of hydrophilic polymers such as poly(ethylene oxide) (PEO) onto polymer surfaces produces a hydrophilic surface with low interfacial tension to aqueous solutions which thereby reduces the adsorption of proteins. The propinquity of protein molecules to the surface is limited by excluded volume effects and decreasing configurational entropy of mobile PEO chains.(21)

Glass, PS, and polyethylene (PE) surfaces, modified through adsorption of amphiphilic PEO block copolymers, substantially diminished the adhesion of albumin, fibrinogen, and blood platelets in comparison to the unmodified surfaces. Aside from

surface modification through adsorption, the surface of a polymer matrix can also be modified by blending with an amphiphilic polymer by which the amphiphile migrates to the surface provided that there is a thermodynamic driving force. The advantage of the blended surface is that the surface can be regenerated as amphiphiles are removed from the surface. The rate at which the amphiphiles desorb or leach from the surface depends on the hydrophobic character of the amphiphile. That is, the more hydrophobic the amphiphile, the slower the leach rate. The toxicity and effect of amphiphiles on elements such as blood plasma is not known. In generating blended surfaces, the concentration of the amphiphilic polymer in the polymer matrix should exceed the critical micelle concentration (CMC). This allows for macrophase separation and micellar morphology formation, which thereby affects the surface properties of the polymer matrix. The degree of surface enrichment depends on the hydrophilicity/ hydrophobicity of the amphiphile in relation to the matrix, the environment, and amphiphile diffusion properties.(21)

The adhesion of human epithelial cells (HepG2) to a Pluronic F68 and collagen pre-conditioned polystyrene (PS) surface depends on the hydrophobicity of the surface. Adhesion of HepG2 cells to bacteriological grade polystyrene (BGPS) was inhibited by pre-conditioning with Pluronic and collagen, whereas adhesion was not prohibited on pre-conditioned tissue culture polystyrene (TCPS) which is less hydrophobic than BGPS. This same adhesion phenomenon was observed when Pluronic F68 was exposed to the surface simultaneously with extracellular matrix (ECM) proteins (e.g., collagen, laminin, and fibronectin) that were directly secreted by the cells. The efficiency of HepG2 cell adhesion to PS is a function of collagen adsorption. The collagen competes with the surfactant.

The BGPS with greater hydrophobicity favors adsorption of Pluronic F68 over collagen, thereby preventing subsequent cell adhesion. However, the less hydrophobic TCPS favors adsorption of collagen over Pluronic F68.(13)

This manner of binding of proteins and human cells to surfaces of differing hydrophobicity was also displayed on oxygen-plasma treated surfaces. A patterned surface with tracks of greater hydrophilicity were generated on polystyrene using photolithography and oxygen plasma treatment. The patterned substrates were pre-conditioned using a solution of Pluronic F68 and extracellular matrix (ECM) proteins (e.g., type I collagen or fibronectin). The adsorption of ECM proteins and subsequent adhesion of mammalian cells occurred preferentially on the tracks with lower hydrophobicity.(12)

Pluronic F68 is a copolymer of polyethylene glycol and polypropylene glycol which has been utilized in animal and insect cell cultures as a means of protecting the cells against shear stress. In 1990, Ramirez and Mutharasan proposed that the protective mechanism of Pluronic F68 was mediated by decreasing the plasma membrane fluidity as a result of direct interaction with the plasma membrane.(41,67) Pluronic F68 has been shown to interact with cell membranes, altering membrane permeability. The addition of the surfactant Pluronic F68 at a concentration of 4g/L to *S. cerevisiae* fermentation medium resulted in an extended lag phase without significantly affecting the biomass concentration.(20,41) In 1996, Laouar et al. indicated that a 1% Pluronic F68 solution did not affect the growth or flocculation of *S. cerevisiae*.(41,52)

Hellung-Larsen et al. (2000) found that Pluronic F68 was toxic at concentrations of 0.2% w/v, but not at lower concentrations of 0.02% and 0.002%, towards low inocula cultures (2 cells/ml) of *Tetrahymena thermophila* in chemically defined medium exposed to a surface. At the lower concentrations, Pluronic F68 protected the cells from death as a result of exposure to the liquid-air interface. Concentrations of 0.001% to 0.1% of Pluronic F68 were also shown to be protective. The investigators found no indication that Pluronic F68 was being absorbed by the cells or was adhering strongly to the cells. In fact, Pluronic F68 had to be present in the solution for the perceived effects to occur. All the tests except that for starvation kinetics were conducted at low inocula concentrations (10, 25, or 100 cells/ml) in order to prevent conditioning of the medium. It had previously been found that 25 cells did not succumb to a surface-mediated cell death upon addition to a medium altered by the death of approximately 100 cells in 1 ml. Hellung-Larsen et al. (2000) believe that during starvation the release of enzymes is not attenuated by Pluronic F68 . The Pluronic serves to stabilize the nonconditioned cells (25 cells/ml) during starvation, prolonging their existence. Pluronic, however, does not have an effect on cultures of higher concentration (conditioned cultures, 10^4 - 10^5 cells/ml). In addition to guarding *T. thermophila* cells from chemical and/or physical stress, Pluronic F68 also garrisoned the toxic effects of calcium and magnesium ions.(41)

PEO-PPO-PEO block copolymers are surfactants that are commercially known as Poloxamers (produced by ICI) and Pluronics (produced by BASF). These block copolymers are employed in the pharmaceutical industry as drug solubilizers, in controlled release systems, and as a covering for burns. As previously indicated, they are also

utilized in bioprocessing to guard microorganisms against mechanical and or chemical stress.(1)

Kayes and Rawlins (1979) generated Langmuir isotherms for the adsorption of seven PEO-PPO-PEO block copolymers (L61, L62, L64, F38, F68, F88, and F108) on PS latex particles. The maximum adsorption occurred at concentrations beyond the apparent critical micelle concentration (CMC). The area per molecule for these seven PEO-PPO-PEO block copolymers (L61, L62, L64, F38, F68, F88, and F108) were found to be 2.85, 3.20, 5.90, 6.51, 15.10, 17.50, and 24.46 nm^2, respectively. The PPO blocks adsorbed to the surface are tightly coiled or form small loops as determined by the molecular area at the interface and corroborated by light scattering and electrophoresis experiments. The adsorption isotherms and adsorbed layer thickness established by dynamic light scattering of several Pluronics on PS latex particles were procured. Increasing PEO block size and bulk polymer concentration resulted in a concurrent increase in the adsorbed layer thickness and specific adsorption. The thickness isotherms have shapes similar to those of high-affinity specific adsorption isotherms, increasing rapidly at low concentrations and leveling out at bulk polymer concentrations.(1,47) The bulk polymer concentration at which the specific adsorption values level out is slightly greater than the CMC of the copolymer. Assuming that all of the segments are in contact with the surface, this plateau concentration is larger than that needed to create a monolayer of the block copolymer. Therefore, the PEO side chains must be extending into the bulk fluid with the PPO segment being in contact with the surface.(1)

As indicated, the conformation of the adsorbed polymer depends on the interactions between the surface and the polymer. Hydrophobic interactions between the apolar PS latex surface and the hydrophobic PPO segments allow for close association of these two components and the extension of the PEO segments into solution. The molar mass of the PEO segments governs the layer thickness. The PEO segments prevent fibrinogen and platelet adhesion through steric repulsion. In contrast to the apolar PS latex surface, a polar silica surface interacts with the Pluronic through hydrogen bonding. This results in very small adlayer thickness which is similar to the adlayer thickness of a PEO homopolymer adsorbed to silica.(1)

As alluded to previously, there are several applications of block copolymer solutions. In the protection of microorganisms, those that don't prohibit cell growth aid in guarding cells from the negative effects of sparging in bioreactors. This protection is due to hydrophilic-lipophilic balance (HLB) where the largest HLB is associated with greater PEO content.(1)

Medical applications utilize Pluronics through the solubilization of drugs in Pluronic copolymer micelles. The application of an insulin vector in conjugation with a micelle containing fluorescein isothiocyanate (FITC) allows permeation of the drug into all tissues including the brain. The conjugation of micelles containing haploperidol to antibodies for a_2-glycoproteins markedly improved the efficacy of the drug. These findings indicate that Pluronic copolymer micelles conjugated to a vector permit the transport of neuropletics across the blood-brain barrier. Pluronic copolymer micelles

containing low molecular weight compounds such as ATP are capable of being transported into intact cells *in vitro*. (1)

The formation of a surface layer on particles using a non-ionic polymer stabilizes the particles, impedes protein binding to the particles, and impedes adhesion of the particles to cells. Repulsion between polymer-coated particles is the results of steric stabilization. Steric stabilization comes about as a consequence of osmotic and elastic contributions on overlap of polymer chains and an elastic contribution due to the loss of configurational entropy between neighboring polymer chains. The grafting of PEO chains to a particle surface results in steric stabilization which prevents the binding of blood components that serve in recognition (opsonins) and also prevent the propinquity necessary for adhesion to macrophages and thereby the engulfment of the particle. The thickness of the steric barrier increases with increasing length of the PEO chain and hence reduces phagocytosis by mouse peritoneal macrophages.(30)

In vivo studies reveal that the type and ratio of PEO/PPO determines the distribution of particles in the organs which is thought to be a function of the steric barrier thickness. However, the surfactant molecules can eventually be removed from the particle surface by blood components and how this may affect the organ distribution *in vivo* is unknown. Therefore, to best understand the effect of the steric barrier *in vivo*, the PEO should be covalently grafted to the particle surface. In *in vitro* studies, the PEO grafted to the particle surface reduced phagocytosis by 87% in comparison to controls by minimizing the particle-cell contact.(30)

The use of the triblock copolymer Poloxamer 238 to coat PS latex particles produces a steric barrier as a result of the PEO chains which float in solution. The hydrophobic portion of the Poloxamer binds to the latex particle, leaving the hydrophilic PEO chain to extend into solution. By adsorbing Poloxamer 238 to PS latex particles grafted with PEO, phagocytosis was reduced by approximately 50%. This decrease in uptake is a consequence of the grafted PEO having spaces between neighboring chains. Thus, although the grafted PEO provides a significant steric barrier, the density of the chains is low enough that the maximum reduction in phagocytosis is not achieved. The Poloxamer adsorbs in the gaps between the grafted PEO chains, thereby increasing the density of the PEO chains and concomitantly the effectiveness of the steric barrier.(30)

APPENDIX B

ORIGINAL DATA

Adhesion of *Candida albicans* strain 1 to pristine polystyrene

Time (min)	Raw Cell Count	Cells/mm^2	Time (min)	Raw Cell Count	Cells/mm^2
3	140	4644.99005	3	99	3284.67153
5	188	6237.55806	5	151	5009.95355
6	234	7763.76908	6	187	6204.37956
7	256	8493.69608	7	238	7896.48308
9	267	8858.65959	9	264	8759.12409
10	281	9323.15859	10	264	8759.12409
12	299	9920.3716	12	339	11247.5116
13	324	10749.8341	13	324	10749.8341
14	324	10749.8341	14	345	11446.5826
15	373	12375.5806	15	361	11977.4386
17	352	11678.8321	17	351	11645.6536
19	391	12972.7936	19	388	12873.2581
21	424	14067.6841	21	417	13835.4346
23	424	14067.6841	23	400	13271.4001
25	402	13337.7571	25	427	14167.2196
30	442	14664.8971	30	468	15527.5382
35	456	15129.3962	35	468	15527.5382
40	430	14266.7551	40	470	15593.8952
45	491	16290.6437	45	476	15792.9662
50	443	14698.0756	50	476	15792.9662
55	464	15394.8242	55	480	15925.6802
60	501	16622.4287	60	478	15859.3232

Time (min)	Average Cells/mm^2
3	3964.83079
5	5623.755806
6	6984.07432
7	8195.089582
9	8808.891838
10	9041.14134
12	10583.94161
13	10749.83411
14	11098.20836
15	12176.50962
17	11662.24287
19	12923.02588
21	13951.55939
23	13669.54214
25	13752.48839
30	15096.21765
35	15328.46715
40	14903.32515
45	16041.80491
50	15245.5209
55	15660.25216
60	16240.87591

Values accurate to three significant figures for reporting purposes. However, the values were carried out to prevent round off errors when performing statistical analysis.

Adhesion of *Candida albicans* strain 1 to Pluronic F127 treated polystyrene

Time (min)	Raw Cell Count	Cells/mm^2	Time (min)	Raw Cell Count	Cells/mm^2
1	0	0	1	0	0
2	0	0	2	0	0
3	1	11.5740741	3	1	11.5740741
4	0	0	4	2	23.1481481
5	0	0	5	2	23.1481481
6	0	0	6	3	34.7222222
7	0	0	7	3	34.7222222
8	0	0	8	2	23.1481481
9	0	0	9	3	34.7222222
10	0	0	10	2	23.1481481
11	0	0	11	2	23.1481481
12	1	11.5740741	12	2	23.1481481
13	1	11.5740741	13	4	46.2962963
14	1	11.5740741	14	4	46.2962963
16	3	34.7222222	16	3	34.7222222
18	1	11.5740741	18	3	34.7222222
20	1	11.5740741	20	4	46.2962963
22	1	11.5740741	22	5	57.8703704
24	1	11.5740741	24	5	57.8703704
26	3	34.7222222	26	7	81.0185185
28	3	34.7222222	28	7	81.0185185
30	5	57.8703704	30	8	92.5925926
35	3	34.7222222	35	5	57.8703704
40	6	69.4444444	40	5	57.8703704
45	6	69.4444444	45	6	69.4444444
50	8	92.5925926	50	7	81.0185185
55	9	104.166667	55	8	92.5925926
60	11	127.314815	60	7	81.0185185

Values accurate to two significant figures for reporting purposes. However, the values were carried out to prevent round off errors when performing statistical analysis.

Adhesion of *Candida albicans* strain 1 to Pluronic F127 treated polystyrene - continued

Time (min)	Raw Cell Count	Cells/mm^2	Average Cells/mm^2
1	0	0	0
2	0	0	0
3	0	0	7.7160494
4	1	11.5740741	11.57407407
5	1	11.5740741	11.57407407
6	1	11.5740741	15.43209877
7	1	11.5740741	15.43209877
8	1	11.5740741	11.57407404
9	1	11.5740741	15.43209877
10	1	11.5740741	11.57407404
11	1	11.5740741	11.57407404
12	1	11.5740741	15.43209877
13	1	11.5740741	23.14814817
14	3	34.7222222	30.86419753
16	1	11.5740741	27.00617283
18	1	11.5740741	19.29012347
20	1	11.5740741	23.14814817
22	2	23.1481481	30.86419753
24	2	23.1481481	30.86419753
26	2	23.1481481	46.29629627
28	2	23.1481481	46.29629627
30	4	46.2962963	65.58641977
35	5	57.8703704	50.154321
40	7	81.0185185	69.44444443
45	7	81.0185185	73.3024691
50	9	104.166667	92.5925927
55	11	127.314815	108.0246915
60	12	138.888889	115.7407408

Values accurate to two significant figures for reporting purposes. However, the values were carried out to prevent round off errors when performing statistical analysis.

Adhesion of *Candida albicans* strain 662 at various locations on polystyrene at 60 minutes.

$$a := \begin{bmatrix} \frac{542}{0.031} & 2.5 \\ \frac{658}{0.032} & 2.5 \\ \frac{778}{0.032} & 2.5 \end{bmatrix} \qquad b := \begin{bmatrix} \frac{238}{0.031} & 1.4 \\ \frac{231}{0.032} & 1.4 \\ \frac{368}{0.032} & 1.4 \end{bmatrix} \qquad c := \begin{bmatrix} \frac{799}{0.031} & 3.6 \\ \frac{843}{0.032} & 3.6 \\ \frac{981}{0.032} & 3.6 \end{bmatrix} \qquad d := \begin{bmatrix} \frac{397}{0.031} & 0.8 \\ \frac{466}{0.032} & 0.8 \\ \frac{935}{0.032} & 0.8 \end{bmatrix}$$

$a1 := a^{<0>}$ $\quad b1 := b^{<0>}$ $\quad c1 := c^{<0>}$ $\quad d1 := d^{<0>}$

$a2 := a^{<1>}$ $\quad b2 := b^{<1>}$ $\quad c2 := c^{<1>}$ $\quad d2 := d^{<1>}$

$$e := \begin{bmatrix} \frac{162}{0.031} & 0.8 \\ \frac{136}{0.032} & 0.8 \\ \frac{426}{0.032} & 0.8 \end{bmatrix} \qquad f := \begin{bmatrix} \frac{825}{0.031} & 4.2 \\ \frac{773}{0.032} & 4.2 \\ \frac{1027}{0.032} & 4.2 \end{bmatrix} \qquad g := \begin{bmatrix} \frac{903}{0.031} & 4.2 \\ \frac{918}{0.032} & 4.2 \\ \frac{1036}{0.032} & 4.2 \end{bmatrix} \qquad t := \begin{bmatrix} 60 \\ 60 \\ 60 \end{bmatrix}$$

$e1 := e^{<0>}$ $\quad f1 := f^{<0>}$ $\quad g1 := g^{<0>}$

$e2 := e^{<1>}$ $\quad f2 := f^{<1>}$ $\quad g2 := g^{<1>}$

For a-g, the first column has units of cells/mm^2, and the second column has units of cm.
For t, the column has units of minutes.

Adhesion of *Candida albicans* strain 662 at various locations at 60 minutes on Pluronic F127 treated polystyrene

$$a := \begin{bmatrix} \frac{33}{0.032} & 2.5 \\ \frac{13}{0.029} & 2.5 \\ \frac{15}{0.03} & 2.5 \end{bmatrix} \quad b := \begin{bmatrix} \frac{10}{0.032} & 1.4 \\ \frac{15}{0.029} & 1.4 \\ \frac{6}{0.03} & 1.4 \end{bmatrix} \quad c := \begin{bmatrix} \frac{28}{0.032} & 3.6 \\ \frac{5}{0.029} & 3.6 \\ \frac{42}{0.03} & 3.6 \end{bmatrix} \quad d := \begin{bmatrix} \frac{10}{0.032} & 0.8 \\ \frac{4}{0.029} & 0.8 \\ \frac{5}{0.03} & 0.8 \end{bmatrix}$$

$a1 := a^{<0>}$ $\quad b1 := b^{<0>}$ $\quad c1 := c^{<0>}$ $\quad d1 := d^{<0>}$

$a2 := a^{<1>}$ $\quad b2 := b^{<1>}$ $\quad c2 := c^{<1>}$ $\quad d2 := d^{<1>}$

$$e := \begin{bmatrix} \frac{3}{0.032} & 0.8 \\ \frac{3}{0.029} & 0.8 \\ \frac{2}{0.03} & 0.8 \end{bmatrix} \quad f := \begin{bmatrix} \frac{46}{0.032} & 4.2 \\ \frac{0}{0.029} & 4.2 \\ \frac{38}{0.03} & 4.2 \end{bmatrix} \quad g := \begin{bmatrix} \frac{34}{0.032} & 4.2 \\ \frac{0}{0.029} & 4.2 \\ \frac{20}{0.03} & 4.2 \end{bmatrix} \quad t := \begin{bmatrix} 60 \\ 60 \\ 60 \end{bmatrix}$$

$e1 := e^{<0>}$ $\quad f1 := f^{<0>}$ $\quad g1 := g^{<0>}$

$e2 := e^{<1>}$ $\quad f2 := f^{<1>}$ $\quad g2 := g^{<1>}$

For a-g, the first column has units of cells/mm^2, and the second column has units of cm. For t, the column has units of minutes.

Adhesion of *Candida albicans* strain 662 to polystyrene (0) and and Pluronic F127 treated polystyrene (1).

Time	CellsPerUnitArea	Surface	Trial
10	3817.374305	0	1
15	6094.967378	0	1
20	8051.772694	0	1
25	9623.632702	0	1
30	11035.09883	0	1
40	13697.63721	0	1
50	15814.83641	0	1
60	17386.69641	0	1
10	6170.484875	0	2
15	9144.975019	0	2
20	10885.36819	0	2
25	12499.18731	0	2
30	14018.07589	0	2
40	16517.91336	0	2
50	19017.75082	0	2
60	20821.43101	0	2
10	7322.668772	0	3
15	10687.98038	0	3
20	12993.84203	0	3
25	15299.70369	0	3
30	17200.48154	0	3
40	20223.02993	0	3
50	22933.97539	0	3
60	24242.70768	0	3
10	30.96807993	1	1
15	43.17608804	1	1
20	309.6807993	1	1
25	340.6488793	1	1
30	371.6169592	1	1
40	495.4892789	1	1
50	805.1700782	1	1
60	1021.946638	1	1
10	0	1	2
15	0	1	2
20	0	1	2
25	68.43254925	1	2
30	68.43254925	1	2
40	136.8650985	1	2
50	513.2441194	1	2
60	444.8115701	1	2
10	100.6053897	1	3
15	167.6756495	1	3
20	268.2810392	1	3
25	301.816169	1	3
30	301.816169	1	3
40	402.4215587	1	3
50	435.9566886	1	3
60	503.0269484	1	3

Percent surface coverage as a function of distance from the first cell layer for biofilms of *Candida albicans* strain 1 on polystyrene at eight locations - Trial 1

$$data := \begin{bmatrix} 0 & 2.65 \\ 4.3 & 18.09 \\ 8.6 & 20.72 \\ 12.9 & 18.01 \\ 17.2 & 9.54 \\ 21.5 & 5.39 \\ 25.8 & 4.73 \\ 30.1 & 3.12 \end{bmatrix}$$

$$data2 := \begin{bmatrix} 0 & 2.50 \\ 4.457 & 23.51 \\ 8.914 & 32.47 \\ 13.374 & 30.44 \\ 17.829 & 22.25 \\ 22.286 & 10.90 \\ 26.743 & 3.87 \\ 31.2 & 0.84 \end{bmatrix}$$

The first column of each data set is distance (microns), and the second column is the percent surface coverage (%). Each data set represents one of the eight locations.

$$data3 := \begin{bmatrix} 0 & 11.49 \\ 4.5 & 23.73 \\ 9.0 & 26.04 \\ 13.5 & 22.63 \\ 18.0 & 18.44 \\ 22.5 & 15.30 \\ 27.0 & 11.67 \\ 31.5 & 10.62 \end{bmatrix}$$

$$data4 := \begin{bmatrix} 0 & 2.11 \\ 4.38 & 18.36 \\ 8.76 & 22.63 \\ 13.14 & 19.63 \\ 17.52 & 10.68 \\ 21.9 & 3.51 \end{bmatrix}$$

$$data5 := \begin{bmatrix} 0 & 1.10 \\ 4.325 & 10.27 \\ 8.65 & 7.79 \\ 12.975 & 3.54 \\ 17.3 & 2.56 \end{bmatrix}$$

$$data6 := \begin{bmatrix} 0 & 2.41 \\ 4.9 & 5.00 \\ 9.8 & 6.13 \\ 14.7 & 6.42 \\ 19.6 & 5.11 \\ 24.5 & 3.14 \\ 29.4 & 2.28 \\ 34.3 & 1.38 \\ 39.2 & 0.62 \end{bmatrix}$$

$$data7 := \begin{bmatrix} 0 & 2.95 \\ 4.467 & 29.07 \\ 8.933 & 33.64 \\ 13.4 & 22.71 \\ 17.867 & 9.43 \\ 22.333 & 2.36 \\ 26.8 & 0.50 \end{bmatrix}$$

$$data8 := \begin{bmatrix} 0 & 4.42 \\ 4.62 & 13.31 \\ 9.24 & 9.22 \\ 13.86 & 6.13 \\ 18.48 & 2.55 \\ 23.1 & 1.03 \end{bmatrix}$$

Percent surface coverage as a function of distance from the first cell layer for biofilms of *Candida albicans* strain 1 on polystyrene at eleven locations - Trial 2

$$data := \begin{bmatrix} 0 & 6.54 \\ 4.825 & 26.19 \\ 9.65 & 25.55 \\ 14.475 & 9.06 \\ 19.3 & 1.39 \end{bmatrix}$$

$$data2 := \begin{bmatrix} 0 & 16.73 \\ 4.175 & 34.90 \\ 8.35 & 20.74 \\ 12.525 & 6.39 \\ 16.7 & 1.38 \end{bmatrix}$$

$$data3 := \begin{bmatrix} 0 & 8.52 \\ 4.217 & 11.67 \\ 8.433 & 11.52 \\ 12.65 & 7.56 \\ 16.867 & 4.15 \\ 21.083 & 1.90 \\ 25.3 & 0.80 \end{bmatrix}$$

$$data4 := \begin{bmatrix} 0 & 12.39 \\ 4.175 & 37.38 \\ 8.35 & 31.82 \\ 12.525 & 11.85 \\ 16.7 & 3.26 \end{bmatrix}$$

$$data5 := \begin{bmatrix} 0 & 5.21 \\ 4.82 & 27.93 \\ 9.64 & 24.46 \\ 14.46 & 10.16 \\ 19.28 & 2.56 \\ 24.1 & 0.30 \end{bmatrix}$$

$$data6 := \begin{bmatrix} 0 & 8.39 \\ 4.733 & 34.15 \\ 9.467 & 21.17 \\ 14.2 & 6.02 \end{bmatrix}$$

$$data7 := \begin{bmatrix} 0 & 8.97 \\ 3.775 & 38.07 \\ 7.55 & 35.74 \\ 11.325 & 13.62 \\ 15.1 & 1.98 \end{bmatrix}$$

$$data8 := \begin{bmatrix} 0 & 14.57 \\ 3.9 & 55.63 \\ 7.8 & 56.53 \\ 11.7 & 22.07 \end{bmatrix}$$

The first column of each data set is distance (microns), and the second column is the percent surface coverage (%). Each data set represents one of the eleven locations.

Percent surface coverage as a function of distance from the first cell layer for biofilms of *Candida albicans* strain 1 on polystyrene at eleven locations - Trial 2 - continued

$$data9 := \begin{bmatrix} 0 & 0.92 \\ 4.325 & 10.16 \\ 8.65 & 8.84 \\ 12.975 & 3.60 \\ 17.3 & 1.41 \end{bmatrix}$$

$$data10 := \begin{bmatrix} 0 & 6.41 \\ 4.54 & 27.25 \\ 9.08 & 24.14 \\ 13.62 & 10.95 \\ 18.16 & 2.94 \\ 22.7 & 0.86 \end{bmatrix}$$

$$data11 := \begin{bmatrix} 0 & 8.17 \\ 4.5 & 37.44 \\ 9.0 & 20.65 \\ 13.5 & 2.81 \end{bmatrix}$$

Percent surface coverage as a function of distance from the first cell layer for biofilms of *Candida albicans* strain 1 on polystyrene at ten locations - Trial 3

$$data := \begin{bmatrix} 0 & 5.55 \\ 4.867 & 16.98 \\ 9.733 & 21.99 \\ 14.6 & 21.12 \\ 19.467 & 15.69 \\ 24.33 & 11.83 \\ 29.2 & 9.13 \\ 34.067 & 5.61 \\ 38.933 & 2.89 \\ 43.8 & 0.81 \end{bmatrix}$$

$$data2 := \begin{bmatrix} 0 & 2.12 \\ 4.825 & 5.79 \\ 9.65 & 6.39 \\ 14.475 & 5.72 \\ 19.3 & 4.17 \\ 24.124 & 3.41 \\ 28.95 & 2.75 \\ 33.775 & 2.69 \\ 38.6 & 1.66 \end{bmatrix}$$

$$data5 := \begin{bmatrix} 0 & 0.83 \\ 5.227 & 5.82 \\ 10.453 & 9.37 \\ 15.68 & 12.44 \\ 20.907 & 13.93 \\ 26.133 & 14.91 \\ 31.36 & 11.92 \\ 36.587 & 6.57 \\ 41.813 & 3.05 \\ 47.04 & 1.92 \\ 52.267 & 0.79 \\ 57.493 & 0.27 \\ 62.72 & 0.22 \\ 67.947 & 0.23 \\ 73.173 & 0.43 \\ 78.4 & 0.28 \end{bmatrix}$$

$$data3 := \begin{bmatrix} 0 & 1.84 \\ 4.862 & 7.25 \\ 9.723 & 10.99 \\ 14.585 & 12.56 \\ 19.446 & 8.21 \\ 24.308 & 3.77 \\ 29.169 & 2.16 \\ 34.031 & 1.25 \\ 38.892 & 0.61 \\ 43.754 & 1.21 \\ 48.615 & 2.06 \\ 53.477 & 1.97 \\ 58.338 & 0.80 \\ 63.2 & 0.21 \end{bmatrix}$$

$$data4 := \begin{bmatrix} 0 & 1.73 \\ 4.9 & 3.83 \\ 9.8 & 5.53 \\ 14.7 & 5.73 \\ 19.6 & 3.35 \\ 24.5 & 1.67 \\ 29.4 & 1.09 \\ 34.3 & 1.16 \end{bmatrix}$$

$$data6 := \begin{bmatrix} 0 & 1.50 \\ 4.821 & 2.04 \\ 9.643 & 2.68 \\ 14.464 & 4.24 \\ 19.286 & 4.87 \\ 24.107 & 5.87 \\ 28.929 & 7.59 \\ 33.75 & 8.78 \\ 38.571 & 8.08 \\ 43.393 & 4.63 \\ 48.214 & 2.22 \\ 53.036 & 1.50 \\ 57.857 & 1.12 \\ 62.679 & 0.71 \\ 67.50 & 0.34 \end{bmatrix}$$

The first column of each data set is distance (microns), and the second column is the percent surface coverage (%). Each data set represents one of the ten locations.

Percent surface coverage as a function of distance from the first cell layer for biofilms of *Candida albicans* strain 1 on polystyrene at ten locations - Trial 3 - continued

$$data8 := \begin{bmatrix} 0 & 1.69 \\ 4.946 & 9.09 \\ 9.892 & 10.52 \\ 14.838 & 7.57 \\ 19.783 & 6.46 \\ 24.729 & 4.98 \\ 29.675 & 4.32 \\ 34.621 & 3.09 \\ 39.567 & 1.35 \\ 44.513 & 1.03 \\ 49.458 & 0.78 \\ 54.404 & 0.53 \\ 59.35 & 0.74 \\ 64.296 & 0.59 \\ 69.242 & 0.69 \\ 74.188 & 0.60 \\ 79.133 & 0.51 \\ 84.079 & 0.30 \\ 89.025 & 0.38 \\ 93.971 & 0.25 \\ 98.917 & 0.23 \\ 103.863 & 0.22 \\ 108.808 & 0.30 \\ 113.754 & 0.32 \\ 118.7 & 0.26 \end{bmatrix}$$

$$data7 := \begin{bmatrix} 0 & 17.70 \\ 4.9 & 26.59 \\ 9.8 & 15.87 \\ 14.7 & 5.53 \\ 19.6 & 3.26 \\ 24.5 & 1.56 \\ 29.4 & 0.67 \\ 34.3 & 0.19 \end{bmatrix}$$

$$data9 := \begin{bmatrix} 0 & 6.68 \\ 4.75 & 13.38 \\ 9.5 & 8.74 \\ 14.25 & 3.40 \\ 19.0 & 2.28 \\ 23.75 & 1.28 \\ 28.5 & 0.55 \end{bmatrix}$$

$$data10 := \begin{bmatrix} 0 & 1.70 \\ 4.742 & 2.67 \\ 9.483 & 3.68 \\ 14.225 & 4.65 \\ 18.967 & 7.06 \\ 23.708 & 9.22 \\ 28.45 & 9.56 \\ 33.192 & 9.66 \\ 37.933 & 9.56 \\ 42.675 & 9.66 \\ 47.417 & 8.69 \\ 52.158 & 7.18 \\ 56.9 & 5.37 \end{bmatrix}$$

Percent surface coverage as a function of distance from the first cell layer for biofilms of *Candida albicans* strain 1 on Pluronic F127 conditioned polystyrene at six locations - Trial 1

$$data := \begin{bmatrix} 0 & 0.65 \\ 3.9 & 0.19 \\ 7.8 & 0.13 \\ 11.7 & 0.18 \end{bmatrix} \qquad data2 := \begin{bmatrix} 0 & 0.78 \\ 4.5 & 1.38 \\ 9.0 & 0.80 \\ 13.5 & 0.32 \end{bmatrix}$$

$$data3 := \begin{bmatrix} 0 & 0.78 \\ 2.9 & 0.53 \\ 5.8 & 0.26 \end{bmatrix} \qquad data4 := (0 \quad 0.54)$$

$$data5 := (0 \quad 0.57) \qquad data6 := (0 \quad 0.98)$$

The first column of each data set is distance (microns), and the second column is the percent surface coverage (%). Each data set represents one of the six locations.

Percent surface coverage as a function of distance from the first cell layer for biofilms of *Candida albicans* strain 1 on Pluronic F127 conditioned polystyrene at six locations - Trial 2

$data := (0 \quad 0.18)$

$data2 := (0 \quad 0.13)$

$data3 := (0 \quad 0.18)$

$data4 := (0 \quad 0.33)$

$data5 := (0 \quad 0.29)$

$data6 := (0 \quad 0.26)$

The first column of each data set is distance (microns), and the second column is the percent surface coverage (%). Each data set represents one of the six locations.

Percent surface coverage as a function of distance from the first cell layer for biofilms of *Candida albicans* strain 1 on Pluronic F127 conditioned polystyrene at seven locations - Trial 3

$$data := \begin{bmatrix} 0 & 0.15 \\ 3.95 & 0.21 \\ 7.9 & 0.12 \end{bmatrix}$$

$$data2 := \begin{bmatrix} 0 & 0.32 \\ 4.5 & 0.58 \\ 9.0 & 0.51 \\ 13.5 & 0.51 \\ 18.0 & 0.42 \\ 22.5 & 0.35 \\ 27.0 & 0.28 \\ 31.5 & 0.11 \end{bmatrix}$$

$$data3 := \begin{bmatrix} 0 & 0.25 \\ 4.06 & 0.71 \\ 8.12 & 1.05 \\ 12.18 & 0.85 \\ 16.24 & 0.40 \\ 20.3 & 0.20 \end{bmatrix}$$

$$data4 := \begin{bmatrix} 0 & 0.34 \\ 4.375 & 2.01 \\ 8.75 & 1.62 \\ 13.125 & 0.25 \\ 17.5 & 0.14 \end{bmatrix}$$

$$data5 := \begin{bmatrix} 0 & 0.55 \\ 3.367 & 0.87 \\ 6.733 & 0.58 \\ 10.1 & 0.20 \end{bmatrix}$$

$$data6 := \begin{bmatrix} 0 & 0.21 \\ 3.35 & 0.20 \\ 6.7 & 0.13 \end{bmatrix}$$

$$data7 := \begin{bmatrix} 0 & 0.95 \\ 4.567 & 0.68 \\ 9.133 & 0.20 \\ 13.7 & 0.12 \end{bmatrix}$$

The first column of each data set is distance (microns), and the second column is the percent surface coverage (%). Each data set represents one of the seven locations.

Percent surface coverage as a function of distance from the first cell layer for biofilms of *Candida albicans* strain 1 on Pluronic F127 conditioned polystyrene at nine locations - Trial 4

$$data := \begin{bmatrix} 0 & 0.43 \\ 3.5 & 0.62 \\ 7.0 & 0.70 \\ 10.5 & 0.61 \end{bmatrix} \qquad data2 := \begin{bmatrix} 0 & 0.39 \\ 4.1 & 0.59 \\ 8.2 & 0.54 \\ 12.3 & 0.32 \end{bmatrix}$$

$$data3 := \begin{bmatrix} 0 & 0.14 \\ 4.75 & 0.25 \\ 9.5 & 0.19 \end{bmatrix} \qquad data4 := \begin{bmatrix} 0 & 0.11 \\ 3.6 & 0.15 \\ 7.2 & 0.12 \\ 10.8 & 0.10 \end{bmatrix}$$

$$data5 := \begin{bmatrix} 0 & 0.12 \\ 4.433 & 0.40 \\ 8.867 & 0.53 \\ 13.3 & 0.28 \end{bmatrix} \qquad data6 := \begin{bmatrix} 0 & 0.27 \\ 4.2 & 1.05 \\ 8.4 & 0.78 \\ 12.6 & 0.24 \end{bmatrix}$$

$$data7 := \begin{bmatrix} 0 & 0.11 \\ 4.3 & 0.93 \\ 8.6 & 1.65 \\ 12.9 & 1.03 \\ 17.2 & 0.46 \end{bmatrix} \qquad data8 := \begin{bmatrix} 0 & 0.18 \\ 3.925 & 1.06 \\ 7.85 & 0.91 \\ 11.775 & 0.29 \\ 15.7 & 0.16 \end{bmatrix}$$

$$data9 := \begin{bmatrix} 0 & 0.14 \\ 4.7 & 0.98 \\ 9.4 & 1.36 \\ 14.1 & 0.83 \\ 18.8 & 0.42 \end{bmatrix}$$

The first column of each data set is distance (microns), and the second column is the percent surface coverage (%). Each data set represents one of the nine locations.

Percent surface coverage as a function of distance from the first cell layer for biofilms of *Candida albicans* strain 662 on polystyrene at seven locations - Trial 1

The first column of each data set is distance (microns), and the second column is the percent surface coverage (%). Each data set represents one of the seven locations.

$$data1 := \begin{bmatrix} 0 & 0.13 \\ 4.783 & 0.21 \\ 9.566 & 0.22 \\ 14.349 & 0.21 \\ 19.132 & 0.17 \\ 23.915 & 0.12 \end{bmatrix}$$

$$data2 := \begin{bmatrix} 0 & 0.11 \\ 4.946 & 0.18 \\ 9.892 & 0.28 \\ 14.838 & 0.39 \\ 19.784 & 0.51 \\ 24.73 & 0.67 \\ 29.676 & 0.81 \\ 34.622 & 0.90 \\ 39.568 & 0.91 \\ 44.514 & 0.84 \\ 49.46 & 0.74 \\ 54.406 & 0.66 \\ 59.352 & 0.63 \\ 64.298 & 0.60 \\ 69.244 & 0.56 \\ 74.19 & 0.51 \\ 79.136 & 0.47 \\ 84.082 & 0.41 \\ 89.028 & 0.37 \\ 93.974 & 0.36 \\ 98.92 & 0.38 \\ 103.866 & 0.38 \\ 108.812 & 0.38 \\ 113.758 & 0.38 \\ 118.704 & 0.40 \\ 123.65 & 0.43 \\ 128.596 & 0.50 \\ 133.542 & 0.67 \\ 138.488 & 0.86 \\ 143.434 & 1.08 \\ 148.38 & 1.28 \end{bmatrix}$$

Percent surface coverage as a function of distance from the first cell layer for biofilms of *Candida albicans* strain 662 on polystyrene at seven locations - Trial 1 - continued

$$data3 := \begin{bmatrix} 0 & 0.30 \\ 4.986 & 0.53 \\ 9.972 & 0.79 \\ 14.958 & 1.02 \\ 19.944 & 1.22 \\ 24.93 & 1.29 \\ 29.916 & 1.26 \\ 34.902 & 1.22 \\ 39.888 & 1.19 \\ 44.874 & 1.12 \\ 49.86 & 1.04 \\ 54.846 & 0.98 \\ 59.832 & 0.86 \\ 64.818 & 0.76 \\ 69.804 & 0.67 \\ 74.79 & 0.53 \\ 79.776 & 0.43 \\ 84.762 & 0.38 \\ 89.748 & 0.38 \\ 94.734 & 0.40 \\ 99.72 & 0.38 \\ 104.706 & 0.40 \\ 109.692 & 0.43 \\ 114.678 & 0.44 \\ 119.664 & 0.40 \\ 124.65 & 0.33 \\ 129.636 & 0.26 \\ 134.622 & 0.18 \\ 139.608 & 0.14 \\ 144.594 & 0.12 \\ 149.58 & 0.11 \end{bmatrix}$$

$$data4 := \begin{bmatrix} 0 & 0.69 \\ 5.189 & 2.67 \\ 10.378 & 1.59 \\ 15.567 & 0.88 \\ 20.756 & 0.73 \\ 25.945 & 1.82 \\ 31.134 & 3.74 \\ 36.323 & 4.18 \\ 41.512 & 3.60 \\ 46.701 & 2.95 \\ 51.89 & 1.91 \\ 57.079 & 1.08 \\ 62.268 & 0.64 \end{bmatrix}$$

$$data5 := \begin{bmatrix} 0 & 0.20 \\ 4.459 & 0.27 \\ 8.918 & 0.17 \end{bmatrix}$$

$$data6 := \begin{bmatrix} 0 & 0.12 \\ 5.229 & 0.23 \\ 10.458 & 0.28 \\ 15.687 & 0.21 \\ 20.916 & 0.18 \\ 26.145 & 0.11 \end{bmatrix}$$

$$data7 := (0 \quad 0.11)$$

Percent surface coverage as a function of distance from the first cell layer for biofilms of *Candida albicans* strain 662 on polystyrene at five locations (could not open last two files) - Trial 2

$$data := \begin{bmatrix} 0 & 0.29 \\ 4.662 & 0.72 \\ 9.324 & 1.04 \\ 13.986 & 1.11 \\ 18.648 & 1.01 \\ 23.31 & 0.79 \\ 27.972 & 0.52 \\ 32.634 & 0.33 \\ 37.296 & 0.20 \end{bmatrix}$$

$$data2 := \begin{bmatrix} 0 & 0.16 \\ 4.824 & 0.37 \\ 9.648 & 0.59 \\ 14.472 & 0.67 \\ 19.296 & 0.59 \\ 24.12 & 0.46 \\ 28.944 & 0.27 \\ 33.768 & 0.16 \end{bmatrix}$$

$$data3 := \begin{bmatrix} 0 & 0.19 \\ 4.702 & 0.41 \\ 9.404 & 0.63 \\ 14.106 & 0.63 \\ 18.808 & 0.48 \\ 23.51 & 0.32 \\ 28.212 & 0.25 \\ 32.914 & 0.22 \\ 37.616 & 0.21 \\ 42.318 & 0.16 \\ 47.02 & 0.13 \end{bmatrix}$$

$$data4 := \begin{bmatrix} 0 & 0.12 \\ 4.702 & 0.25 \\ 9.404 & 0.35 \\ 14.106 & 0.29 \\ 18.808 & 0.20 \\ 23.51 & 0.12 \end{bmatrix}$$

$$data5 := \begin{bmatrix} 0 & 0.11 \\ 4.702 & 0.53 \\ 9.404 & 1.43 \\ 14.105 & 2.11 \\ 18.808 & 2.05 \\ 23.51 & 1.49 \\ 28.212 & 0.98 \\ 32.914 & 0.64 \\ 37.615 & 0.38 \\ 42.318 & 0.22 \\ 47.02 & 0.16 \\ 51.722 & 0.13 \end{bmatrix}$$

The first column of each data set is distance (microns), and the second column is the percent surface coverage (%). Each data set represents one of the five locations.

Percent surface coverage as a function of distance from the first cell layer for biofilms of *Candida albicans* strain 662 on polystyrene at seven locations - Trial 3

$$data := \begin{bmatrix} 0 & 3.97 \\ 5.027 & 6.37 \\ 10.054 & 9.06 \\ 15.081 & 8.97 \\ 20.108 & 6.96 \\ 25.135 & 3.91 \\ 30.162 & 1.83 \\ 35.189 & 1.15 \\ 40.216 & 1.16 \\ 45.243 & 1.27 \\ 50.27 & 1.30 \\ 55.297 & 1.02 \\ 60.324 & 0.89 \\ 65.351 & 0.78 \\ 70.378 & 0.78 \\ 75.405 & 0.69 \\ 80.432 & 0.51 \\ 85.459 & 0.37 \\ 90.486 & 0.38 \\ 95.513 & 0.29 \\ 100.54 & 0.20 \\ 105.567 & 0.17 \\ 110.594 & 0.16 \\ 115.621 & 0.15 \\ 120.648 & 0.16 \\ 125.675 & 0.19 \\ 130.702 & 0.17 \\ 135.729 & 0.15 \\ 140.756 & 0.15 \\ 145.783 & 0.15 \\ 150.81 & 0.15 \\ 155.837 & 0.16 \end{bmatrix}$$

The first column of each data set is distance (microns), and the second column is the percent surface coverage (%). Each data set represents one of the seven locations.

$$data2 := \begin{bmatrix} 0 & 0.12 \\ 4.864 & 0.76 \\ 9.728 & 1.65 \\ 14.592 & 2.04 \\ 19.456 & 1.73 \\ 24.32 & 1.23 \\ 29.184 & 1.00 \\ 34.048 & 0.92 \\ 38.912 & 0.64 \\ 43.776 & 0.50 \\ 48.64 & 0.35 \\ 53.504 & 0.42 \\ 58.368 & 0.52 \\ 63.232 & 0.67 \\ 68.096 & 0.82 \\ 72.96 & 0.83 \\ 77.824 & 0.77 \\ 82.688 & 0.84 \\ 87.552 & 0.87 \end{bmatrix}$$

Percent surface coverage as a function of distance from the first cell layer for biofilms of *Candida albicans* strain 662 on polystyrene at seven locations - Trial 3 - continued

$$data3 := \begin{bmatrix} 0 & 0.14 \\ 4.946 & 1.00 \\ 9.892 & 1.87 \\ 14.838 & 2.32 \\ 19.784 & 2.16 \\ 24.73 & 1.66 \\ 29.676 & 1.08 \\ 34.622 & 0.95 \\ 39.568 & 0.94 \\ 44.514 & 0.93 \\ 49.46 & 0.90 \\ 54.406 & 0.82 \\ 59.352 & 0.70 \\ 64.298 & 0.60 \\ 69.244 & 0.37 \\ 74.19 & 0.16 \\ 79.136 & 0.12 \end{bmatrix}$$

$$data4 := \begin{bmatrix} 0 & 0.26 \\ 4.864 & 1.15 \\ 9.728 & 1.95 \\ 14.592 & 2.22 \\ 19.456 & 2.18 \\ 24.32 & 1.75 \\ 29.184 & 1.02 \\ 34.048 & 0.54 \\ 38.912 & 0.38 \end{bmatrix}$$

$$data7 := \begin{bmatrix} 0 & 0.13 \\ 4.743 & 1.94 \\ 9.486 & 3.65 \\ 14.229 & 2.94 \\ 18.972 & 1.80 \\ 23.715 & 1.31 \\ 28.458 & 1.18 \\ 33.201 & 0.90 \\ 37.944 & 0.65 \\ 42.687 & 0.51 \\ 47.43 & 0.36 \\ 52.173 & 0.34 \\ 56.916 & 0.35 \end{bmatrix}$$

$$data6 := \begin{bmatrix} 0 & 0.76 \\ 4.459 & 4.89 \\ 8.918 & 9.05 \\ 13.377 & 11.44 \\ 17.836 & 11.11 \\ 22.295 & 11.03 \\ 26.754 & 10.59 \\ 31.213 & 10.37 \\ 35.672 & 9.49 \end{bmatrix}$$

$$data5 := \begin{bmatrix} 0 & 0.25 \\ 4.094 & 2.00 \\ 8.188 & 4.18 \\ 12.282 & 4.94 \\ 16.376 & 3.99 \end{bmatrix}$$

Percent surface coverage as a function of distance from the first cell layer for biofilms of *Candida albicans* strain 662 on polystyrene at seven locations - Trial 4

The first column of each data set is distance (microns), and the second column is the percent surface coverage (%). Each data set represents one of the seven locations.

$$\text{data} := \begin{bmatrix} 0 & 0.11 \\ 4.824 & 0.13 \\ 9.648 & 0.16 \\ 14.472 & 0.20 \\ 19.296 & 0.23 \\ 24.12 & 0.25 \\ 28.944 & 0.28 \\ 33.768 & 0.32 \\ 38.592 & 0.38 \\ 43.416 & 0.44 \\ 48.24 & 0.47 \\ 53.064 & 0.45 \\ 57.888 & 0.38 \\ 62.712 & 0.28 \\ 67.536 & 0.18 \\ 72.36 & 0.12 \end{bmatrix}$$

$$\text{data2} := \begin{bmatrix} 0 & 0.14 \\ 5.027 & 0.22 \\ 10.054 & 0.29 \\ 15.081 & 0.28 \\ 20.108 & 0.22 \\ 25.135 & 0.16 \\ 30.162 & 0.12 \\ 35.189 & 0.11 \\ 40.216 & 0.12 \\ 45.243 & 0.12 \\ 50.27 & 0.11 \\ 55.297 & 0.10 \\ 60.324 & 0.13 \\ 65.351 & 0.15 \\ 70.378 & 0.23 \\ 75.405 & 0.37 \\ 80.432 & 0.58 \\ 85.459 & 0.67 \\ 90.486 & 0.74 \\ 95.513 & 0.62 \\ 100.54 & 0.44 \\ 105.567 & 0.28 \\ 110.594 & 0.19 \\ 115.621 & 0.13 \\ 120.648 & 0.11 \end{bmatrix}$$

Percent surface coverage as a function of distance from the first cell layer for biofilms of *Candida albicans* strain 662 on polystyrene at seven locations - Trial 4 - continued

$$data3 := \begin{bmatrix} 0 & 0.11 \\ 4.946 & 0.44 \\ 9.892 & 0.71 \\ 14.838 & 0.21 \\ 19.784 & 0.13 \\ 24.73 & 0.12 \\ 29.676 & 0.11 \end{bmatrix}$$

$$data4 := \begin{bmatrix} 0 & 0.14 \\ 4.864 & 1.12 \\ 9.728 & 2.92 \\ 14.592 & 3.65 \\ 19.456 & 2.75 \\ 24.32 & 1.24 \\ 29.184 & 0.53 \\ 34.048 & 0.23 \\ 38.912 & 0.13 \end{bmatrix}$$

$$data5 := \begin{bmatrix} 0 & 0.38 \\ 4.743 & 2.31 \\ 9.486 & 4.17 \\ 14.229 & 3.79 \\ 18.972 & 2.07 \\ 23.715 & 0.95 \\ 28.458 & 0.55 \\ 33.201 & 0.45 \\ 37.944 & 0.41 \\ 42.687 & 0.45 \\ 47.43 & 0.41 \\ 52.173 & 0.38 \\ 56.916 & 0.29 \\ 61.659 & 0.19 \\ 66.402 & 0.11 \end{bmatrix}$$

$$data6 := \begin{bmatrix} 0 & 0.27 \\ 4.986 & 1.48 \\ 9.972 & 2.17 \\ 14.958 & 2.02 \\ 19.944 & 1.60 \\ 24.93 & 1.30 \\ 29.916 & 1.03 \\ 34.902 & 0.93 \\ 39.888 & 1.11 \\ 44.874 & 1.44 \\ 49.86 & 1.82 \\ 54.846 & 1.91 \\ 59.832 & 1.79 \\ 64.818 & 1.29 \\ 69.804 & 0.95 \\ 74.79 & 0.67 \\ 79.776 & 0.51 \\ 84.762 & 0.42 \\ 89.748 & 0.39 \\ 94.734 & 0.33 \\ 99.72 & 0.25 \\ 104.706 & 0.18 \end{bmatrix}$$

$$data7 := \begin{bmatrix} 0 & 0.30 \\ 4.540 & 1.05 \\ 9.08 & 1.80 \\ 13.62 & 1.96 \\ 18.16 & 1.42 \\ 22.7 & 0.82 \\ 27.24 & 0.40 \end{bmatrix}$$

Percent surface coverage as a function of distance from the first cell layer for biofilms of *Candida albicans* strain 662 on polystyrene at seven locations - Trial 5

The first column of each data set is distance (microns), and the second column is the percent surface coverage (%). Each data set represents one of the seven locations.

$$data := \begin{bmatrix} 0 & 0.16 \\ 4.864 & 0.31 \\ 9.728 & 0.31 \\ 14.592 & 0.23 \\ 19.456 & 0.20 \\ 24.32 & 0.18 \\ 29.184 & 0.17 \\ 34.048 & 0.17 \\ 38.912 & 0.18 \\ 43.776 & 0.17 \\ 48.64 & 0.18 \\ 53.504 & 0.16 \\ 58.368 & 0.17 \\ 63.232 & 0.20 \\ 68.096 & 0.18 \\ 72.96 & 0.16 \\ 77.824 & 0.16 \\ 82.688 & 0.16 \\ 87.552 & 0.15 \\ 92.416 & 0.14 \\ 97.28 & 0.13 \\ 102.144 & 0.12 \\ 107.008 & 0.11 \end{bmatrix}$$

$$data2 := (0 \quad 0)$$

Percent surface coverage as a function of distance from the first cell layer for biofilms of *Candida albicans* strain 662 on polystyrene at seven locations - Trial 5 - continued

$$data3 := \begin{bmatrix} 0 & 0.14 \\ 4.986 & 0.29 \\ 9.972 & 0.35 \\ 14.958 & 0.28 \\ 19.944 & 0.21 \\ 24.93 & 0.15 \\ 29.916 & 0.11 \end{bmatrix}$$

$$data4 := \begin{bmatrix} 0 & 0.11 \\ 4.783 & 0.39 \\ 9.566 & 0.67 \\ 14.349 & 0.53 \\ 19.132 & 0.21 \\ 23.915 & 0.11 \end{bmatrix}$$

$$data5 := \begin{bmatrix} 0 & 0.11 \\ 4.986 & 0.20 \\ 9.972 & 0.22 \\ 14.958 & 0.24 \\ 19.944 & 0.22 \\ 24.93 & 0.27 \\ 29.916 & 0.43 \\ 34.902 & 0.59 \\ 39.888 & 0.60 \\ 44.874 & 0.51 \\ 49.86 & 0.36 \\ 54.846 & 0.26 \\ 59.832 & 0.20 \\ 64.818 & 0.28 \\ 69.804 & 0.35 \\ 74.79 & 0.34 \\ 79.776 & 0.26 \\ 84.762 & 0.23 \\ 89.748 & 0.18 \\ 94.734 & 0.13 \end{bmatrix}$$

$$data6 := \begin{bmatrix} 0 & 0.17 \\ 4.905 & 0.38 \\ 9.81 & 0.38 \\ 14.715 & 0.24 \\ 19.62 & 0.14 \\ 24.525 & 0.11 \end{bmatrix}$$

$$data7 := \begin{bmatrix} 0 & 0.12 \\ 4.864 & 0.19 \\ 9.728 & 0.27 \\ 14.592 & 0.23 \\ 19.456 & 0.21 \\ 24.32 & 0.17 \\ 29.184 & 0.13 \\ 34.048 & 0.11 \end{bmatrix}$$

Percent surface coverage as a function of distance from the first cell layer for biofilms of *Candida albicans* strain 662 on polystyrene at seven locations - Trial 6

$$data := \begin{bmatrix} 0 & 0.19 \\ 4.946 & 0.32 \\ 9.892 & 0.33 \\ 14.838 & 0.21 \\ 19.784 & 0.18 \\ 24.73 & 0.16 \\ 29.676 & 0.15 \\ 34.622 & 0.15 \\ 39.568 & 0.15 \\ 44.514 & 0.16 \\ 49.46 & 0.21 \\ 54.406 & 0.28 \\ 59.352 & 0.31 \\ 64.298 & 0.30 \\ 69.244 & 0.28 \\ 74.19 & 0.21 \\ 79.136 & 0.13 \\ 89.028 & 0.11 \end{bmatrix}$$

$$data2 := \begin{bmatrix} 0 & 0.13 \\ 4.824 & 0.15 \\ 9.648 & 0.20 \\ 14.472 & 0.22 \\ 19.296 & 0.21 \\ 24.12 & 0.12 \end{bmatrix}$$

$$data5 := \begin{pmatrix} 0 & 0.11 \end{pmatrix}$$

$$data3 := \begin{bmatrix} 0 & 0.18 \\ 5.148 & 0.33 \\ 10.296 & 0.25 \\ 15.444 & 0.18 \\ 20.592 & 0.14 \\ 25.74 & 0.12 \\ 30.888 & 0.12 \\ 36.036 & 0.12 \\ 41.184 & 0.11 \\ 46.332 & 0.17 \\ 51.48 & 0.19 \\ 56.628 & 0.23 \\ 61.776 & 0.32 \\ 66.924 & 0.31 \\ 72.072 & 0.21 \\ 77.22 & 0.16 \\ 82.368 & 0.15 \\ 87.516 & 0.15 \\ 92.664 & 0.11 \end{bmatrix}$$

$$data6 := \begin{bmatrix} 0 & 0.21 \\ 4.986 & 0.50 \\ 9.972 & 1.68 \\ 14.958 & 2.53 \\ 19.944 & 1.93 \\ 24.93 & 0.95 \\ 29.916 & 0.46 \\ 34.902 & 0.25 \\ 39.888 & 0.20 \\ 44.874 & 0.17 \\ 49.86 & 0.14 \\ 54.846 & 0.11 \end{bmatrix}$$

$$data4 := \begin{bmatrix} 0 & 0.13 \\ 4.419 & 0.35 \\ 8.838 & 0.29 \\ 13.257 & 0.14 \\ 17.676 & 0.12 \\ 22.095 & 0.11 \end{bmatrix}$$

$$data7 := \begin{bmatrix} 0 & 0.35 \\ 4.581 & 0.57 \\ 9.162 & 0.50 \\ 13.743 & 0.33 \\ 18.324 & 0.20 \\ 22.905 & 0.15 \\ 27.486 & 0.12 \end{bmatrix}$$

The first column of each data set is distance (microns), and the second column is the percent surface coverage (%). Each data set represents one of the seven locations.

Percent surface coverage as a functin of distance from the first cell layer for biofilms of *Candida albicans* strain 662 on polystyrene at seven locations - Trial 7

$$data := \begin{bmatrix} 0 & 0.11 \\ 4.864 & 0.14 \\ 9.728 & 0.18 \\ 14.592 & 0.22 \end{bmatrix}$$

$$data2 := \begin{bmatrix} 0 & 0.13 \\ 4.378 & 0.22 \\ 8.756 & 0.33 \\ 13.134 & 0.40 \\ 17.512 & 0.10 \end{bmatrix}$$

$$data3 := (0 \quad 0)$$

$$data4 := \begin{bmatrix} 0 & 0.11 \\ 4.864 & 0.26 \\ 9.728 & 0.54 \\ 14.592 & 0.98 \\ 19.456 & 1.29 \\ 24.32 & 1.24 \\ 29.184 & 1.01 \\ 34.048 & 0.69 \\ 38.912 & 0.48 \\ 43.776 & 0.41 \\ 48.64 & 0.33 \\ 53.504 & 0.30 \\ 58.368 & 0.25 \\ 63.232 & 0.21 \\ 68.096 & 0.19 \\ 72.96 & 0.22 \\ 77.824 & 0.22 \\ 82.688 & 0.20 \\ 87.552 & 0.16 \\ 92.416 & 0.14 \\ 97.28 & 0.13 \\ 102.144 & 0.13 \\ 107.008 & 0.13 \\ 111.872 & 0.13 \\ 116.736 & 0.11 \end{bmatrix}$$

$$data5 := (0 \quad 0)$$

$$data6 := \begin{bmatrix} 0 & 0.11 \\ 4.986 & 0.10 \end{bmatrix}$$

$$data7 := \begin{bmatrix} 0 & 0.11 \\ 4.946 & 0.13 \\ 9.892 & 0.15 \\ 14.838 & 0.14 \\ 19.784 & 0.14 \\ 24.73 & 0.15 \\ 29.676 & 0.14 \\ 34.622 & 0.13 \\ 39.568 & 0.12 \\ 44.514 & 0.11 \end{bmatrix}$$

The first column of each data set is distance (microns), and the second column is the percent surface coverage (%). Each data set represents one of the seven locations.

Percent surface coverage as a function of distance from the first cell layer for biofilms of *Candida albicans* strain 662 on Pluronic F127 conditioned polystyrene - Trial 1

no cells were observed at any of the six locations - unable to open file for seventh location.

Percent surface coverage as a function of distance from the first cell layer for biofilms of *Candida albicans* strain 662 on Pluronic F127 conditioned polystyrene - Trial 2

$$data := \begin{pmatrix} 0 & 0 \end{pmatrix}$$

$$data2 := \begin{pmatrix} 0 & 0.10 \end{pmatrix}$$

$$data3 := \begin{bmatrix} 0 & 0.11 \\ 4.946 & 0.12 \\ 9.892 & 0.11 \end{bmatrix}$$

$$data4 := \begin{pmatrix} 0 & 0.10 \end{pmatrix}$$

$$data5 := \begin{pmatrix} 0 & 0.10 \end{pmatrix}$$

$$data6 := \begin{pmatrix} 0 & 0.10 \end{pmatrix}$$

$$data7 := \begin{pmatrix} 0 & 0 \end{pmatrix}$$

The first column of each data set is distance (microns), and the second column is the percent surface coverage (%). Each data set represents one of the seven locations.

Percent surface coverage as a function of distance from the first cell layer for biofilms of *Candida albicans* strain 662 on Pluronic F127 conditioned polystyrene at seven locations - Trial 3

$$data := \begin{bmatrix} 0 & 0.11 \\ 4.459 & 0.11 \\ 8.918 & 0.10 \end{bmatrix}$$

$$data2 := \begin{bmatrix} 0 & 0.14 \\ 4.662 & 0.15 \\ 9.324 & 0.13 \\ 13.986 & 0.11 \end{bmatrix}$$

$$data3 := \begin{bmatrix} 0 & 0.11 \\ 4.905 & 0.12 \\ 9.81 & 0.12 \\ 14.715 & 0.12 \\ 19.62 & 0.12 \\ 24.525 & 0.15 \\ 29.43 & 0.15 \\ 34.335 & 0.13 \end{bmatrix}$$

$$data4 := \begin{bmatrix} 0 & 0.12 \\ 4.905 & 0.11 \\ 9.81 & 0.10 \end{bmatrix}$$

$$data5 := \begin{bmatrix} 0 & 0.11 \\ 4.581 & 0.11 \\ 9.162 & 0.10 \end{bmatrix}$$

$$data6 := \begin{bmatrix} 0 & 0.11 \\ 4.702 & 0.11 \\ 9.404 & 0.10 \end{bmatrix}$$

$$data7 := (0 \quad 0.11)$$

The first column of each data set is distance (microns), and the second column is the percent surface coverage (%). Each data set represents one of the seven locations.

Percent surface coverage as a function of distance from the first cell layer for biofilms of *Candida albicans* strain 662 on Pluronic F127 conditioned polystyrene at seven locations - Trial 4

$$data := \begin{bmatrix} 0 & 0.11 \\ 4.419 & 0.11 \\ 8.838 & 0.10 \end{bmatrix} \qquad data2 := \begin{bmatrix} 0 & 0.18 \\ 4.5 & 0.28 \\ 9.0 & 0.31 \\ 13.5 & 0.26 \\ 18.0 & 0.16 \end{bmatrix}$$

$$data3 := \begin{bmatrix} 0 & 0.12 \\ 4.702 & 0.15 \\ 9.404 & 0.16 \\ 14.106 & 0.14 \\ 18.808 & 0.12 \\ 23.51 & 0.11 \end{bmatrix} \qquad data4 := \begin{bmatrix} 0 & 0.15 \\ 4.783 & 0.22 \\ 9.566 & 0.10 \end{bmatrix}$$

$$data5 := \begin{bmatrix} 0 & 0.11 \\ 4.094 & 0.12 \\ 9.808 & 0.12 \end{bmatrix} \qquad data6 := \begin{bmatrix} 0 & 0.14 \\ 5.108 & 0.23 \\ 10.216 & 0.26 \\ 15.324 & 0.10 \end{bmatrix}$$

$$data7 := \begin{bmatrix} 0 & 0.11 \\ 4.946 & 0.13 \\ 9.892 & 0.16 \\ 14.838 & 0.16 \\ 19.784 & 0.15 \end{bmatrix}$$

The first column of each data set is distance (microns), and the second column is the percent surface coverage (%). Each data set represents one of the seven locations.

Growth curves for suspension cultures of *Candida alibcans* strain 1 in reduced and nonreduced medium with and without the presence of Pluronic F127. The first column represents time (minutes), and the second column represents log(cells/ml).

$$data := \begin{bmatrix} 120 & \log(8.50\cdot10^5) \\ 240 & \log(7.75\cdot10^5) \\ 365 & \log(3.33\cdot10^6) \\ 435 & \log(4.30\cdot10^6) \\ 720 & \log(6.50\cdot10^6) \\ 1385 & \log(6.00\cdot10^6) \\ 1440 & \log(1.00\cdot10^7) \end{bmatrix} \qquad data2 := \begin{bmatrix} 120 & \log(8.00\cdot10^5) \\ 240 & \log(8.75\cdot10^5) \\ 365 & \log(3.03\cdot10^6) \\ 405 & \log(5.68\cdot10^6) \\ 720 & \log(9.00\cdot10^6) \\ 1440 & \log(7.25\cdot10^6) \end{bmatrix}$$

No Pluronic F127

Reduced medium

$$dataN := \begin{bmatrix} 120 & \log(1.8\cdot10^6) \\ 230 & \log(4.85\cdot10^6) \\ 410 & \log(4.33\cdot10^7) \\ 660 & \log(1.88\cdot10^8) \\ 1178 & \log(3.13\cdot10^8) \\ 1485 & \log(3.13\cdot10^8) \end{bmatrix}$$

No Pluronic F127

Nonreduced medium

$$dataCa1 := \begin{bmatrix} 120 & \log(5.50\cdot10^5) \\ 255 & \log(1.275\cdot10^6) \\ 365 & \log(1.35\cdot10^6) \\ 395 & \log(3.175\cdot10^6) \\ 710 & \log(2.325\cdot10^6) \\ 1395 & \log(2.925\cdot10^6) \\ 1440 & \log(3.05\cdot10^6) \end{bmatrix} \qquad data2Ca1 := \begin{bmatrix} 120 & \log(9.25\cdot10^5) \\ 255 & \log(1.175\cdot10^6) \\ 365 & \log(2.275\cdot10^6) \\ 395 & \log(4.40\cdot10^6) \\ 710 & \log(2.275\cdot10^6) \\ 1395 & \log(2.60\cdot10^6) \\ 1440 & \log(2.35\cdot10^6) \end{bmatrix}$$

1000 μl of 4% Pluronic F127 solution

Reduced medium

Growth curves for suspension cultures of *Candida albicans* strain 662 in reduced and nonreduced medium with and without the presence of Pluronic F127. The first column represents time (minutes), and the second column represents log(cells/ml).

$$\text{data} := \begin{bmatrix} 120 & \log(1.40\cdot10^{6}) \\ 255 & \log(5.95\cdot10^{6}) \\ 365 & \log(8.25\cdot10^{6}) \\ 395 & \log(9.00\cdot10^{6}) \\ 710 & \log(1.00\cdot10^{7}) \\ 1395 & \log(8.25\cdot10^{6}) \\ 1440 & \log(1.00\cdot10^{7}) \end{bmatrix} \qquad \text{data2} := \begin{bmatrix} 120 & \log(1.60\cdot10^{6}) \\ 255 & \log(6.05\cdot10^{6}) \\ 365 & \log(7.75\cdot10^{6}) \\ 395 & \log(9.50\cdot10^{6}) \\ 710 & \log(7.25\cdot10^{6}) \\ 1395 & \log(4.00\cdot10^{6}) \\ 1440 & \log(1.00\cdot10^{7}) \end{bmatrix}$$

No Pluronic F127

Reduced medium

$$\text{dataN} := \begin{bmatrix} 125 & \log(2.55\cdot10^{6}) \\ 240 & \log(8.68\cdot10^{6}) \\ 330 & \log(3.63\cdot10^{7}) \\ 450 & \log(1.23\cdot10^{8}) \\ 700 & \log(3.46\cdot10^{8}) \\ 1250 & \log(4.36\cdot10^{8}) \\ 1420 & \log(4.20\cdot10^{8}) \end{bmatrix}$$

No Pluronic F127

Nonreduced Medium

$$\text{dataCa662} := \begin{bmatrix} 120 & \log(1.425\cdot10^{6}) \\ 255 & \log(4.375\cdot10^{6}) \\ 365 & \log(5.675\cdot10^{6}) \\ 395 & \log(4.30\cdot10^{6}) \\ 710 & \log(5.45\cdot10^{6}) \\ 1395 & \log(4.65\cdot10^{6}) \\ 1440 & \log(5.675\cdot10^{6}) \end{bmatrix} \qquad \text{data2Ca662} := \begin{bmatrix} 120 & \log(1.575\cdot10^{6}) \\ 255 & \log(3.325\cdot10^{6}) \\ 365 & \log(4.20\cdot10^{6}) \\ 395 & \log(3.725\cdot10^{6}) \\ 710 & \log(5.65\cdot10^{6}) \\ 1395 & \log(4.30\cdot10^{6}) \\ 1440 & \log(4.35\cdot10^{6}) \end{bmatrix}$$

1000 ml of 4% Pluronic F127 solution

Reduced medium

Growth curves for suspension cultures of *Candida albicans* strain 1 in GYEP medium with and without Pluronic F127. The units for t (time) are minutes, and ca_ is in terms of log(cells/ml).

$$t1 := \begin{bmatrix} 120 \\ 230 \\ 410 \\ 660 \\ 1178 \\ 1485 \end{bmatrix} \qquad ca1 := \begin{bmatrix} \log(1.8\cdot10^{6}) \\ \log(4.85\cdot10^{6}) \\ \log(4.33\cdot10^{7}) \\ \log(1.88\cdot10^{8}) \\ \log(3.13\cdot10^{8}) \\ \log(3.13\cdot10^{8}) \end{bmatrix}$$

No Pluronic F127

$$t2 := \begin{bmatrix} 125 \\ 235 \\ 355 \\ 460 \\ 730 \\ 1295 \\ 1455 \end{bmatrix} \qquad ca1pl1 := \begin{bmatrix} \log(2.88\cdot10^{6}) \\ \log(9.55\cdot10^{6}) \\ \log(3.75\cdot10^{7}) \\ \log(1.04\cdot10^{8}) \\ \log(2.70\cdot10^{8}) \\ \log(4.10\cdot10^{8}) \\ \log(4.36\cdot10^{8}) \end{bmatrix}$$

10 μl of 4% Pluronic F127 solution

$$t3 := \begin{bmatrix} 135 \\ 240 \\ 360 \\ 460 \\ 715 \\ 1330 \\ 1440 \end{bmatrix} \qquad ca1pl2 := \begin{bmatrix} \log(1.78\cdot10^{6}) \\ \log(6.5\cdot10^{6}) \\ \log(2.88\cdot10^{7}) \\ \log(7.73\cdot10^{7}) \\ \log(2.84\cdot10^{8}) \\ \log(4.10\cdot10^{8}) \\ \log(4.55\cdot10^{8}) \end{bmatrix}$$

1000 ml of 4% Pluronic F127 solution

Growth curves for suspension cultures of *Candida albicans* strain 662 in GYEP medium with and without Pluronic F127. The units for t (time) are minutes, and ca_ is in terms of log(cells/ml).

$$t1 := \begin{bmatrix} 125 \\ 240 \\ 330 \\ 450 \\ 700 \\ 1250 \\ 1420 \end{bmatrix} \qquad ca2 := \begin{bmatrix} \log(2.55 \cdot 10^{6}) \\ \log(8.68 \cdot 10^{6}) \\ \log(3.63 \cdot 10^{7}) \\ \log(1.23 \cdot 10^{8}) \\ \log(3.46 \cdot 10^{8}) \\ \log(4.36 \cdot 10^{8}) \\ \log(4.20 \cdot 10^{8}) \end{bmatrix}$$

No Pluronic F127

$$t2 := \begin{bmatrix} 115 \\ 225 \\ 340 \\ 445 \\ 715 \\ 1280 \\ 1440 \end{bmatrix} \qquad ca2pl1 := \begin{bmatrix} \log(1.38 \cdot 10^{6}) \\ \log(7.63 \cdot 10^{6}) \\ \log(3.85 \cdot 10^{7}) \\ \log(8.73 \cdot 10^{7}) \\ \log(4.54 \cdot 10^{8}) \\ \log(4.64 \cdot 10^{8}) \\ \log(4.28 \cdot 10^{8}) \end{bmatrix}$$

10 μl of 4% Pluronic F127 solution

$$t3 := \begin{bmatrix} 115 \\ 230 \\ 340 \\ 445 \\ 700 \\ 1310 \\ 1420 \end{bmatrix} \qquad ca2pl2 := \begin{bmatrix} \log(2.43 \cdot 10^{6}) \\ \log(1.10 \cdot 10^{7}) \\ \log(2.83 \cdot 10^{7}) \\ \log(1.07 \cdot 10^{8}) \\ \log(4.11 \cdot 10^{8}) \\ \log(5.20 \cdot 10^{8}) \\ \log(4.89 \cdot 10^{8}) \end{bmatrix}$$

1000 ml of 4% Pluronic F127 solution

Hydrophobic microsphere assay for *Candida albicans* strain 1 (HMA1) and *Candida albicans* strain 662 (HMA2). The numbers represent the percent of cells which have three or more attached microspheres.

$$HMA1 := \begin{bmatrix} 10.5 \\ 0.26 \\ 0.25 \\ 11.9 \end{bmatrix} \qquad HMA2 := \begin{bmatrix} 0.51 \\ 6.1 \\ 2.6 \end{bmatrix}$$

$mean(HMA1) = 5.7275$

$Stdev(HMA1) = 6.344895$

$median(HMA1) = 5.38$

$$\frac{3 \cdot (5.7275 - 5.38)}{6.344895} = 0.164305$$

Slightly positively skewed.

$mean(HMA2) = 3.07$

$Stdev(HMA2) = 2.824482$

$median(HMA2) = 2.6$

$$\frac{3 \cdot (3.07 - 2.6)}{2.824482} = 0.499$$

Slightly positively skewed.

Atomic Concentration Table

File: pscs2 silanized coverslip coated with PS
Area: 1 Lens: Minimum Area Omni-Focus Source:Monochromated

Element	Area (cts-eV/s)	Sensitivity Factor	Concentration (%)
C1s	268380	16.518	95.35
O1s	24145	39.890	3.55
Si2p	2936	15.733	1.10

Atomic Concentration Table

File: pscs22 silanized coverslip coated with PS cleaned with bleach
Area: 1 Lens: Minimum Area Omni-Focus Source:Monochromated

Element	Area (cts-eV/s)	Sensitivity Factor	Concentration (%)
C1s	278572	16.518	96.72
O1s	18130	39.890	2.61
Si2p	1646	15.733	0.60
Cl2p	560	42.872	0.07

Atomic Concentration Table

File: ps723c12 polystyrene, treated with 180 W plasma
Area: 1 Lens: Minimum Area Omni-Focus Source:Monochromated

Element	Area (cts-eV/s)	Sensitivity Factor	Concentration (%)
C1s	68668	16.518	82.46
O1s	35265	39.890	17.54

Atomic Concentration Table

File: beam183 18 shot O beam treated polystyrene
Area: 1 Lens: Minimum Area Omni-Focus Source:Monochromated

Element	Area (cts-eV/s)	Sensitivity Factor	Concentration (%)
C1s	95413	16.518	83.06
O1s	46993	39.890	16.94

Atomic Concentration Table

File: kwob62 polystyrene sample 6
Area: 1 Lens: Minimum Area Omni-Focus Source:Monochromated

Element	Area (cts-eV/s)	Sensitivity Factor	Concentration (%)
C1s	103780	16.518	98.40
O1s	4073	39.890	1.60

Atomic Concentration Table

File: kw825982 Pluronic f127 treated Polystyrene
Area: 1 Lens: Minimum Area Omni-Focus Source:Monochromated

Element	Area (cts-eV/s)	Sensitivity Factor	Concentration (%)
C1s	156050	16.518	89.60
O1s	43730	39.890	10.40

APPENDIX C

CALCULATIONS

Growth rate calculations for *Candida albicans* strain 1 and *Candida albicans* strain 662 in reduced GYEP medium.

$$\mathbf{data} := \begin{bmatrix} 120 & \log(8.50\cdot10^{5}) \\ 240 & \log(7.75\cdot10^{5}) \\ 365 & \log(3.33\cdot10^{6}) \\ 435 & \log(4.30\cdot10^{6}) \\ 720 & \log(6.50\cdot10^{6}) \\ 1385 & \log(6.00\cdot10^{6}) \\ 1440 & \log(1.00\cdot10^{7}) \end{bmatrix} \qquad \mathbf{data2} := \begin{bmatrix} 120 & \log(8.00\cdot10^{5}) \\ 255 & \log(8.75\cdot10^{5}) \\ 365 & \log(3.03\cdot10^{6}) \\ 405 & \log(5.68\cdot10^{6}) \\ 720 & \log(9.00\cdot10^{6}) \\ 1440 & \log(7.25\cdot10^{6}) \end{bmatrix}$$

$$\mathbf{Time} := \mathbf{data}^{<0>} \qquad \mathbf{Concentration} := \mathbf{data}^{<1>} - \log(8.50\cdot10^{5})$$

$$\mathbf{Time2} := \mathbf{data2}^{<0>} \qquad \mathbf{Concentration2} := \mathbf{data2}^{<1>} - \log(8.00\cdot10^{5})$$

$$\mathbf{dataCa662} := \begin{bmatrix} 120 & \log(1.40\cdot10^{6}) \\ 255 & \log(5.95\cdot10^{6}) \\ 365 & \log(8.25\cdot10^{6}) \\ 395 & \log(9.00\cdot10^{6}) \\ 710 & \log(1.00\cdot10^{7}) \\ 1395 & \log(8.25\cdot10^{6}) \\ 1440 & \log(1.00\cdot10^{7}) \end{bmatrix} \qquad \mathbf{data2Ca662} := \begin{bmatrix} 120 & \log(1.60\cdot10^{6}) \\ 255 & \log(6.05\cdot10^{6}) \\ 365 & \log(7.75\cdot10^{6}) \\ 395 & \log(9.50\cdot10^{6}) \\ 710 & \log(7.25\cdot10^{6}) \\ 1395 & \log(4.00\cdot10^{6}) \\ 1440 & \log(1.00\cdot10^{7}) \end{bmatrix}$$

$$\mathbf{TimeCa662} := \mathbf{data}^{<0>} \qquad \mathbf{ConcentrationCa662} := \mathbf{dataCa662}^{<1>} - \log(1.40\cdot10^{6})$$

$$\mathbf{Time2Ca662} := \mathbf{data2Ca662}^{<0>} \qquad \mathbf{Concentration2Ca662} := \mathbf{data2Ca662}^{<1>} - \log(1.60\cdot10^{6})$$

Growth rate calculations for *Candida albicans* strain 1 and *Candida albicans* strain 662 in reduced GYEP medium - continued.

$$\mathbf{y1} := \begin{bmatrix} 0.11 \\ 0.11 \\ 0.11 \end{bmatrix} \quad \mathbf{x1} := \begin{bmatrix} 0 \\ 800 \\ 1600 \end{bmatrix} \quad \mathbf{y2} := \begin{bmatrix} 0.411 \\ 0.411 \\ 0.411 \end{bmatrix} \quad \mathbf{x2} := \begin{bmatrix} 0 \\ 800 \\ 1600 \end{bmatrix}$$

$$\mathbf{y3} := \begin{bmatrix} -0.10 \\ 0.11 \end{bmatrix} \quad \mathbf{x3} := \begin{bmatrix} 143 \\ 143 \end{bmatrix} \quad \mathbf{y4} := \begin{bmatrix} -0.10 \\ 0.11 \end{bmatrix} \quad \mathbf{x4} := \begin{bmatrix} 270 \\ 270 \end{bmatrix}$$

$$\mathbf{y5} := \begin{bmatrix} -0.10 \\ 0.411 \end{bmatrix} \quad \mathbf{x5} := \begin{bmatrix} 205 \\ 205 \end{bmatrix} \quad \mathbf{y6} := \begin{bmatrix} -0.10 \\ 0.411 \end{bmatrix} \quad \mathbf{x6} := \begin{bmatrix} 331 \\ 331 \end{bmatrix}$$

CA1

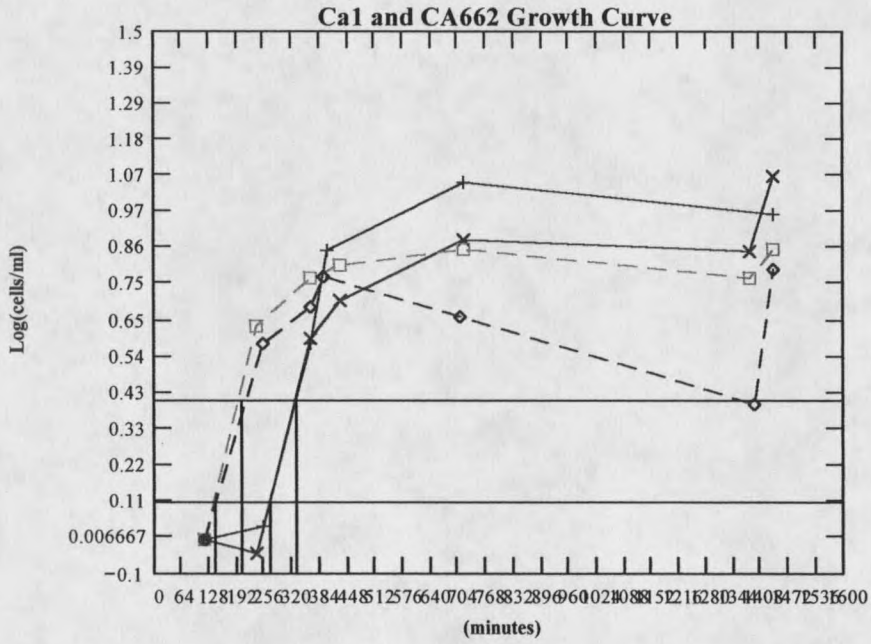
$331 - 270 = 61$

$\mu = 0.01136$

CA662

$205 - 143 = 62$

$\mu = 0.01118$



Shear Rate - Calculations - For Fluid Flow Between Two Flat Parallel Plates

Average velocity of the fluid

$$v_x = \frac{\mathring{V}}{2\delta W}$$

where $\mathring{V}$ is the volume flow rate
δ is one-half of the distance between the plates
W is the width of the plates

Shear Rate

$$\frac{dv_x}{dy} = \frac{3\, v_x}{\delta}$$

Old Flow Cell

Viton gasket: 1/32 ″ thick

Width of flow channel as determined by design of viton gasket: 1.5 cm

$$v_x = \frac{1.5\ \text{ml}}{\text{min}} \cdot \frac{\text{cm}^3}{2 \cdot 0.0396875\ \text{cm} \cdot 1.5\ \text{cm} \cdot \text{ml}}$$

$v_x = 12.5984252$ cm/min

$$\frac{dv_x}{dy} = \frac{3 \cdot 12.6\ \text{cm}}{\text{min} \cdot 0.0396875\ \text{cm} \cdot 60\ \text{sec}} = 15.87\ \text{sec}^{-1}$$

New Flow Cell

Channel thickness: 1/16 ″

Width of flow channel: 1 cm

$$v_x = \frac{3.44\ \text{ml}}{\text{min}} \cdot \frac{\text{cm}^3}{2 \cdot 0.079375\ \text{cm} \cdot 1\ \text{cm} \cdot \text{ml}}$$

$v_x = 21.66929134$ cm/min

$$\frac{dv_x}{dy} = \frac{3 \cdot 21.7\ \text{cm}}{\text{min} \cdot 0.079375\ \text{cm} \cdot 60\ \text{sec}} = 13.65\ \text{sec}^{-1}$$

Fluid-flow and mass transfer characteristics of the Kynar flow cell system.

Volume of Flow Cell

$l := 5.1\ cm$

$w := 1.0\ cm$

$h := 0.15875\ cm$

$l \cdot w \cdot h = 8.1 \cdot 10^{-7} \cdot m^3$

Mean Residence Time

$l := 5.1\ cm$

$v := 21.66929134 \cdot \frac{cm}{min}$

$\Theta := \frac{l}{v}$

$\Theta = 14.12 \cdot s$

Reynolds Number

$D := 1.5875 \cdot 10^{-3}\ m$

$vx := 21.66929134\ \frac{cm}{min}$

$\rho := 997\ \frac{kg}{m^3}$

$\eta := 8.57 \cdot 10^{-4}\ Pa \cdot s$

$Nre := \frac{D \cdot vx \cdot \rho}{\eta}$

$Nre = 6.67$

Entry Length

$Le := 0.05 \cdot Nre \cdot D$

$Le = 5.3 \cdot 10^{-4} \cdot m$

Reynolds Number for a Particle in a Fluid

$Dp := 0.0005\ cm$

$vx := 21.66929134\ \frac{cm}{min}$

$\rho := 997\ \frac{kg}{m^3}$

$\eta := 8.57 \cdot 10^{-4}\ Pa \cdot s$

$Nrep := \frac{Dp \cdot vx \cdot \rho}{\eta}$

$Nrep = 0.02$

Terminal Settling Velocity

$g = 9.807 \cdot m \cdot s^{-2}$

$Dp = 5 \cdot 10^{-6} \cdot m$

$\rho p := 1001\ \frac{kg}{m^3}$

$\rho = 997 \cdot kg \cdot m^{-3}$

$\eta = 8.57 \cdot 10^{-4} \cdot kg \cdot m^{-1} \cdot s^{-1}$

$ut := \frac{g \cdot Dp^2 \cdot (\rho p - \rho)}{18 \cdot \eta}$

$ut = 6.4 \cdot 10^{-8} \cdot m \cdot s^{-1}$

Fluid-flow and mass transfer characteristics of the Kynar flow cell system - continued.

Liquid Diffusion Coefficient

$KbT := 4.1124 \cdot 10^{-17} \frac{kg \cdot cm^2}{s^2}$

$\eta = 8.57 \cdot 10^{-4} \cdot kg \cdot m^{-1} \cdot s^{-1}$

$Rp := \frac{Dp}{2}$

$Df := \frac{KbT}{6 \cdot \pi \cdot \eta \cdot Rp}$

$Df = 1.02 \cdot 10^{-13} \cdot m^2 \cdot s^{-1}$

Diffusion Distance

$Df = 1.018 \cdot 10^{-13} \cdot m^2 \cdot s^{-1}$

$t1 := 14\ sec$ (Mean Residence Time)

$Dd1 := \sqrt{Df \cdot t1}$

$Dd1 = 1.2 \cdot 10^{-6} \cdot m$

$t2 := 3600\ sec$ (Time Length of Experiment)

$Dd2 := \sqrt{Df \cdot t2}$

$Dd2 = 1.9 \cdot 10^{-5} \cdot m$

APPENDIX D

STATISTICAL ANALYSIS

Data from adhesion of *C. albicans* strain 662 on polystyrene as a function of distance from the inlet. This data was given previously in Appendix B. Cells per unit area has units of cells per square millimeter, and distance has units of centimeters. Regression analysis was performed on the data set. The results of the analysis are given in the following pages.

	C1	C2	C3	C4	C5
	Cell Per Unit Area	Distance	Log(Cells Per Unit Area)	SRES1	SRES2
1	17386.7	2.5	4.24022	-0.50233	0.18308
2	7634.7	1.4	3.88279	-1.34996	-1.09037
3	25630.9	3.6	4.40876	-0.09000	0.09981
4	12735.3	0.8	4.10501	1.09723	1.25611
5	5196.8	0.8	3.71574	-1.03793	-1.62680
6	26465.0	4.2	4.42267	-0.88671	-0.51879
7	28967.1	4.2	4.46191	-0.18402	-0.23069
8	20821.4	2.5	4.31851	0.42771	0.73740
9	7309.7	1.4	3.86390	-1.43968	-1.22673
10	26675.5	3.6	4.42611	0.19685	0.22438
11	14745.9	0.8	4.16867	1.66670	1.72759
12	4303.5	0.8	3.63382	-1.29094	-2.23345
13	24460.4	4.2	4.38846	-1.44968	-0.76999
14	29048.7	4.2	4.46313	-0.16110	-0.22172
15	24242.7	2.5	4.38458	1.35412	1.20520
16	11467.0	1.4	4.05945	-0.29207	0.18474
17	30568.2	3.6	4.48527	1.26581	0.64914
18	13274.3	0.8	4.12301	1.24989	1.38944
19	32001.6	4.2	4.50517	0.66820	0.08703
20	32282.1	4.2	4.50896	0.74697	0.11487

Regression Analysis: Log(Cells Per Unit Area) versus Distance

The regression equation is
Log(Cells Per Unit Area) = 3.80 + 0.164 Distance

Predictor	Coef	SE Coef	T	P
Constant	3.80412	0.06825	55.74	0.000
Distance	0.16409	0.02323	7.06	0.000

S = 0.1449 R-Sq = 73.5% R-Sq(adj) = 72.0%

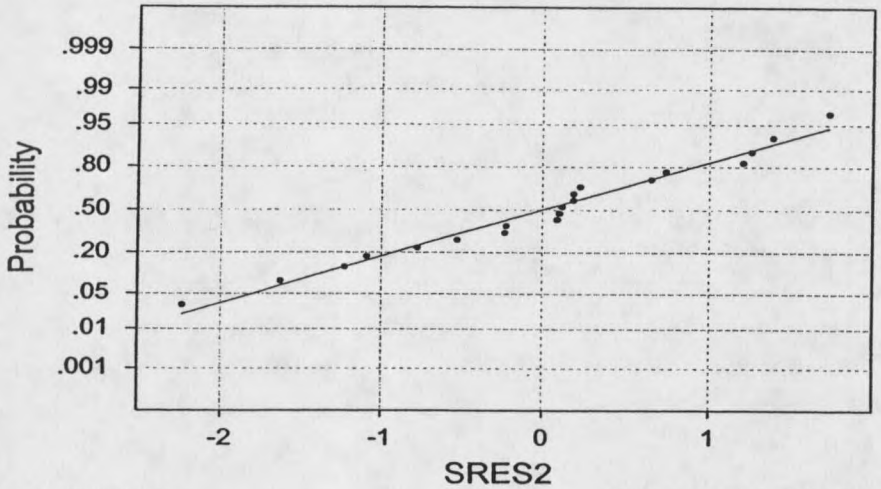
Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	1.0476	1.0476	49.88	0.000
Residual Error	18	0.3780	0.0210		
Total	19	1.4257			

Unusual Observations

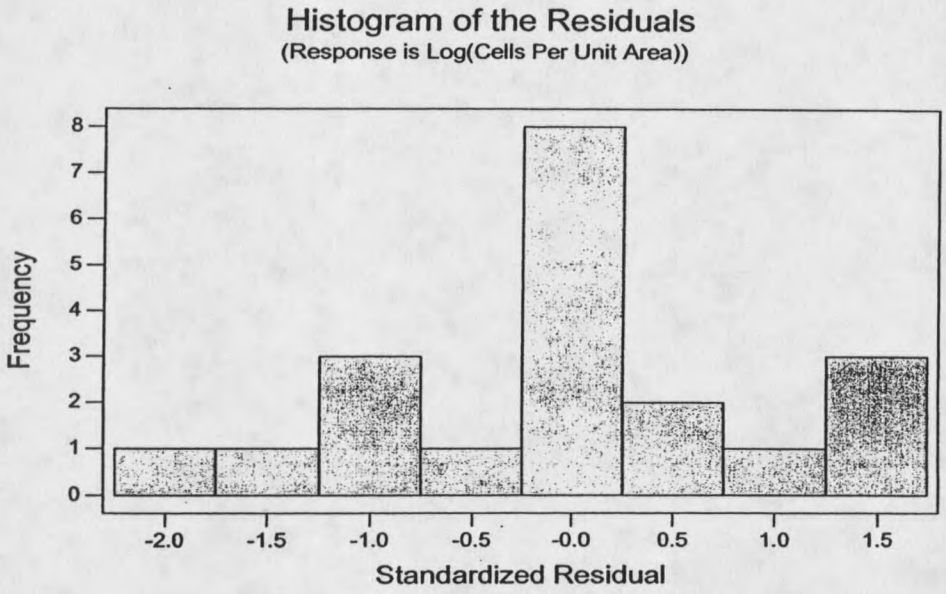
Obs	Distance	Log(Cell	Fit	SE Fit	Residual	St Resid
12	0.80	3.6338	3.9354	0.0526	-0.3016	-2.23R

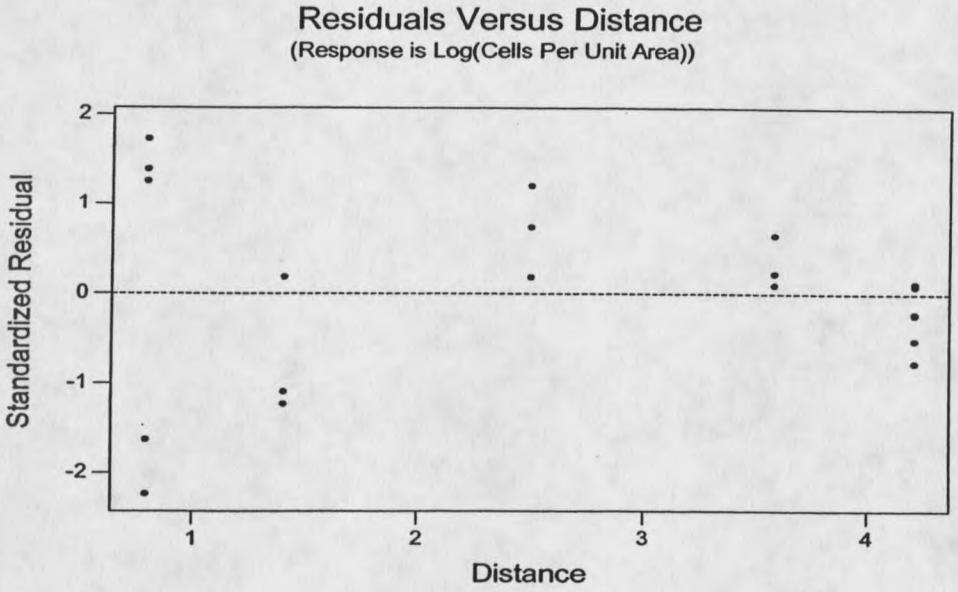
R denotes an observation with a large standardized residual

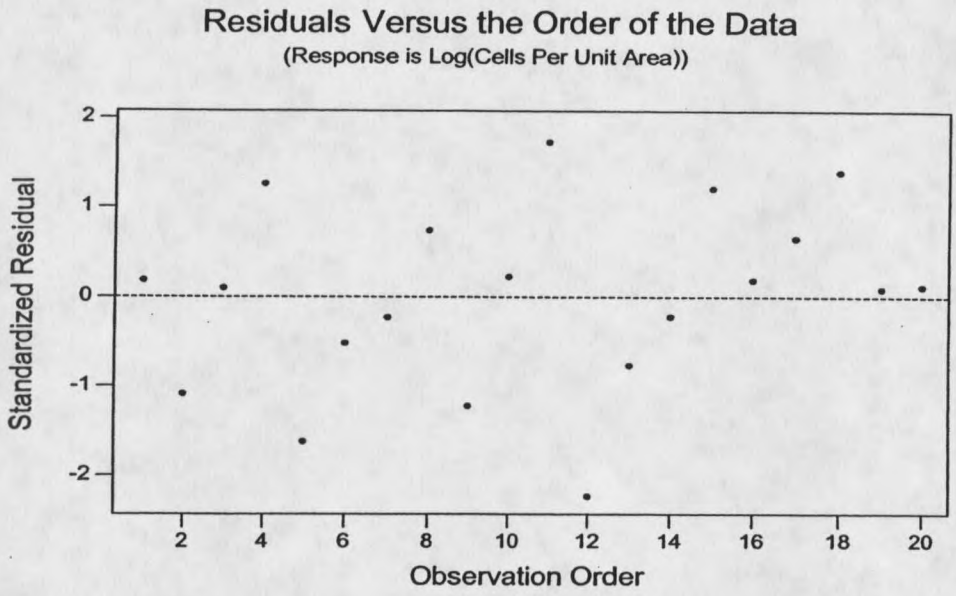


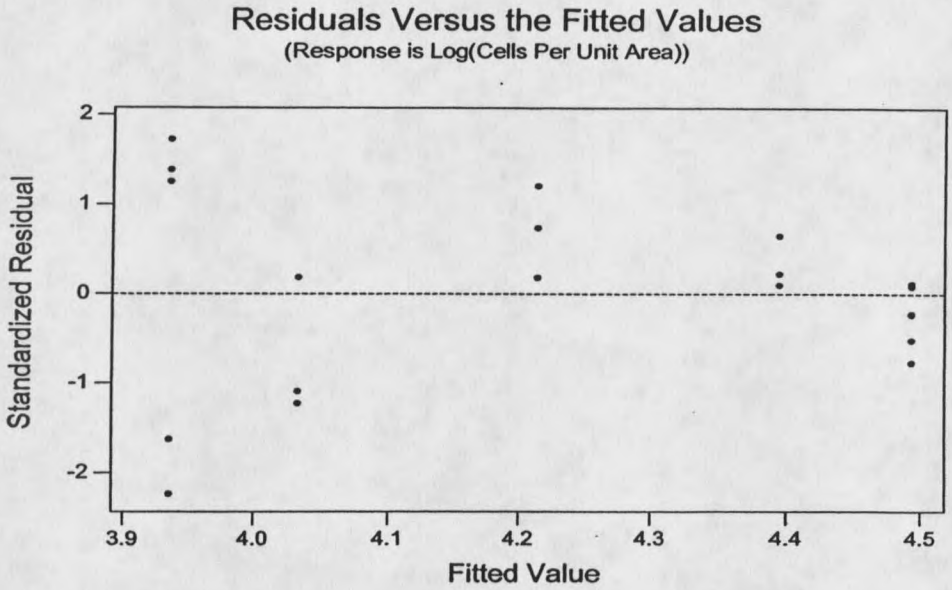
Average: -0.0029865
StDev: 1.03497
N: 20

Anderson-Darling Normality Test
A-Squared: 0.260
P-Value: 0.675


Histogram of the Residuals
(Response is Log(Cells Per Unit Area))
Frequency
0
1
2
3
4
5
6
7
8
-2.0
-1.5
-1.0
-0.5
-0.0
0.5
1.0
1.5
Standardized Residual


Residuals Versus Distance
(Response is Log(Cells Per Unit Area))
Standardized Residual
2
1
0
-1
-2
1
2
3
4
Distance


Residuals Versus the Order of the Data
(Response is Log(Cells Per Unit Area))
Standardized Residual
2
1
0
-1
-2
2
4
6
8
10
12
14
16
18
20
Observation Order

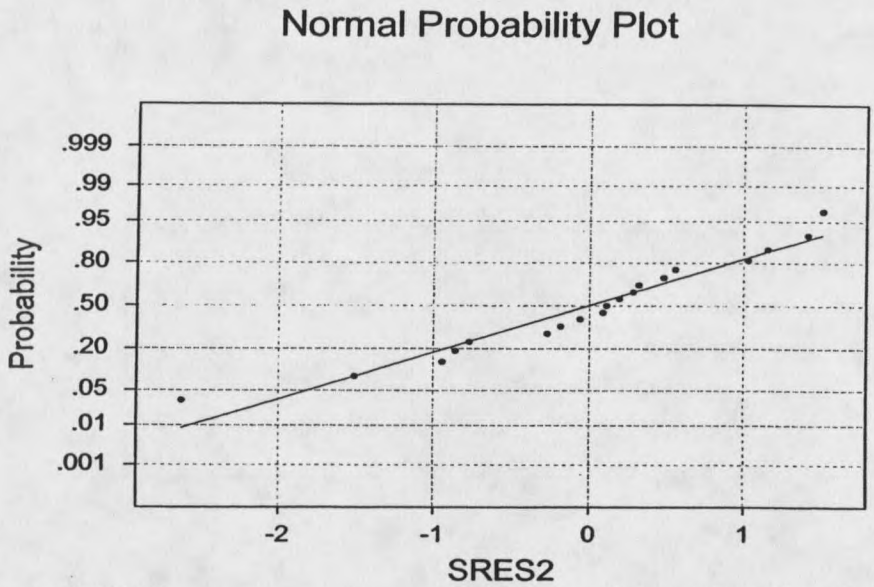

Residuals Versus the Fitted Values
(Response is Log(Cells Per Unit Area))
Standardized Residual
2
1
0
-1
-2
3.9
4.0
4.1
4.2
4.3
4.4
4.5
Fitted Value

Data from adhesion of *C. albicans* strain 662 on Pluronic F127 treated polystyrene as a function of distance from the inlet. This data was given previously in Appendix B. Cells per unit area has units of cells per square millimeter, and distance has units of centimeters. Regression analysis was performed on the data set. The results of the analysis are given in the following pages.

	C1	C2	C3
	Cells Per Unit Area	Distance	Log (Cells Per Unit Area)
1	1021.95	2.5	3.00943
2	309.68	1.4	2.49091
3	867.11	3.6	2.93807
4	309.68	0.8	2.49091
5	92.90	0.8	1.96804
6	1424.53	4.2	3.15367
7	1052.91	4.2	3.02239
8	444.81	2.5	2.64818
9	513.24	1.4	2.71032
10	171.08	3.6	2.23320
11	136.87	0.8	2.13629
12	102.65	0.8	2.01135
13	0.00	4.2	*
14	0.00	4.2	*
15	503.03	2.5	2.70159
16	201.21	1.4	2.30365
17	1408.48	3.6	3.14875
18	167.68	0.8	2.22447
19	67.07	0.8	1.82653
20	1274.33	4.2	3.10528
21	670.70	4.2	2.82653

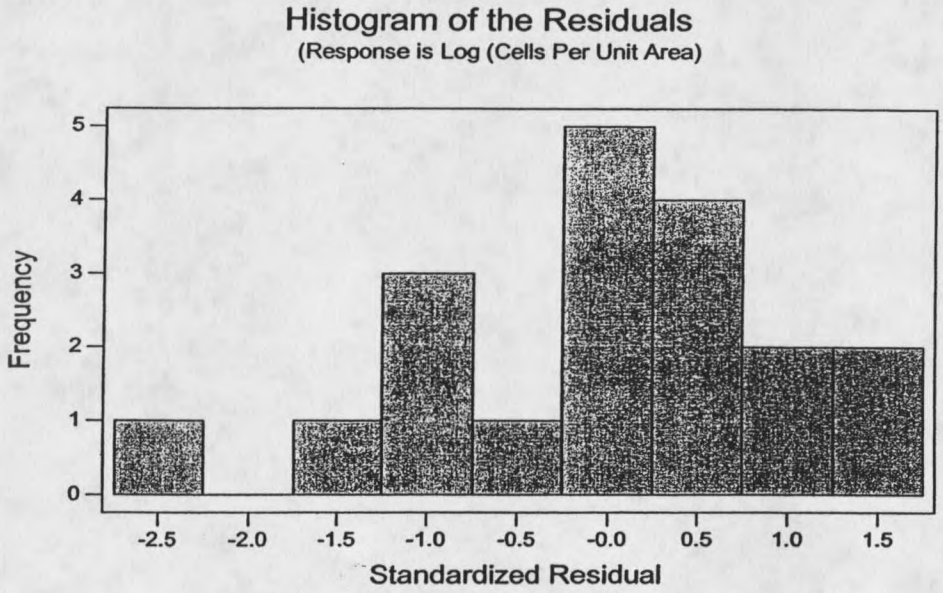
Regression Analysis: Log (Cells Per Unit Area) versus Distance

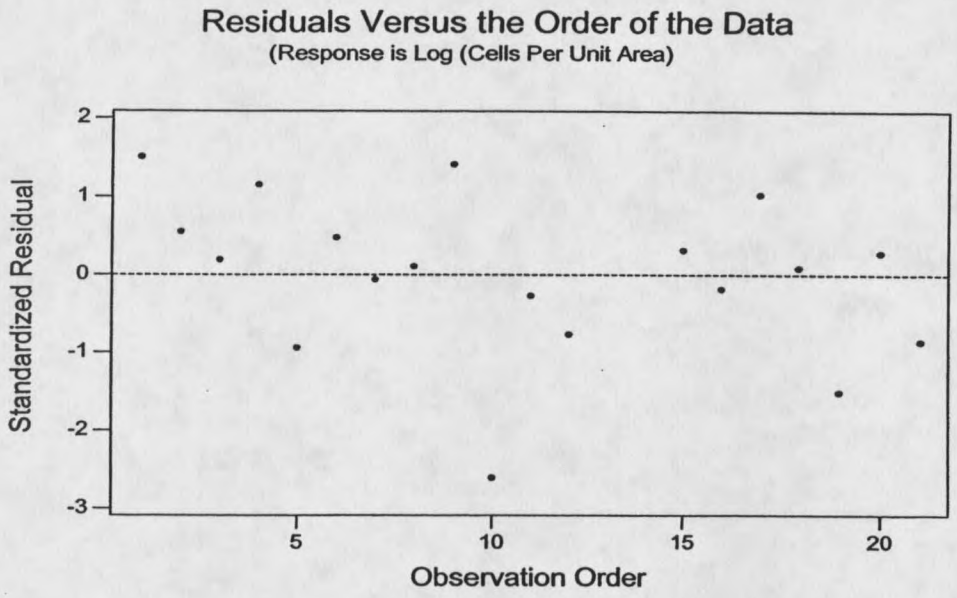
```
The regression equation is
Log (Cells Per Unit Area) = 2.01 + 0.245 Distance

19 cases used 2 cases contain missing values

Predictor        Coef     SE Coef        T       P
Constant       2.0069      0.1198    16.75   0.000
Distance      0.24532     0.04448     5.52   0.000

S = 0.2652              R-Sq = 64.2%        R-Sq(adj) = 62.0%
PRESS = 1.47183         R-Sq(pred) = 55.87%

Analysis of Variance

Source            DF        SS        MS        F        P
Regression         1    2.1396    2.1396    30.42    0.000
Residual Error    17    1.1956    0.0703
Total             18    3.3351

Unusual Observations
Obs   Distance   Log (Cel       Fit    SE Fit    Residual    St Resid
 10       3.60     2.2332    2.8900    0.0833     -0.6568      -2.61R

R denotes an observation with a large standardized residual
```

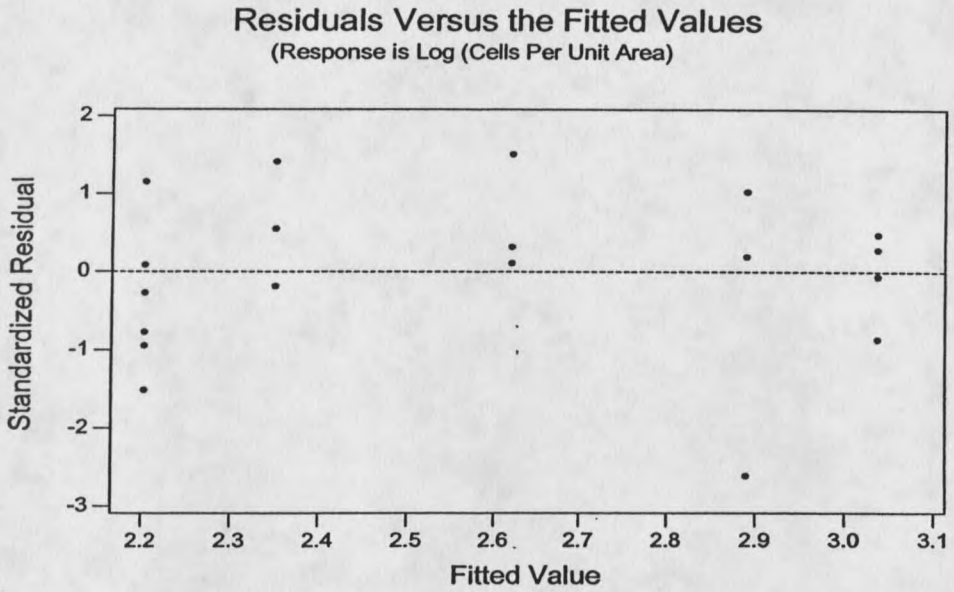


Average: -0.0051855
StDev: 1.02347
N: 19

Anderson-Darling Normality Test
A-Squared: 0.330
P-Value: 0.485


Histogram of the Residuals
(Response is Log (Cells Per Unit Area)
Frequency
0
1
2
3
4
5
-2.5
-2.0
-1.5
-1.0
-0.5
-0.0
0.5
1.0
1.5
Standardized Residual


Residuals Versus the Order of the Data
(Response is Log (Cells Per Unit Area)
Standardized Residual
2
1
0
-1
-2
-3
5
10
15
20
Observation Order


Residuals Versus the Fitted Values
(Response is Log (Cells Per Unit Area)
Standardized Residual
2
1
0
-1
-2
-3
2.2
2.3
2.4
2.5
2.6
2.7
2.8
2.9
3.0
3.1
Fitted Value

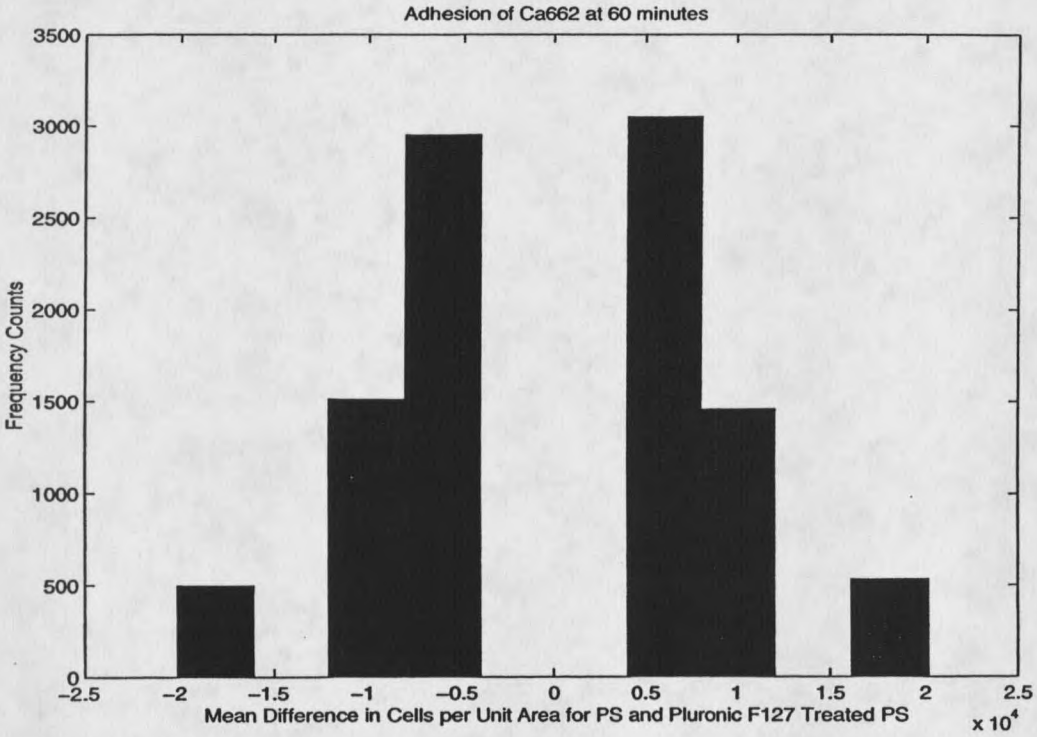
Matlab® program used to perform a randomization test on adhesion data. The program is used to determined whether there is a significant difference in the number of cells (*C. albicans* strain 662) that adhered to Pluronic F127 treated polystyrene in comparison to unmodified polystyrene after 60 minutes at location A.

```
format long;
load AdhD6.m;
Y=AdhD6;
k=0;
n=10000;
D=zeros(n,1);
while k < 10000
    j = 0;
    h = 0;
    k = k+1;
    X=rand(6,1);
    [Z,I] = sort(X);
    A=zeros(3,1);
    B=zeros(3,1);
    Ma=0;
    Mb=0;
    for i = 1:6
        if I(i,1) <= 3
           j = j+1;
           A(j,1) = Y(i,1);
        else
           h = h+1;
           B(h,1) = Y(i,1);
        end
    end
    Ma = mean(A);
    Mb = mean(B);
    D(k,1) = Ma - Mb;
end
D
```

Results from performing the randomization test on the adhesion data for *C. albicans* strain 662 on Pluronic F127 treated polystyrene and unmodified polystyrene using the Matlab® program. Z = prctile(D,P) returns a value that is greater the P percent of the values in D.

```
MATLAB Command Window
July 12, 2002                                                    Page 1
                                                                 2:11:31 PM
>> P=[0,1,2,3,4,5,10,20,30,40,50,60,70,80,90,95,96,97,98,99,100]

P =

  Columns 1 through 12

     0     1     2     3     4     5    10    20    30    40    50    60

  Columns 13 through 21

    70    80    90    95    96    97    98    99   100

>> Z=prctile(D,P)

Z =

   1.0e+04 *

  Columns 1 through 3

  -2.01603499811667  -2.01603499811667  -2.01603499811667

  Columns 4 through 6

  -2.01603499811667  -2.01603499811667  -0.92505167998333

  Columns 7 through 9

  -0.89045703401000  -0.88657600879000  -0.65759370212333

  Columns 10 through 12

  -0.43338961601000   0.42950859079000   0.46798426198333

  Columns 13 through 15

   0.66147472734333   0.69606937331667   0.92505167998333

  Columns 16 through 18

   2.01603499811667   2.01603499811667   2.01603499811667

  Columns 19 through 21

   2.01603499811667   2.01603499811667   2.01603499811667

>>
```

```
>> Z=prctile(D,P)

Z =

     2.016034998116667e+04

>> P=[0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100];
>> Z=prctile(D,P)

Z =

   1.0e+04 *

  Columns 1 through 3

  -2.01603499811667  -0.89045703401000  -0.88657600879000

  Columns 4 through 6

  -0.65759370212333  -0.43338961601000   0.42950859079000

  Columns 7 through 9

   0.46798426198333   0.66147472734333   0.69606937331667

  Columns 10 through 11

   0.92505167998333   2.01603499811667
```

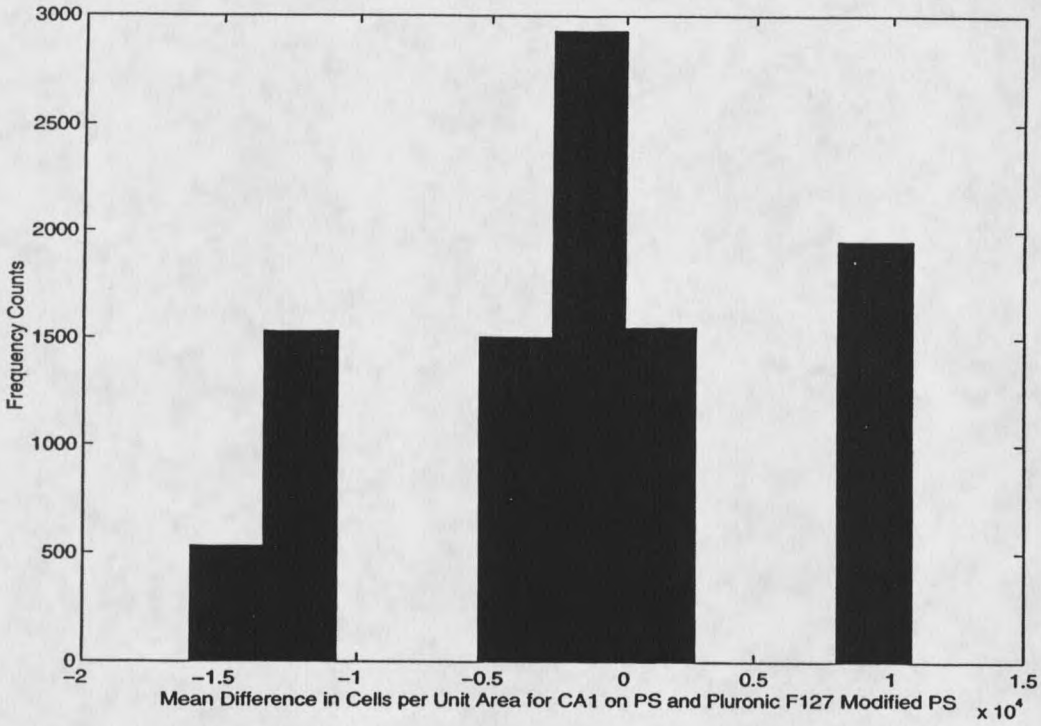

Adhesion of Ca662 at 60 minutes
Frequency Counts
3500
3000
2500
2000
1500
1000
500
0
−2.5
−2
−1.5
−1
−0.5
0
0.5
1
1.5
2
2.5
Mean Difference in Cells per Unit Area for PS and Pluronic F127 Treated PS
x 10^4

Matlab® program used to perform a randomization test on adhesion data. The program is used to determined whether there is a significant difference in the number of cells (*C. albicans* strain 1) that adhered to Pluronic F127 treated polystyrene in comparison to unmodified polystyrene after 60 minutes at location A.


```
format long;
load AdhD5.m;
Y=AdhD5;
k=0;
n=10000;
D=zeros(n,1);
while k < 10000
    j = 0;
    h = 0;
    k = k+1;
    X=rand(6,1);
    [Z,I] = sort(X);
    A=zeros(3,1);
    B=zeros(2,1);
    Ma=0;
    Mb=0;
    for i = 1:5
    if I(i,1) <= 3
       j = j+1;
       A(j,1) = Y(i,1);
    else
       h = h+1;
       B(h,1) = Y(i,1);
    end
    end
    Ma = mean(A);
    Mb = mean(B);
    D(k,1) = Ma - Mb;
end
D
```

Results from performing the randomization test on the adhesion data for *C. albicans* strain 1 on Pluronic F127 treated polystyrene and unmodified polystyrene using the Matlab® program. Z = prctile(D,P) returns a value that is greater the P percent of the values in D.

```
MATLAB Command Window                                                    Page 1
July 12, 2002                                                            2:41:52 PM
>> P=[0,1,2,3,4,5,6,7,8,9,10,20,30,40,50,60,70,80,90,91,92,93,94,95,96,97,98,99,100];
>> Z=prctile(D,P)

Z =

   1.0e+04 *

  Columns 1 through 3

  -1.61251352091667  -1.61251352091667  -1.61251352091667

  Columns 4 through 6

  -1.61251352091667  -1.61251352091667  -1.61251352091667

  Columns 7 through 9

  -1.08041024851667  -1.08041024851667  -1.08041024851667

  Columns 10 through 12

  -1.08041024851667  -1.08041024851667  -1.07655222381667

  Columns 13 through 15

  -0.30151282216667  -0.23888520333333  -0.23406267245833

  Columns 16 through 18

  -0.02775166481667   0.01926401048333   0.02312203518333

  Columns 19 through 21

   1.07211549541667   1.07597352012500   1.07597352012500

  Columns 22 through 24

   1.07597352012500   1.07597352012500   1.07645577320833

  Columns 25 through 27

   1.07693802629167   1.07693802629167   1.07693802629167

  Columns 28 through 29

   1.07693802629167   1.07693802629167

>> [n,x]=hist(D)

n =

  Columns 1 through 6

        532        1530           0           0        1506        2930

  Columns 7 through 10
```

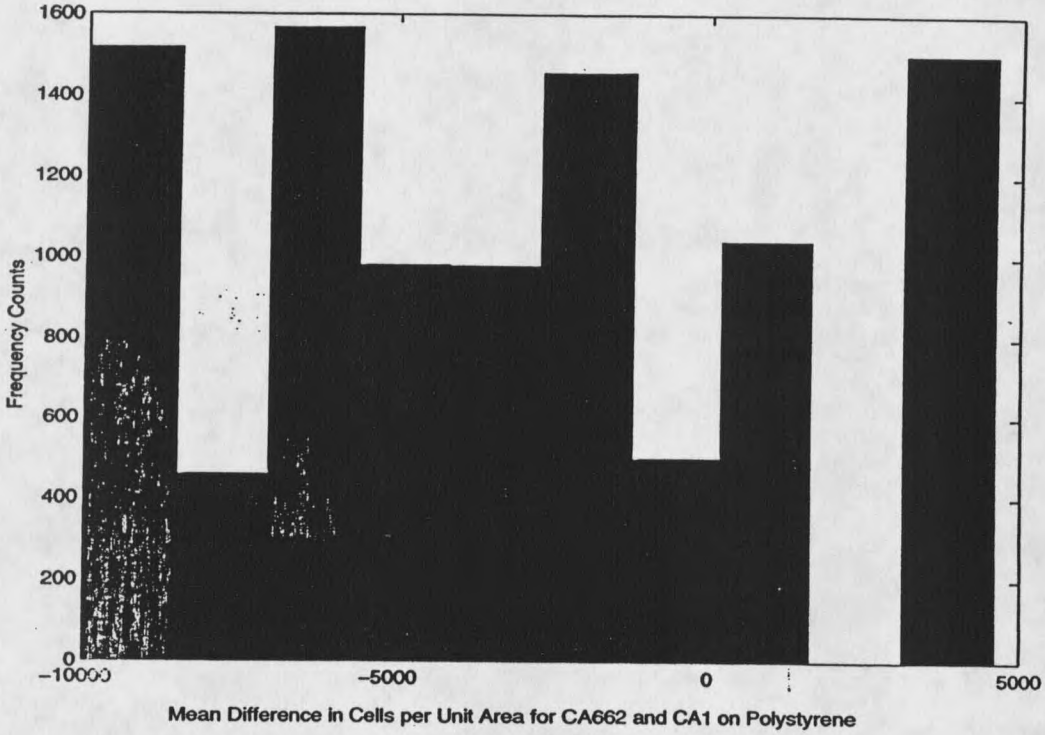

3000
2500
2000
1500
1000
500
0
Frequency Counts
−2
−1.5
−1
−0.5
0
0.5
1
1.5
Mean Difference in Cells per Unit Area for CA1 on PS and Pluronic F127 Modified PS
x 10^4

Matlab® program used to perform a randomization test on adhesion data. The program is used to determined whether there is a significant difference in the number of *C. albicans* strain 1 cells that adhered to polystyrene in comparison to the number of *C. albicans* strain 662 cells that adhered to polystyrene.


```
format long;
load AdhD4.m;
Y=AdhD4;
k=0;
n=10000;
D=zeros(n,1);
while k < 10000
    j = 0;
    h = 0;
    k = k+1;
    X=rand(6,1);
    [Z,I] = sort(X);
    A=zeros(3,1);
    B=zeros(2,1);
    Ma=0;
    Mb=0;
    for i = 1:5
    if I(i,1) <= 3
       j = j+1;
       A(j,1) = Y(i,1);
    else
       h = h+1;
       B(h,1) = Y(i,1);
    end
    end
    Ma = mean(A);
    Mb = mean(B);
    D(k,1) = Ma - Mb;
end
D
```

Results from performing the randomization test on the adhesion data for *C. albicans* strain 1 on polystyrene and for *C. albicans* strain 662 on polystyrene using the Matlab® program. Z = prctile(D,P) returns a value that is greater the P percent of the values in D.

```
MATLAB Command Window                                          Page 1
July 12, 2002                                                  2:33:33 PM
>> P=[0,10,20,30,40,41,42,43,44,45,46,47,48,49,50,60,70,80,90,100];
>> Z=prctile(D,P)

Z =

   1.0e+03 *

  Columns 1 through 3

  -9.98969440000000  -8.97144559333333  -6.68162252666666

  Columns 4 through 6

  -6.17211072000000  -4.90950841333333  -4.40077141333334

  Columns 7 through 9

  -4.40077141333334  -4.40077141333334  -4.40077141333334

  Columns 10 through 12

  -4.40077141333334  -3.89125960666667  -3.89125960666667

  Columns 13 through 15

  -3.89125960666667  -3.89125960666667  -3.89125960666667

  Columns 16 through 18

  -2.41008465000000  -0.19591051666667   1.07690049166667

  Columns 19 through 20

   3.93917932500000   4.57606908333333

>>
```


1600
1400
1200
1000
800
600
400
200
0
Frequency Counts
-10000
-5000
0
5000
Mean Difference in Cells per Unit Area for CA662 and CA1 on Polystyrene

Maximum Percent Surface Coverage for CA1 Biofilms

Max % Surface Coverage	Surface	Max % Surface Coverage	Surface
20.72	PS	0.54	PLPS
32.47	PS	1.38	PLPS
26.04	PS	0.58	PLPS
22.63	PS	0.99	PLPS
10.27	PS	0.65	PLPS
6.42	PS	0.78	PLPS
33.64	PS	0.18	PLPS
13.31	PS	0.13	PLPS
26.19	PS	0.33	PLPS
34.9	PS	0.18	PLPS
11.67	PS	0.26	PLPS
37.38	PS	0.29	PLPS
27.93	PS	0.58	PLPS
34.15	PS	1.05	PLPS
38.07	PS	2.01	PLPS
56.53	PS	0.87	PLPS
37.44	PS	0.21	PLPS
27.25	PS	0.95	PLPS
10.16	PS	0.7	PLPS
21.99	PS	0.59	PLPS
6.39	PS	0.25	PLPS
12.56	PS	0.15	PLPS
14.91	PS	0.53	PLPS
8.78	PS	1.05	PLPS
26.59	PS	1.65	PLPS
10.52	PS	1.06	PLPS
13.38	PS	1.36	PLPS
9.66	PS		

Maximum Percent Surface Coverage for CA662 Biofilms

Location A		Location B	
Max. % Surface Coverage	Surface	Max. % Surface Coverage	Surface
0.22	PS	0.91	PS
1.11	PS	0.67	PS
9.07	PS	2.04	PS
0.47	PS	0.74	PS
0.31	PS	0	PS
0.33	PS	0.22	PS
0.22	PS	0.4	PS
0	PLPS	0	PLPS
0	PLPS	0.1	PLPS
0.11	PLPS	0.15	PLPS
0.11	PLPS	0.31	PLPS

P(diff > 1.6207) = 0.00303

P(diff > 0.5714) = 0.02727

Location C		Location D	
Max. % Surface Coverage	Surface	Max. % Surface Coverage	Surface
1.29	PS	4.18	PS
0.63	PS	0.35	PS
2.32	PS	2.22	PS
0.71	PS	3.65	PS
0.35	PS	0.67	PS
0.33	PS	0.35	PS
0	PS	1.29	PS
0	PLPS	0	PLPS
0.12	PLPS	0.1	PLPS
0.15	PLPS	0.12	PLPS
0.16	PLPS	0.22	PLPS

P(diff > 0.6968) = 0.01515

P(diff > 1.7057) = 0.00303

Maximum Percent Surface Coverage for CA662 Biofilms - Continued

Location E		Location F	
Max. % Surface Coverage	Surface	Max. % Surface Coverage	Surface
0.27	PS	0.28	PS
2.11	PS	0.59	PS
4.94	PS	11.44	PS
4.17	PS	2.17	PS
0.6	PS	0.38	PS
0.11	PS	2.53	PS
0	PS	0.11	PS
0	PLPS	0	PLPS
0.1	PLPS	0.1	PLPS
0.11	PLPS	0.11	PLPS
0.12	PLPS	0.26	PLPS

P(diff > 1.6604) = 0.02727

P(diff > 2.3825) = 0.00909

Location G	
Max. % Surface Coverage	Surface
0.11	PS
3.65	PS
1.96	PS
0.27	PS
0.57	PS
0.15	PS
0	PLPS
0	PLPS
0.11	PLPS
0.16	PLPS

P(diff > 1.0508) = 0.01905

Data from growth of *C. albicans* strain 662 and strain 1 in reduced medium as a function of time. This data was given previously in Appendix B. Analysis of variance (ANOVA) was performed on the data set. The results of the analysis are given in the following pages. Time has units of minutes, and concentration is in terms of cells per milliliter. Strain 1, *C. albicans* strain 1; strain 2, *C. albicans* strain 662. Pluronic 0, Pluronic F127 absent; Pluronic 1, Pluronic F127 present (1 ml of 4% Pluronic F127 in 100 ml of solution). The adjusted concentration was discussed previously in the Experimental Procedures section of this dissertation.

	C1	C2	C3	C4	C5	C6	C7
	Time	Concentration	Trial	Strain	Pluronic	Adj Concentration	Ln(Concentration)
1	120.00	850000	1	1	0	0	13.6530
2	251.25	775000	1	1	0	-75000	13.5606
3	365.00	3325000	1	1	0	2475000	15.0170
4	401.25	4300000	1	1	0	3450000	15.2741
5	712.50	6500000	1	1	0	5650000	15.6873
6	1440.00	10000000	1	1	0	9150000	16.1181
7	120.00	800000	2	1	0	0	13.5924
8	251.25	875000	2	1	0	75000	13.6820
9	365.00	3025000	2	1	0	2225000	14.9224
10	401.25	5675000	2	1	0	4875000	15.5516
11	712.50	9000000	2	1	0	8200000	16.0127
12	1440.00	7250000	2	1	0	6450000	15.7965
13	120.00	550000	1	1	1	0	13.2177
14	251.25	1275000	1	1	1	725000	14.0585
15	365.00	1350000	1	1	1	800000	14.1156
16	401.25	3175000	1	1	1	2625000	14.9708
17	712.50	2325000	1	1	1	1775000	14.6592
18	1440.00	3050000	1	1	1	2500000	14.9307
19	120.00	925000	2	1	1	0	13.7375
20	251.25	1175000	2	1	1	250000	13.9768
21	365.00	2275000	2	1	1	1350000	14.6375
22	401.25	4400000	2	1	1	3475000	15.2971
23	712.50	2275000	2	1	1	1350000	14.6375
24	1440.00	2350000	2	1	1	1425000	14.6699
25	120.00	1400000	1	2	0	0	14.1520
26	251.25	5950000	1	2	0	4550000	15.5989
27	365.00	8250000	1	2	0	6850000	15.9257
28	401.25	9000000	1	2	0	7600000	16.0127
29	712.50	10000000	1	2	0	8600000	16.1181
30	1440.00	10050000	1	2	0	8650000	16.1231
31	120.00	1600000	2	2	0	0	14.2855
32	251.25	6050000	2	2	0	4450000	15.6156
33	365.00	7750000	2	2	0	6150000	15.8632
34	401.25	9500000	2	2	0	7900000	16.0668
35	712.50	7250000	2	2	0	5650000	15.7965
36	1440.00	10000000	2	2	0	8400000	16.1181
37	120.00	1425000	1	2	1	0	14.1697
38	251.25	4375000	1	2	1	2950000	15.2914
39	365.00	5675000	1	2	1	4250000	15.5516
40	401.25	4300000	1	2	1	2875000	15.2741
41	712.50	5450000	1	2	1	4025000	15.5111
42	1440.00	5675000	1	2	1	4250000	15.5516
43	120.00	1575000	2	2	1	0	14.2698
44	251.25	3325000	2	2	1	1750000	15.0170
45	365.00	4200000	2	2	1	2625000	15.2506
46	401.25	3725000	2	2	1	2150000	15.1306
47	712.50	5650000	2	2	1	4075000	15.5472
48	1440.00	4350000	2	2	1	2775000	15.2857

	C8	C9	C10	C11	C12	C13
	Ln(Adj Concentration)	Log(Adj Concentration)	Time*Pluronic	Time*Strain	Strain*Pluronic	Adj Conc
1	0.0000	0.00000	0.00	120.00	0	1.0000
2	0.0000	4.87506	0.00	251.25	0	1.0000
3	1.3640	6.39358	0.00	365.00	0	3.9118
4	1.6211	6.53782	0.00	401.25	0	5.0587
5	2.0343	6.75205	0.00	712.50	0	7.6469
6	2.4651	6.96142	0.00	1440.00	0	11.7647
7	0.0000	0.00000	0.00	120.00	0	1.0000
8	0.0896	4.87506	0.00	251.25	0	1.0937
9	1.3300	6.34733	0.00	365.00	0	3.7810
10	1.9592	6.68797	0.00	401.25	0	7.0936
11	2.4203	6.91381	0.00	712.50	0	11.2492
12	2.2041	6.80956	0.00	1440.00	0	9.0621
13	0.0000	0.00000	120.00	120.00	1	1.0000
14	0.8408	5.86034	251.25	251.25	1	2.3182
15	0.7879	5.90309	365.00	365.00	1	2.1988
16	1.7531	6.41913	401.25	401.25	1	5.7725
17	1.4415	6.24920	712.50	712.50	1	4.2270
18	1.7130	6.39794	1440.00	1440.00	1	5.5456
19	0.0000	0.00000	120.00	120.00	1	1.0000
20	0.2393	5.39794	251.25	251.25	1	1.2704
21	0.9000	6.13033	365.00	365.00	1	2.4596
22	1.5596	6.54095	401.25	401.25	1	4.7569
23	0.9000	6.13033	712.50	712.50	1	2.4596
24	0.9324	6.15381	1440.00	1440.00	1	2.5406
25	0.0000	0.00000	0.00	240.00	0	1.0000
26	1.4469	6.65801	0.00	502.50	0	4.2499
27	1.7737	6.83569	0.00	730.00	0	5.8926
28	1.8607	6.88081	0.00	802.50	0	6.4282
29	1.9661	6.93450	0.00	1425.00	0	7.1428
30	1.9711	6.93702	0.00	2880.00	0	7.1786
31	0.0000	0.00000	0.00	240.00	0	1.0000
32	1.3301	6.64836	0.00	502.50	0	3.7814
33	1.5777	6.78888	0.00	730.00	0	4.8438
34	1.7813	6.89763	0.00	802.50	0	5.9376
35	1.0000	6.75205	0.00	1425.00	0	2.7183
36	1.5110	6.92428	0.00	2880.00	0	4.5313
37	0.0000	0.00000	120.00	240.00	2	1.0000
38	1.1217	6.46982	251.25	502.50	2	3.0701
39	1.3819	6.62839	365.00	730.00	2	3.9825
40	1.1044	6.45864	401.25	802.50	2	3.0174
41	1.3414	6.60477	712.50	1425.00	2	3.8244
42	1.3819	6.62839	1440.00	2880.00	2	3.9825
43	0.0000	0.00000	120.00	240.00	2	1.0000
44	0.7472	6.24304	251.25	502.50	2	2.1111
45	0.9808	6.41913	365.00	730.00	2	2.6666
46	0.8608	6.33244	401.25	802.50	2	2.3651
47	1.2774	6.61013	712.50	1425.00	2	3.5873
48	1.0159	6.44326	1440.00	2880.00	2	2.7618

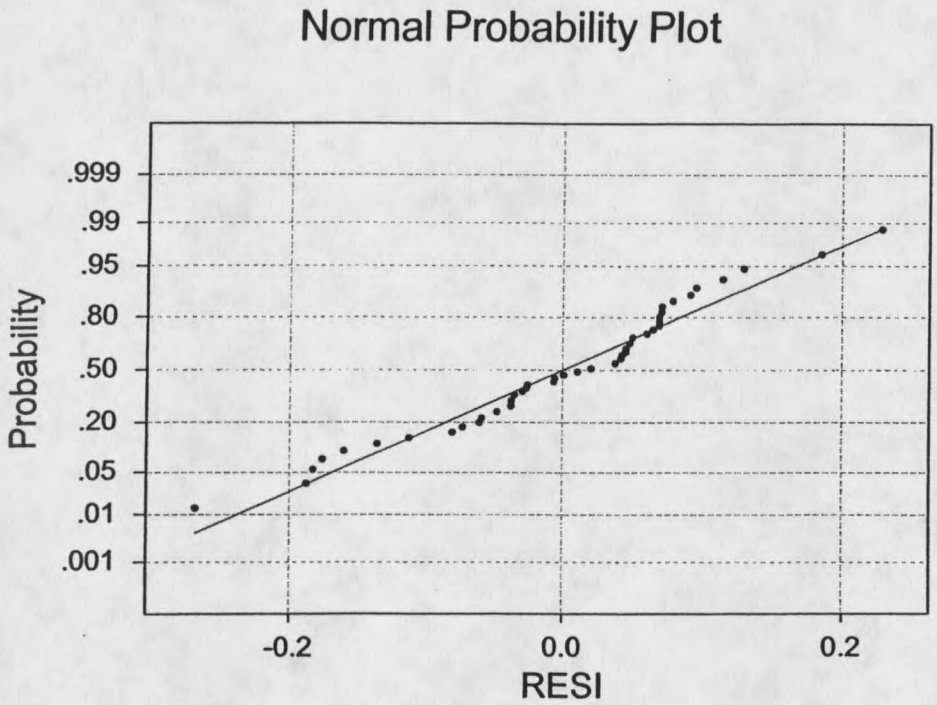
	C14	C15	C16	C17	C18	C19
			Log(Adj Conc)			RESI5
1			0.00000			-0.037718
2			0.00000			-0.160244
3			0.59238			-0.029391
4			0.70403			-0.187637
5			0.88349			-0.025678
6			1.07058			0.069959
7			0.00000			0.048306
8			0.03891			-0.035306
9			0.57761			0.041868
10			0.85087			0.045223
11			1.05112			0.227985
12			0.95723			0.042633
13			0.00000			-0.048306
14			0.36515			0.185377
15			0.34218			-0.073593
16			0.76136			0.070213
17			0.62604			-0.026581
18			0.74395			0.070196
19			0.00000			0.037718
20			0.10393			0.010173
21			0.39087			0.061116
22			0.67733			0.072201
23			0.39087			-0.175726
24			0.40494			-0.182789
25			0.00000			-0.048306
26			0.62838			0.080126
27			0.77031			-0.006690
28			0.80809			0.045436
29			0.85387			0.065620
30			0.85604			0.000601
31			0.00000			0.037718
32			0.57766			0.115425
33			0.68519			-0.005787
34			0.77361			0.096978
35			0.43429			-0.267927
36			0.65622			-0.113193
37			0.00000			-0.037718
38			0.48715			-0.059466
39			0.60015			0.050324
40			0.47963			-0.061322
41			0.58256			0.072039
42			0.60015			0.092760
43			0.00000			0.048306
44			0.32450			-0.136084
45			0.42596			-0.037847
46			0.37384			-0.081092
47			0.55477			0.130268
48			0.44120			0.019833

ANOVA: Log(Adj Conc) versus Time, Stain, Pluronic, Trial

Factor	Type	Levels	Values				
Time	fixed	6	120.00	251.25	365.00	401.25	712.50
			1440.00				
Strain	fixed	2	1	2			
Pluronic	fixed	2	0	1			
Trial	random	2	1	2			

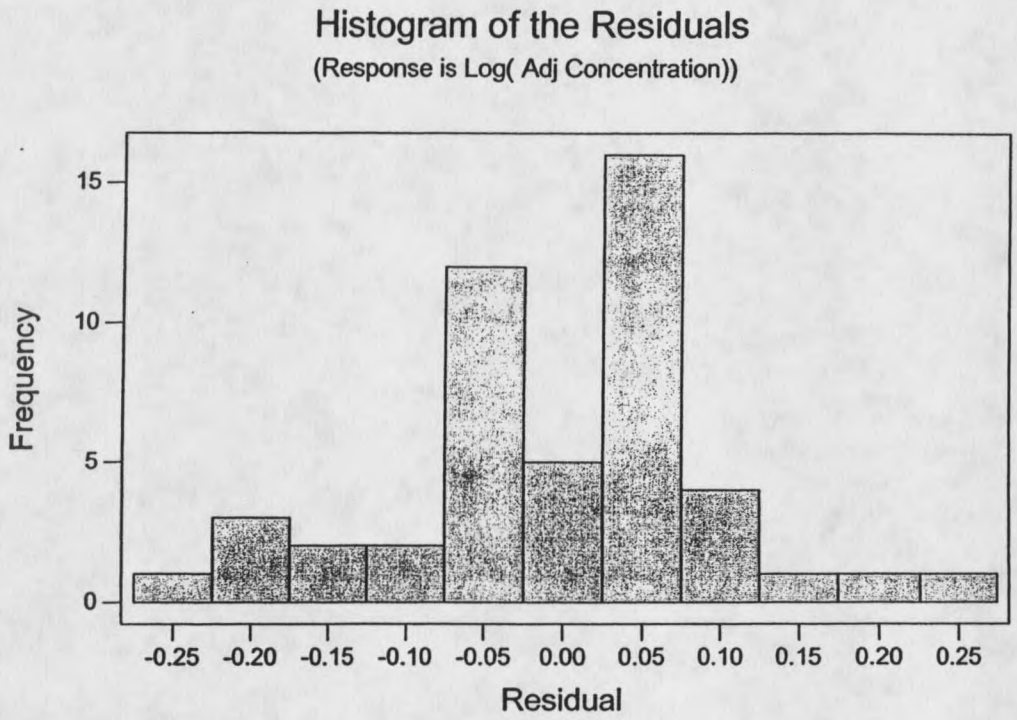
Analysis of Variance for Log(Adj Concentration)

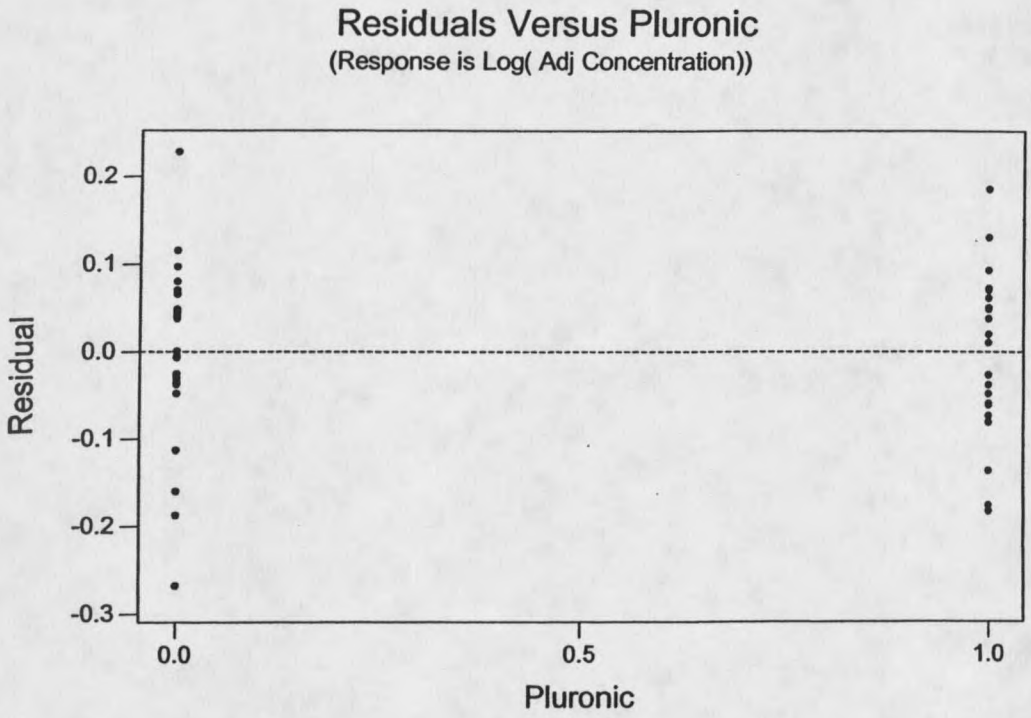
Source	DF	SS	MS	F	P
Time	5	3.15024	0.63005	38.98	0.000
Strain	1	0.00302	0.00302	0.19	0.669
Pluronic	1	0.34907	0.34907	21.60	0.000
Trial	1	0.08880	0.08880	5.49	0.026
Time*Pluronic	5	0.20451	0.04090	2.53	0.052
Time*Strain	5	0.44581	0.08916	5.52	0.001
Strain*Pluronic	1	0.00135	0.00135	0.08	0.775
Error	28	0.45256	0.01616		
Total	47	4.69537			

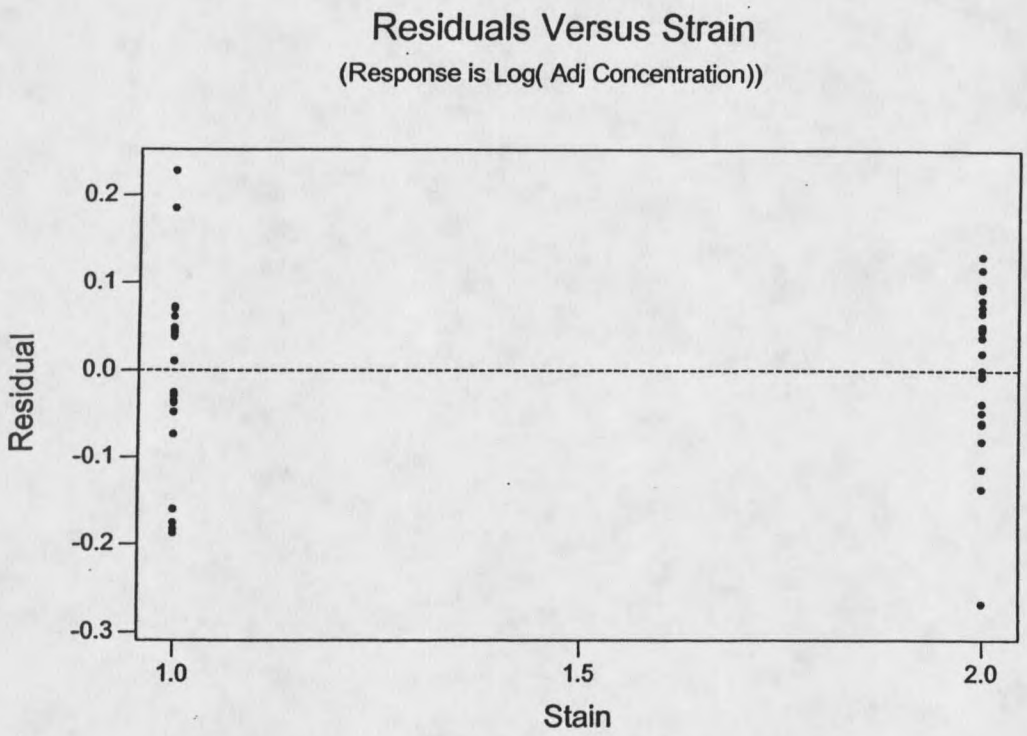


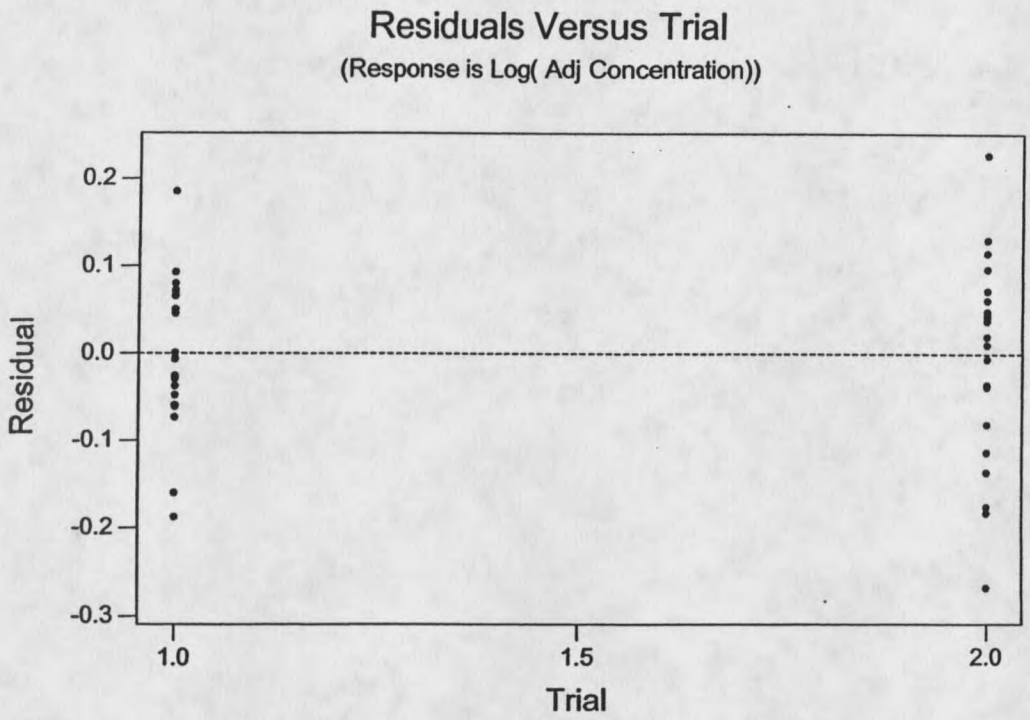
Average: -0.0000000
StDev: 0.0981274
N: 48

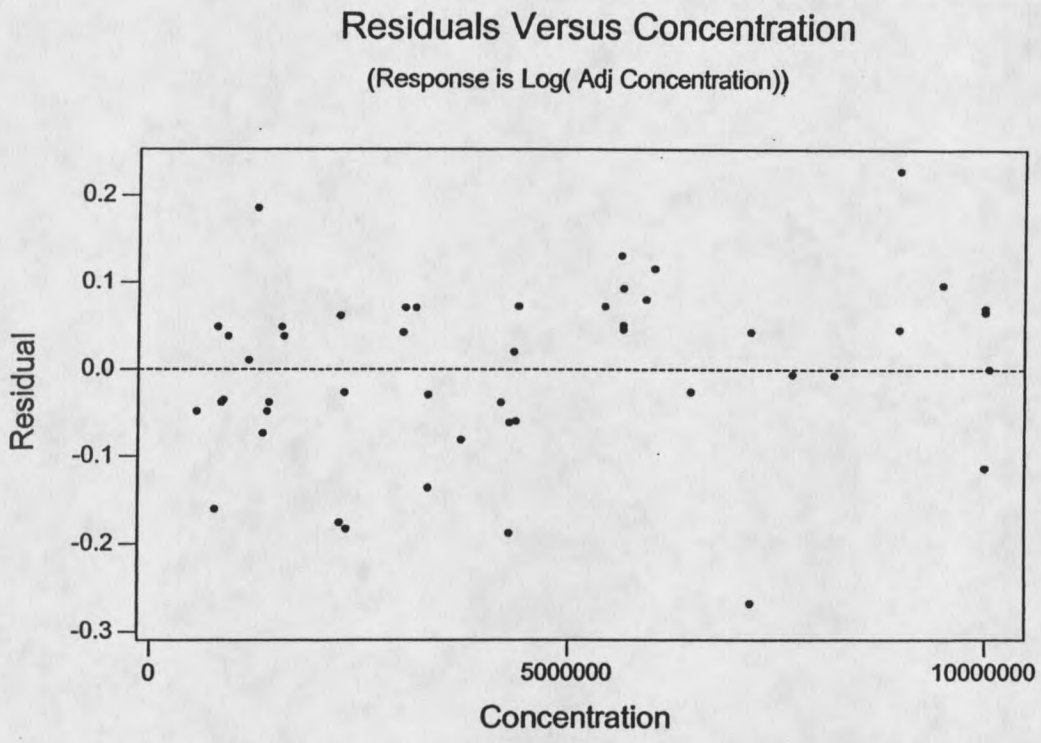
Anderson-Darling Normality Test
A-Squared: 0.711
P-Value: 0.059


Histogram of the Residuals
(Response is Log(Adj Concentration))
Frequency
15
10
5
0
-0.25
-0.20
-0.15
-0.10
-0.05
0.00
0.05
0.10
0.15
0.20
0.25
Residual


Residuals Versus Pluronic
(Response is Log(Adj Concentration))
Residual
0.2
0.1
0.0
-0.1
-0.2
-0.3
0.0
0.5
1.0
Pluronic


Residuals Versus Strain
(Response is Log(Adj Concentration))
Residual
0.2
0.1
0.0
-0.1
-0.2
-0.3
1.0
1.5
2.0
Stain


Residuals Versus Trial
(Response is Log(Adj Concentration))
Residual
0.2
0.1
0.0
-0.1
-0.2
-0.3
1.0
1.5
2.0
Trial


Residuals Versus Concentration
(Response is Log(Adj Concentration))
Residual
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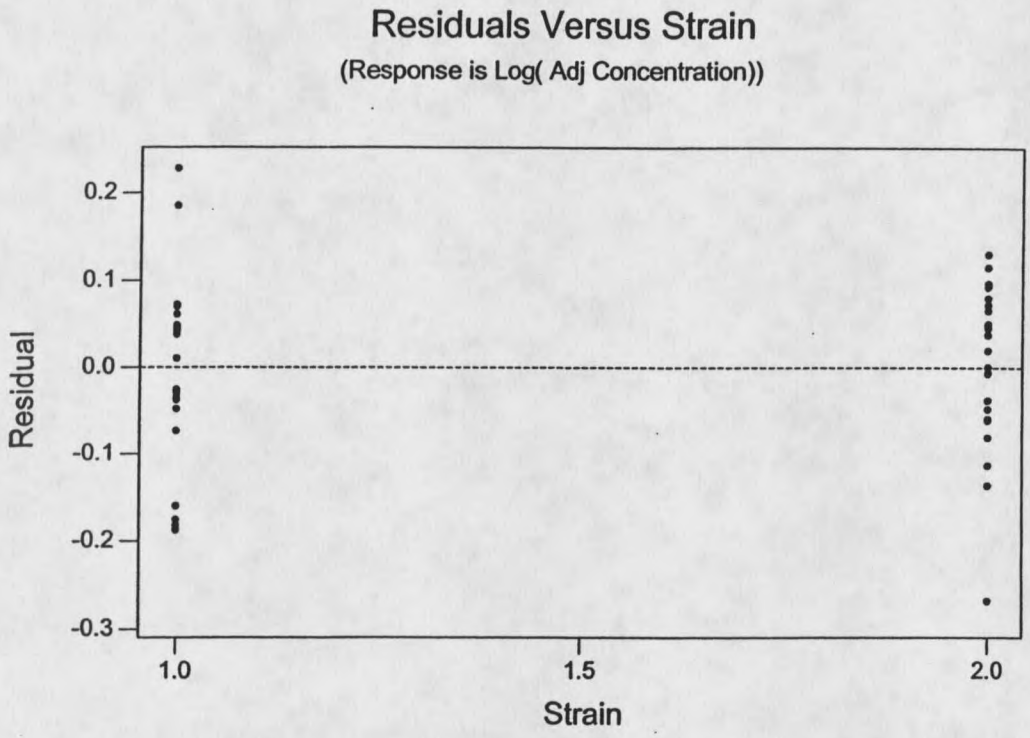
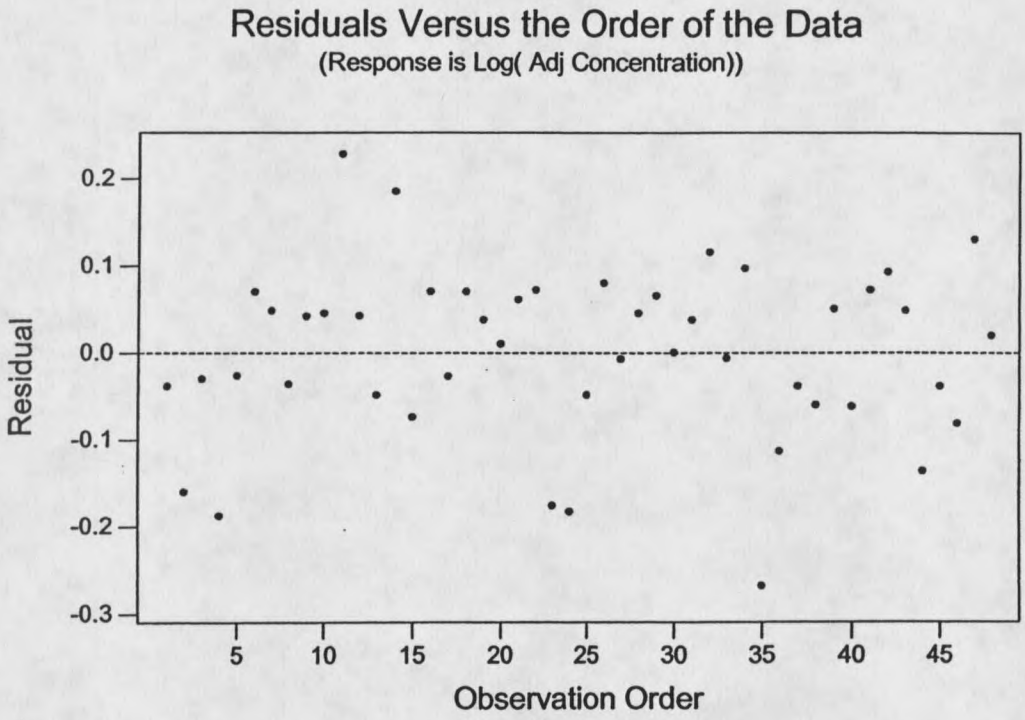
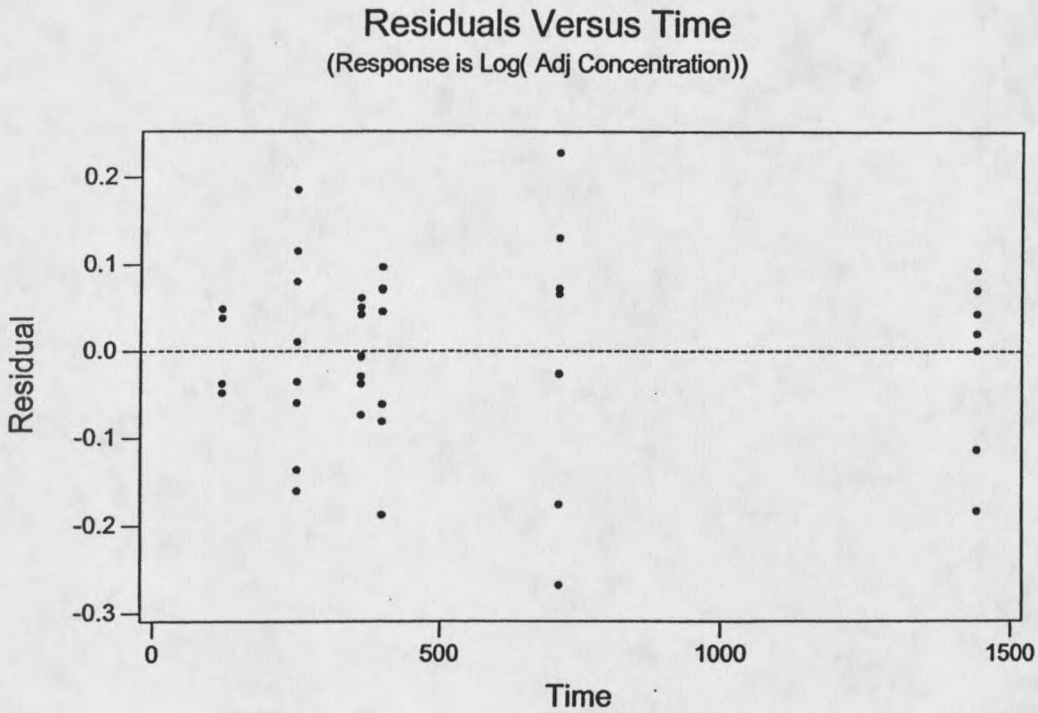
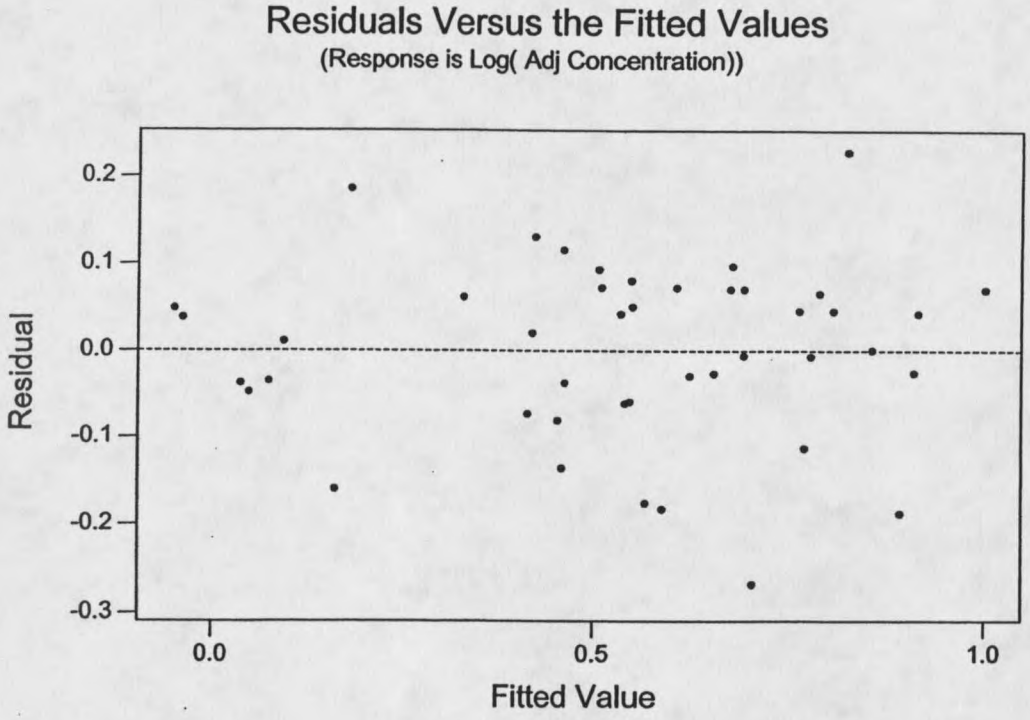
0.1
0.0
-0.1
-0.2
-0.3
0
5000000
10000000
Concentration


Residuals Versus Strain
(Response is Log(Adj Concentration))
Residual
0.2
0.1
0.0
-0.1
-0.2
-0.3
1.0
1.5
2.0
Strain


Residuals Versus the Order of the Data
(Response is Log(Adj Concentration))
Residual
0.2
0.1
0.0
-0.1
-0.2
-0.3
5
10
15
20
25
30
35
40
45
Observation Order


Residuals Versus Time
(Response is Log(Adj Concentration))
Residual
0.2
0.1
0.0
-0.1
-0.2
-0.3
0
500
1000
1500
Time


Residuals Versus the Fitted Values
(Response is Log(Adj Concentration))
Residual
0.2
0.1
0.0
-0.1
-0.2
-0.3
0.0
0.5
1.0
Fitted Value