# Characteristics of biofilm formation by *Candida albicans*

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*Summary*

A variety of manifestations of *Candida albicans* infections are associated with the formation of biofilms on the surface of biomaterials. Cells in biofilms display phenotypic traits that are dramatically different from their free-floating planktonic counterparts, such as increased resistance to anti-microbial agents and protection form host defenses. Here, we describe the characteristics of *C. albicans* biofilm development using a 96 well microtitre plate model, microscopic observations and a colorimetric method based on the use of a modified tetrazolium salt (2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide, XTT) to monitor metabolic activities of cells within the biofilm. *C. albicans* biofilm formation was characterized by initial adherence of yeast cells (0-2 h), followed by germination and micro-colony formation (2-4 h), filamentation (4-6 h), monolayer development (6-8 h), proliferation (8-24 h) and maturation (24-48 h). The XTT-reduction assay showed a linear relationship between cellular density of the biofilm and metabolic activity. Serum and saliva pre-conditioning films increased the initial attachment of *C. albicans*, but had minimal effect on subsequent biofilm formation. Scanning electron microscopy and confocal scanning laser microscopy were used to visualize *C. albicans* biofilms. Mature *C. albicans* biofilms consisted of a dense network of yeasts cells and hyphal elements embedded within exopolymeric material. *C. albicans* biofilms displayed a complex three dimensional structure which demonstrated spatial heterogeneity and a typical architecture showing microcolonies with ramifying water channels. Antifungal susceptibility testing demonstrated the increased resistance of sessile *C. albicans* cells against clinically used fluconazole and amphotericin B as compared to their planktonic counterparts.

*Key words* *Candida albicans*, Biofilm, Models

# Características de la formación de biopelículas por *Candida albicans*

*Resumen*

Diferentes manifestaciones clínicas de infecciones producidas por *Candida albicans* están asociadas con la formación de biopelículas en la superficie de los biomateriales utilizados en la práctica clínica. Las células que forman parte de estas biopelículas exhiben fenotipos diferentes en comparación con las células planctónicas crecidas en condiciones típicas de laboratorio (cultivos líquidos), tales como la elevada resistencia hacia los agentes antimicrobianos y protección contra las defensas del huésped. En el presente estudio describimos las características de la formación de biopelículas por *C. albicans* utilizando un modelo basado en placas de microtitulación de 96 pocillos, junto con observaciones microscópicas y un método colorimétrico basado en la reducción de un producto derivado del formazán (XTT) que permite la detección de la actividad metabólica de las células que forman parte de la biopelícula. La formación de biopelícilas por *C. albicans* se caracterizó por la adherencia inicial de formas levaduriformes (0-2 h), seguida por germinación y formación de micro-colonias (2-4 h), filamentación (4-6 h), crecimiento confluente (6-8 h), proliferación (8-24 h) y envejecimiento (24-48 h). El ensayo de reducción del XTT demostró una excelente correlación entre la densidad cellular de la biopelícula y su actividad metabólica.

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La formación de películas de suero y saliva incrementaron la fase inicial de adherencia de *C. albicans*, pero tuvieron una influencia mínima en el subsecuente desarrollo de biopelículas. También se utilizaron técnicas de microscopía electrónica de barrido y microscopía confocal que demostraron que las biopelículas de *C. albicans* consisten en una densa red de formas levaduriformes y filamentosas encapsuladas dentro de un material extracelular. Las biopelículas de *C. albicans* exhibieron una estructura tridimensional compleja, con una elevada heterogeneidad espacial y una típica arquitectura de microcolonias con ramales de canalizaciones de agua. Por último, los ensayos de sensibilidad a fluconazol y anfotericina B demostraron la elevada resistencia de las células de *C. albicans* presentes en biopelículas hacia estos antifúngicos utilizados en el tratamiento de la candidiasis.

*Palabras clave* *Candida albicans*, Biopelícula, Modelos

*Candida albicans* is a dimorphic fungus that can be either commensal or an opportunistic pathogen with the ability to cause a variety of infections, ranging from superficial to life threatening. Predisposing factors for *C. albicans* infections includes immunosuppressive therapy, antibiotic therapy, use of indwelling devices and intravenous catheters, HIV-infection, diabetes and old age [1,2]. Nosocomial infections due to *Candida* are becoming increasingly important. The most recent surveys have shown *Candida* to be the third or fourth most commonly isolated bloodstream pathogen from U.S. hospitals, now surpassing Gram-negative rods in frequency [3-6]. Importantly, yeasts (mainly *C. albicans* but also other *Candida* species such as *Candida parapsilosis*) are the third leading cause of catheter-related infections, with the second highest colonization to infection rate and the overall highest crude mortality [7,8].

*C. albicans* is capable of invading virtually every site on the body, including deep tissues and organs, superficial sites such as skin, nails and mucosa. Superficial infections, such as acute pseudomembranous infections of the oral cavity or vagina, are some of the most frequently encountered [1,2]. Furthermore, biomaterials such as stents, shunts, prostheses (voice, heart valve, knee, etc), implants (lens, breast, denture, etc.) endotracheal tubes, pacemakers, and various types of catheters to name a few, have all been shown to support colonization and biofilm formation by *Candida* [9-25].

To colonize, yeast cells must first adhere to either host cells and tissues or biomaterial surfaces that are coated with a glycoproteinaceous conditioning film [26-29]. The conditioning film is formed on the surface of biomaterials and tissue substratum immediately following implantation because biomedical devices are usually surrounded by body fluids such as urine, blood, saliva and synovial fluid [30,31]. Initial attachment of cells is closely followed by proliferation and biofilm formation [32-34]. Moreover, sessile cells in biofilms display properties that are dramatically different to their planktonic (free living) counterparts [35-38]. Two consequences of biofilm growth with profound clinical implications are the markedly enhanced resistance to antimicrobial agents and the protection from host defenses [39-46].

The study of biofilms is increasing at a rapid pace, particularly in the case of bacterial biofilm research, with all focus on fungal biofilms being somewhat neglected. *C. albicans* biofilm development and characteristics have been reported elsewhere [32,33,47-54], and they appear to share several properties with bacterial biofilms, including their structural heterogeneity, the presence of exopolymeric material and their decreased susceptibility to antimicrobials and biocides [34,50,55-57]. However, unlike typical bacterial biofilm development, *C. albicans* is capable of dimorphic switching from a yeast form to a filamentous form, which gives *C. albicans* biofilms unique developmental characteristics [48,53,58]. It is increasingly obvious that *C. albicans* biofilm infections are an escalating clinical problem, and with a limited arsenal of treatment therapies the future looks pessimistic unless research interests in this area are initiated. It was therefore the purpose of this study to investigate the characteristics of *C. albicans* biofilm development in respect to morphology, adherence and antifungal susceptibility.

## MATERIALS AND METHODS

*Organisms. C. albicans* 3153A was used in the course of this study, and was stored on Sabouraud dextrose slopes (BBL, Cockeysville, Md) at –70 °C. *C. albicans* 3153A was propagated in yeast peptone dextrose (YPD) medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose [US Biological, USA]). Batches of medium (20 ml) were inoculated from YPD agar plates containing freshly grown *C. albicans*, and incubated overnight in an orbital shaker at 30 °C. *C. albicans* 3153A grew in the budding-yeast phase under these conditions. Cells were harvested and washed in sterile phosphate buffered saline (PBS: 10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4 [Sigma, USA]). Cells were resuspended in RPMI-1640 supplemented with L-glutamine and buffered with morpholinepropanesulfonic acid (MOPS) (Angus Buffers and Chemicals, USA) and adjusted to the desired cellular density by counting in a haematocytometer (see below).

*Biofilm growth kinetics on the surface of wells of microtiter plates. C. albicans* biofilms were formed on commercially available pre-sterilized, polystyrene, flat-bottomed, 96-well microtitre plates (Corning Incorporated, USA). Biofilms were formed by pipetting standardised cell suspensions (100 $\mu$l of a suspension containing $10^6$ cells/ml in RPMI-1640) into selected wells of microtitre plates and incubated over a series of time intervals (2, 4, 6, 8, 24, 48 h) at 37 °C. After biofilm formation the medium was aspirated and non-adherent cells removed by thoroughly washing the biofilms three times in sterile PBS. A semi-quantitative measure of biofilm formation was calculated using an 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay, essentially as described before [59,60]. Briefly, XTT (Sigma) was prepared as a saturated solution at 0.5 g/l in Ringer's lactate. This solution was filter sterilized through a 0.22 $\mu$m-pore size filter, aliquoted, and

then stored at –70 °C. Prior to each assay, an aliquot of stock XTT was thawed, and menadione (Sigma, 10 mM prepared in acetone) dispensed to a final concentration of 1 $\mu$M. A 100 $\mu$l aliquot of XTT/menadione was then added to each pre-washed biofilm, and to control wells to measure background XTT levels. The plates were then incubated in the dark for 1 h at 37 °C and the colorimetric change (a reflection of the metabolic activity of the biofilm) was measured in a microtitre plate reader at 490nm (Bechmark Microplate Reader, BioRad, USA). Microscopic examinations of biofilms formed in microtiter plates were performed by light microscopy using an inverted microscope.

*Preconditioning films with serum and saliva.* Serum was collected from patients at the University Hospital, University of Texas Health Science Centre at San Antonio, who tested negatively for both HIV- and hepatitis B-infection. The serum was pooled, aliquoted and stored at –70 °C. Saliva was collected from several volunteers who chewed parafilm to stimulate their salivary glands for 1 h and collected in 50 ml Falcon tubes on ice, then centrifuged at 3,000 g. The saliva was pooled, aliquoted, and stored at –70 °C. Serum and saliva were prepared at 50% (v/v) in sterile PBS, and were individually dispensed (50 $\mu$l) into six wells of a microtiter plate. The serum and saliva solution were then incubated in the wells overnight at 4 °C. Excess serum and saliva were then aspirated, and the adsorbed conditioning film washed once in sterile PBS. *C. albicans* 3153A was washed in PBS and re-suspended at a concentration of 1 x $10^8$ cells per milliliter in RPMI-1640, and dispensed (100 $\mu$l per well) into the 96-well microtiter plates and incubated for 30 min, 4 h and 24 h at 37 °C. For each time point the wells were aspirated and washed three times in sterile PBS, and adhesion measured by the XTT reduction assay and by light microscopy. Experiments were performed twice with six replicates for each condition, with similar results. The effect of serum and saliva conditioning films on *C. albicans* adherence and biofilm formation as compared to control wells was assessed by using the Student's t test. $P < 0.05$ was considered statistically significant. The analyses were performed using GraphPad Prism version 3.00 for Windows (GraphPad Software, USA).

*Scanning Electron Microscopy.* For scanning electron microscopy (SEM), *C. albicans* biofilms were formed on polymethylmethacrylate (PMMA) discs (diameter 25 mm), which were prepared by combining Dentsply® repair material/lucitone® Fas-Por™ E pourable denture base liquid with Dentsply® repair material powder at the ratio of 7.5 ml monomer liquid to 10 g powder, and mixed thoroughly. The PMMA solution was then quickly poured into Teflon moulds and allowed to polymerize into rigid discs. The discs were then washed in sterile distilled $H_2O$ to remove toxic monomer residues and sterilised with 70% v/v alcohol. Biofilms were formed on PMMA discs within 6 well cell culture plates (Corning) by dispensing standardised cell suspensions (4 ml of a suspension containing 1.0 x $10^6$ cells/ml in RPMI-1640) onto appropriate discs at 37 °C. The discs were removed at the selected time intervals (2, 4, 6, 8, 24 and 48 h) and washed as described above. The biofilms were either air-dried or placed in fixative (4% formaldehyde v/v, 1% glutaraldehyde v/v in phosphate buffered saline) overnight. The samples were rinsed in 0.1 M phosphate buffer (2 x 3 min) and then placed in 1% Zetterquist's osmium for 30 min. The samples were subsequently dehydrated in a series of ethanol washes (70% for 10 min, 95% for 10 min, 100% for 20 min), then treated (2 x 5 min) with hexamethyldisilizane (HMDS: Polysciences Inc., USA), and finally air dried in a desiccator. The specimens were coated with gold/palladium (40%/60%). After processing, samples were observed in a scanning electron microscope (Leo 435 VP) in high vacuum mode at 15kV. The images were processed for display using Photoshop software (Adobe, Mountain View, Calif.).

*Confocal laser scanning microscopy (CLSM).* *C. albicans* biofilms were formed as described above but using 15 mm diameter sterile thermanox plastic cell culture coverslips (Nunc, Denmark). After incubation at 37 °C for different periods of time they were washed with PBS and stained using the LIVE/DEAD fluorescent staining (Molecular Probes, USA). Stained biofilms were observed with an Olympus FV-500 Laser Scanning Confocal Microscope, using a 488 argon ion laser. Serial sections in the *xy* plane were obtained at 1 $\mu$m intervals along the *z* axis. Three-dimensional reconstructions of imaged biofilms were obtained by the resident software. The images were processed for display using the Adobe PhotoShop program (Adobe Systems Inc., USA).

*Antifungal Susceptibility Testing.* Two clinically used antifungal agents were used in this study, fluconazole (Pfizer, Inc., USA) and amphotericin B (Bristol-Myers Squibb, USA). Fluconazole was prepared at stock concentrations of 1024 $\mu$g/ml in RPMI-1640 (Angus Buffers and Chemicals) and amphotericin B was prepared in antibiotic medium 3 (Difco Laboratories). Antifungal susceptibility testing to determine minimum inhibitory concentrations (MICs) of planktonic cells was performed by using the National Committee for Clinical Laboratory Standards (NCCLS) M-27A broth microdilution method with reading of endpoints at 48 h [61]. For antifungal susceptibility testing of sessile (biofilm) cells, biofilms were formed by pipetting standardised cell suspensions (100 $\mu$l) into selected wells of the microtitre plate, as described above, which were then incubated for 48 h at 37 °C. The biofilms were then washed thoroughly three times with sterile PBS before the addition of antifungal agents in serially double diluted concentrations and incubated for a further 48 h at 37 °C. A series of antifungal free wells were also included to serve as controls. Sessile minimum inhibitory concentrations were determined at 50% inhibition (SMIC50) and at 80% inhibition (SMIC80) compared to drug-free control wells using the XTT-reduction assay described above.

## RESULTS

In vitro *biofilm formation by* C. albicans. The kinetics of adherence and subsequent biofilm formation by *C. albicans* 3153A on the surface of polystyrene wells over 48 h, as determined by the colorimetric XTT-reduction assay, are illustrated in figure 1. The production of the soluble colored formazan salt from sessile cells, a direct reflection of cellular metabolic activity, increased over time with the increased sessile cellular density. The biofilms were highly metabolically active in the first 8 h, but as the biofilm matured and the complexity increased (24 to 48 h) the metabolic activity reached a plateau, but remained high probably reflecting the increased number of cells that constituted the mature biofilm. Experiments were performed in sets of eight replicates on three separate occasions, with similar results obtained in all experiments.

Light microscopy observations performed in parallel (Figure 2) demonstrated how the biofilm was emanated from small micro-colonies comprised predominantly of budding yeast cells (2-4 h). After 4 h the budding yeast cells began to filament, forming pseudo-hyphae and eventually true-hyphae. After 8 h, hyphae from neighboring

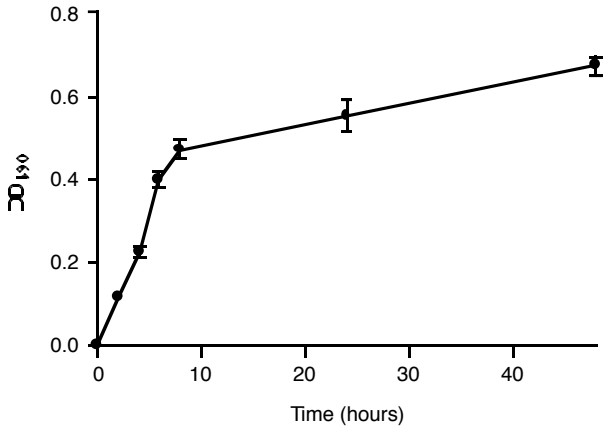


Figure 1. Kinetics of *C. albicans* biofilm formation as determined by XTT readings. The different phases of biofilm development according to both metabolic reading and microscopic observations are indicated.

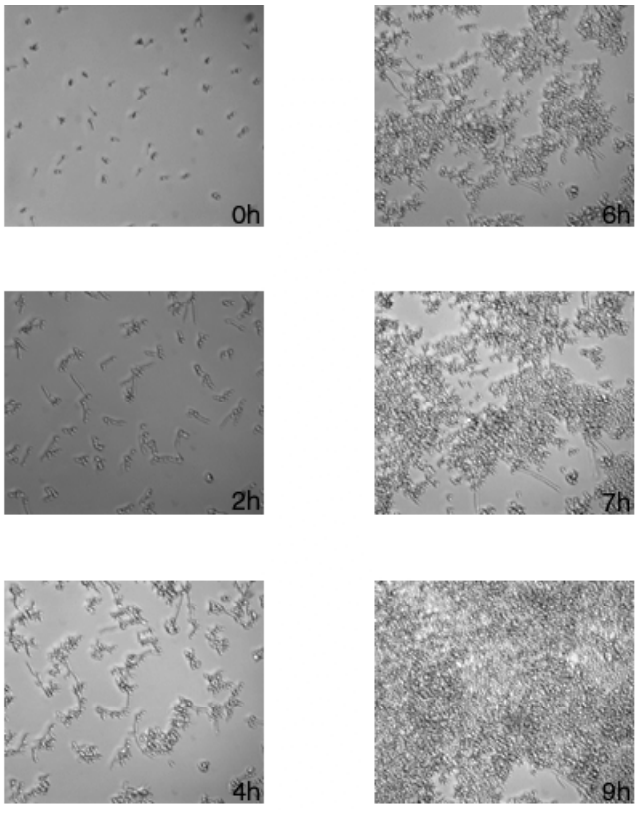


Figure 2. Time-lapse microscopy of *C. albicans* biofilm formation. The different panels show the same field with photomicrographs taken at set time intervals. (To see the corresponding movie, please contact JL López-Ribot at ribot@uthscsa.edu).

micro-colonies, comprised from principally budding yeast cells, merged into an intricate network of spatially dispersed filamentous forms that intertwined to form a coherent monolayer of woven-like structures. As biofilm maturation occurred (24 and 48 h growth), the complexity of the biofilm increased into a multilayered biofilm matrix with all fungal morphologies being present in the final biofilm structure.

*Effect of serum and saliva conditioning films on* C. albicans *adhesion and biofilm formation*. The effect of serum and saliva conditioning films on *C. albicans* adherence and biofilm formation is shown in figure 3. When serum was provided as a conditioning film the level of *C. albicans* adherence was significantly elevated in comparison to that observed in untreated wells. The effect was most clearly demonstrated during early adherence at 30 min there was a 240% compared to control values adherence when compared to the control ($p < 0.0001$). After 4 h and 24 h there was a smaller difference observed between biofilm formation in the presence or absence of the serum pellicle (representing 138% and 123% of the corresponding values for binding to polystyrene respectively), which did not represent statistically significant differences. In contrast to serum, the effect of saliva preconditioning films was minimal. Initial adhesion at 30 min was marginally increased with only 10% increase when compared to the control, which was not statistically significant ($p = 0.8332$). Moreover, after 4 h and 24 h there was only a minimal effect in biofilm metabolic activity, with differences not being statistically significant.

*SEM and CSLM visualization of* C. albicans *biofilms*. Biofilm formation by *C. albicans* on acrylic discs was monitored by SEM (Figures 4 and 5). Despite its destructive nature, SEM observations provided useful information on the different cellular morphologies present in the biofilm structure. Initial adherence of yeast cells was followed by germ tube formation and subsequent development of hyphae (Figures 4 and 5). Mature biofilms consisted of a dense network of cells of all morphologies, deeply embedded in matrix consisting of expolymeric material, which was better preserved when the biofilms samples were air-dried (Figure 4) rather than fixed (Figure 5) prior to processing for SEM.

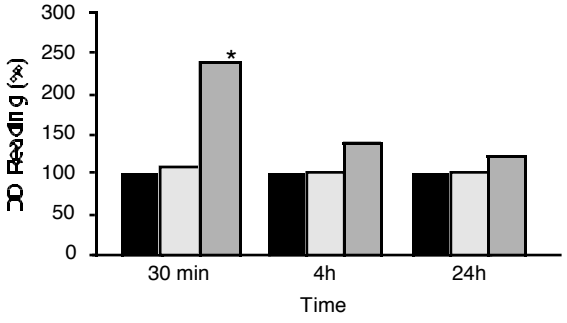


Figure 3. Effect of serum and saliva pre-conditioning films on the formation of *C. albicans* biofilms. Values are expressed as average percent OD readings for XTT assays as compared to control wells (considered 100%). Results are from a single experiment performed with six replicate wells. Asterisk denotes statistically significant differences.
■ Control; ■ Saliva; ■ Serum.

The non-invasive CSLM technique enabled imaging of intact biofilms and visualization of the three dimensional distribution of labelled *C. albicans* cells in the context of the complex biofilm community. Significant channeling and porosity were observed. Overall, results indicated that mature *C. albicans* biofilms displayed a typical microcolony/water channel architecure with extensive spatial heterogeneity. Figure 6 shows a three dimensional reconstruction of a 24 h old, 70 $\mu$m thick *C. albicans* biofilm resulting from the compilation of a series of individual *xy* sections taken across the *z* axis.

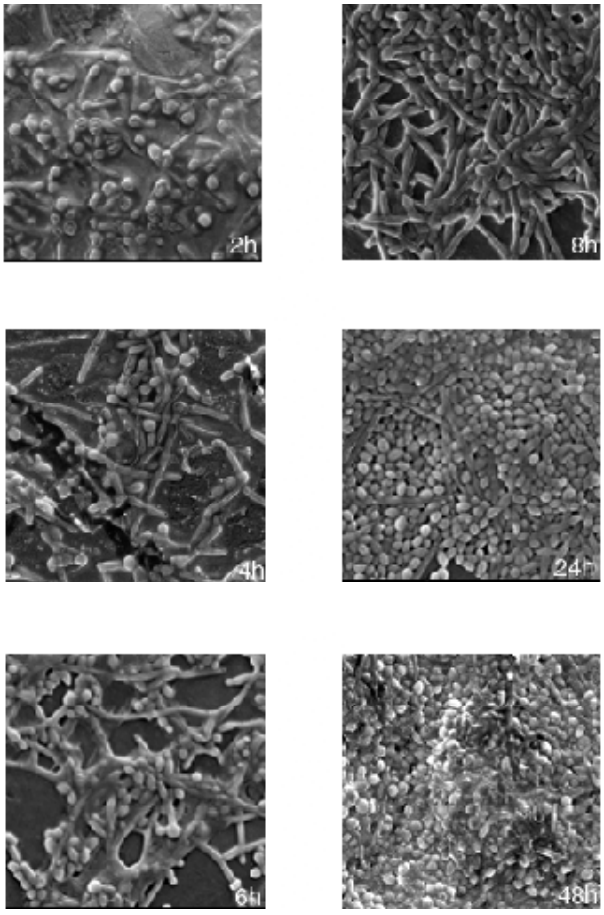


Figure 4. SEM images of *C. albicans* biofilm formation on PMMA disks over a period of times (2, 4, 6, 8, 24 and 48 h). Biofilm samples were not fixed to maximize the preservation of exopolymeric material.

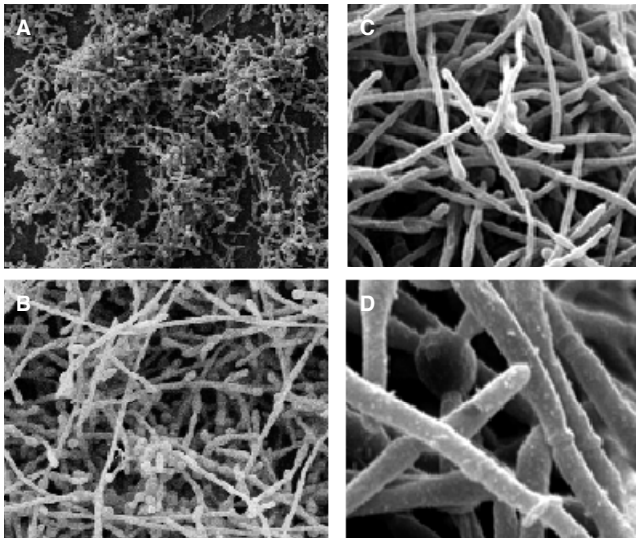


Figure 5. SEM images of mature (48h) *C. albicans* biofilms formed on PMMA. The same biofilm area is shown at different magnifications: Panel A, 100X; panel B, 500X; panel C, 1,500X and panel D, 500X. Samples were fixed prior to processing for SEM.

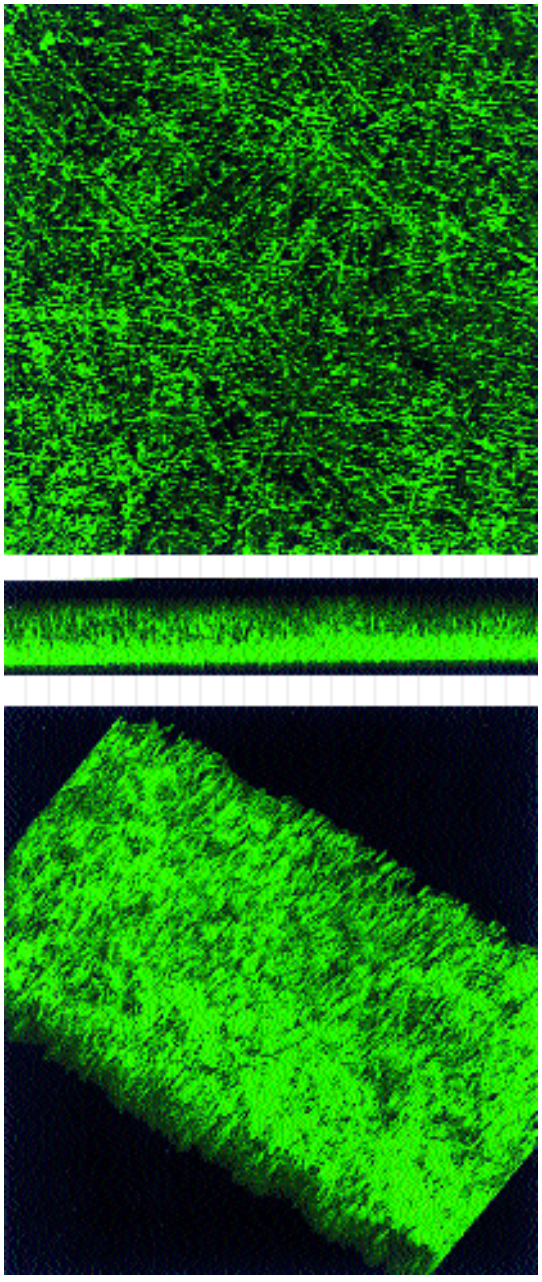

Figure 6. Three-dimensional reconstruction of a *C. albicans* 24h biofilm using confocal microscopy and the associated software for the compilation of *xy* optical sections taken across the *z* axis. A, view from the top; B, lateral view to show biofilm thickness (70 $\mu$m); C, rotated view to provide a global perspective of the biofilm. Note that *C. albicans* biofilms demonstrated structural heterogeneity and displayed a three dimensional architecture characterized by a presence of microcolonies with ramifying water channels. (To see the corresponding movie, showing the rotating 3D reconstruction, please contact JL López-Ribot at ribot@uthscsa.edu).

*Susceptibility testing of* C. albicans *biofilms against clinically used antifungal agents*. The *in vitro* activity of clinically used fluconazole and amphotericin B against pre-formed *C. albicans* biofilms was assessed using the modified XTT-reduction assay. Experiments revealed the increased resistance of sessile *C. albicans* cells compared to their planktonic counterparts (Table 1). The antifungal agents tested showed less activity against 48 h biofilms compared to planktonic MIC's, as the SMIC50s and SMIC80s were generally much greater than the concentration of antifungal required to inhibit planktonic cells. Data revealed that *C. albicans* biofilms were intrinsically resistant to fluconazole (MICs >1024 μg/ml), and the activity of this azole derivative against biofilms was reduced up to 250 times compared with its activity against planktonic cultures. Amphotericin B demonstrated certain activity against *C. albicans* biofilms, as indicated by SMIC80s (1 μg/ml), but this concentration is generally regarded as resistance already, due to the high toxicity displayed by this drug [62]. Importantly, complete killing of cells within the biofilms was never achieved, as reflected by residual metabolic activity of biofilms at concentrations up to 16 μg/ml.

**Table 1.** Antifungal susceptibility testing of *C. albicans* 3153A under planktonic (MIC) and biofilm (SMIC) growing conditions. Values are in μg/ml.

| | Planktonic MIC | Biofilm SMIC50 | Biofilm SMIC80 |
|---|---|---|---|
| Fluconazole MIC's | 4 | >1024 | >1024 |
| Amphotericin B MIC's | 0.5 | 0.5 | 1.0 |

## DISCUSSION

*C. albicans* is an opportunistic pathogen that has become one of the leading causes of nosocomial infections [3,5,6]. These data are partially related to the fact that this organism has a high propensity to adhere to a variety of surfaces, such as living tissue substratum and biomaterials, subsequently forming spatially organized communities of sessile cells that possess phenotypic traits that differ substantially from their planktonic counterparts. Communities of cells such as these are referred to as microbial biofilms and are typically notorious for being resistant to antimicrobials agents and immune factors [39-46]. However, these observations are on the whole limited to work performed on bacterial biofilms, with scant literature reflecting fungal biofilm research [32,47,54,55, 57,63]. An understanding of the complexities of *C. albicans* biofilm development and phenotypic characteristics will allow us to develop antifungal agents and treatment strategies aimed at eradicating and preventing *Candida* biofilms, thereby reducing the incidence of *C. albicans* infections.

*C. albicans* biofilms and their associated models have been described, e.g. a simple static biomaterial-specific disc model [48] and a perfused biofilm fermenter model [55]. The results and observations from these investigations have given an excellent insight into *C. albicans* biofilm development and some important associated characteristics. We have developed an alternative model that permits high sample throughput potential that is based on a 96-well microtiter plate system. We have previously demonstrated that this system allows the growth of multiple independent biofilms, which showed no statistically significant differences to one another when assessed by an XTT metabolic reduction assay (Ramage *et al*, 2001, submitted). This model, therefore, allows us to more easily observe and assess multiple isolates and multiple experimental parameters in a reproducible manner. The XTT-reduction assay described herein, developed on the basis of the study by Tellier and coworkers [60], was used to assess the metabolic activity of biofilms grown over a range of time intervals. It was clearly shown that an increasing cellular density (biofilm formation) was directly related to increased colorimetric measurements with the XTT-reduction assay, as demonstrated by figure 1. Hawser [64,65] previously indicated this relationship by comparing total viable cell counts with XTT readings.

*C. albicans* biofilm developmental characteristics was assessed using an inverted light microscope at 200 x magnification. Our observations were similar to those described by Baille and Douglas [47,48]. Initially, there was a period of adherence (0-2 h) and subsequent microcolony formation (2-4 h). Dimorphic switching occurred thereafter with a transition from budding-yeast forms to filamentous pseudo- and true-hyphal forms (4-6 h). Micro-colonies then became interlinked by the hyphal extensions, forming a confluent monolayer (6-8 h). The complexity of the biofilm increased with time, taking on a three-dimensional architecture with spatial heterogeneity as it matured (8–48 h). The biofilm after 24 h and 48 h consisted of a mixture of yeast cells, pseudohypahe and true hyphae. Filamentous forms were the most important factor in the three-dimensional architecture, with yeast cells located in the basal layer as also reported by Baille and Douglas [47,48]. We have assessed *C. albicans* mutants that are deficient in the production of hyphae and have demonstrated an inability to form three dimensional biofilms (unpublished results). Therefore, the dimorphic switching observed in this species is a pivotal factor for biofilm formation and the pathogenic potential of *C. albicans* [53,58].

In addition to the cell-specific factors that are integral for adhesion and biofilm formation, other features can also play important defined roles. For example, biomaterial surface physico-chemistry and surface roughness has been reported to play a role in bacterial adherence [26,66]. The effect of pre-conditioning films has also been reported to have a positive effect on initial adhesion and biofilm formation [15,52,67]. In this study we have shown that the presence of serum or salivary pellicles, which are normally found in the oral environment, increased the initial adherence of *C. albicans* cells to polystyrene microtiter plates. Our results indicated that biological conditioning films, particularly serum, may help provide receptor binding sites for planktonic *C. albicans*. Other investigators have previously shown that presence of serum and salivary pellicles can potentiate *C. albicans* colonization of acrylic strips and denture lining materials [49,51,52,54,63,68,69]. We have not yet assessed biomaterial surface properties in relation to biofilm developmental characteristics, this subject area has however has been adequately reviewed by Radford and coworkers [15]. *C. albicans* biofilm formation is a complex process dependant upon multiple variables, including proteinaceous preconditioning films during early adhesion events.

Following initial adhesion, the structural integrity and developmental characteristics of *C. albicans* biofilms was monitored using scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) techniques. The increased magnification and resolution power associated with the SEM technique permitted a

more detailed examination of biofilms. *C. albicans* biofilms mature biofilms consist of a mixture of yeast and filamentous forms embedded within exopolymeric material. The exopolymeric material was better preserved when biofilm preparations were air-dried, whereas in fixed biofilms only its remains were visible attached to the surface of the cells (compare figures 4 and 5). Hawser *et al*. [33] also described the production of extracellular matrix polymers by *C. albicans* biofilms. The non-destructive CSLM technique (Figure 5) permitted *in situ* visualization of hydrated biofilms and demonstrated that *C. albicans* biofilms possess structural heterogeneity and display a typical micro-colony/water channel architecture similar to what has been described for bacterial biofilms [38]. This structural complexity that was observed may represent an optimal spatial arrangement for influx of nutrients, disposal of waste products and establishment of micro-niches throughout the biofilm. CSLM provides the added advantage over all other analytical techniques in that it can used to accurately measure the depths of biofilms. In this study, a mean biofilm depth of 70 $\mu$m was measured for 24 h biofilms. Detailed information of this nature can also be used for comparative studies of biofilms formed under different environmental conditions.

Currently, there is a well described method by the NCCLS (M-27A) to determine antifungal susceptibilities for yeast planktonic cultures [61]. Nevertheless, the susceptibility data generated from this approach does not account for the intrinsic resistance exhibited by sessile cells. For example, Hawser and Douglas [57] reported that a range of antifungals were between 30 and 2000 times less active against *C. albicans* biofilms than that of the planktonic MIC's. In agreement, here we have demonstrated the intrinsic resistance of *C. albicans* biofilms to fluconazole (up to 250 times), the most commonly used antifungal agent for the treatment of OPC, and their increased resistance to clinically used amphotericin B. Although amphotericin B exhibited a certain degree of activity against biofilms as indicated by SMIC50s, the SMIC80 values already fell into the resistant range according to interpretative break points [61,62]. Moreover, even at higher concentrations (up to 512 $\mu$g/ml) sterility was never achieved. The disparity between planktonic and sessile minimum inhibitory concentrations from an identical isolate may therefore explain why antifungal treatment may be ineffective in some instances and partially explain the lack of absolute correlation between clinical (*in vivo*) and mycological (*in vitro*) resistance.

We have shown that *C. albicans* can form biofilms with relative ease and simplicity. However, macroscopic and microscopic evaluation of these biofilms by different techniques has revealed a level of complexity that could not have been mediated by random cell division. Importantly, *C. albicans* biofilms formed on different types of biomaterials showed similar architectural features and properties. We have shown that these communities of cells are metabolically active and encased in an extrapolymeric substance. Moreover, they resist the actions of antifungal agents at concentrations that would kill them when free-floating. The spatial heterogeneity and complexity observed in the intact biofilms would lead us to consider that cell-cell communication and highly regulated gene-expression systems are present. Our observations corroborate those by Baille and Douglas [47] and provide an excellent platform from which to initiate research in more focussed avenues, such as the use of genetic approaches to study biofilm formation and the biofilm life style of *C. albicans*.

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