A seed and feed model for the formation of *Candida albicans* biofilms under flow conditions using an improved modified Robbins device

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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. In order to maintain their niche these adherent populations need to withstand the continuous bathing action of physiological fluids (saliva, blood), which also provide water and nutrients to the fungal cells. Thus, it was the aim of this study to examine and further characterize the development of *C. albicans* biofilms under shear forces and a flow of replenishing nutrients, emulating the conditions that fungal cells would normally encounter within the host. An improved modified Robbins device (MRD) was designed to hold six poly methyl methacrylate (PMMA) plugs of 25 mm in diameter. A "seed and feed" model of biofilm formation was then implemented for which the apparatus was initially seeded with a *C. albicans* cell suspension to allow initial adhesion of fungal cells to the biomaterial. Following this initial step, sterile medium was then pumped through the MRD at a constant flow rate. Scanning electron microscopy (SEM) and confocal scanning laser microscopy (CSLM) demonstrated a high degree of heterogeneity associated with the structure of biofilms formed under flowing conditions using the MRD. In addition, these biofilms displayed a complex three dimensional architecture and increased production of exopolymeric material.

Key words *Candida albicans*, Biofilm, Flow-through, Robbins device

Formación de biopelículas de *Candida albicans* en condiciones de flujo utilizando un aparato de Robbins modificado mejorado

Resumen

Diferentes manifestaciones clínicas de infecciones producidas por *Candida albicans* están asociadas con la formación de biopelículas en la superficie de biomateriales utilizados en la práctica clínica. Para mantener su nicho, estas poblaciones adheridas deben ser capaces de soportar la acción constante de lavado ejercida por fluidos fisiológicos (sangre, saliva) que también proveen agua y nutrientes a las células fúngicas. Por lo tanto, el objetivo de este estudio fue examinar y caracterizar el desarrollo de biopelículas de *C. albicans* bajo fuerzas de flujo y condiciones de reaprovisionamiento de nutrientes, imitando así las condiciones que las células fúngicas encuentran normalmente dentro del cuerpo humano. A tal efecto hemos diseñado y construido una versión mejorada del llamado *aparato de Robbins modificado,* capaz de albergar un total de seis discos de polimetilmetracrilato (PMMA) de 25 mm de diámetro. Seguidamente implementamos un modelo de biopelícula en el que, primeramente, "sembramos" las células fúngicas estáticamente, dejándolas adherirse al substrato, seguido de un periodo de "alimentación" durante el cual circulamos medio de cultivo a través del aparato a un flujo continuo. Para caracterizar las biopelículas resultantes utilizamos técnicas de microscopía electrónica de barrido y microscopía confocal, que demostraron un elevado grado de heterogeneidad asociado con la estructura de las biopelículas, así como una arquitectura tridimensional altamente compleja y una mayor producción de matriz exocelular.

Palabras clave *Candida albicans*, Biopelículas, Condiciones de flujo, Aparato de Robbins

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Introduction

Candida albicans remains the fungal species most commonly associated with adherent biofilm communities on both host tissues and medical devices, and a majority of infections caused by this opportunistic fungal pathogen are associated with a biofilm etiology [17,18]. Biofilms are defined as structured communities of cells attached to a surface and encased within a matrix of exopolymeric material and *C. albicans* biofilms possess unique developmental and architectural characteristics that are in marked contrast to their free-floating planktonic counterparts [6,18,21]. Biofilm formation, regardless of the anatomical site, carries important clinical repercussions including serving as a reservoir for persistent sources of infectious microorganisms, increased resistance to antifungal agents and protection from host defenses [10]. Biofilms are now thought to account for 65% of all microbial infections, which is in all likelihood a conservative estimate [7]. As a result, microbial biofilm research, and specifically *C. albicans* biofilm research of these structures, has now become a thriving field of study. However, with increasing research activity the variety of biofilm models used to conduct the research has expanded to suit the individual researcher needs. This is often at the expense of more complex and time-consuming clinically relevant models, which take into account both physical and environmental factors that *C. albicans* encounters inside the human host. For example, *C. albicans* is subject to physical forces from biological fluids, such as serum, saliva and urine, which are either associated with mucosal membranes or indwelling biomedical devices [9,14,17]. Shear force and replenishing nutrients play a key role in biofilm development and are knowm to alter biofilm growth and architecture [4,5].

In the present study we have designed and used an improved modified Robbins device (MRD) for the formation of *C. albicans* biofilms under flow conditions. The resulting biofilms were characterized in regards to their prominent architectural features.

Materials and methods

Organism and culture conditions

C. albicans 3153A strain was used during the course of this study. It was stored on Sabouraud dextrose slopes (BBL, Cockeysville, Md) at -70 ºC. It was routinely propagated in yeast peptone dextrose (YPD) medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose [US Biological, Swampscott, MA]). 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 ºC. *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, St Louis, MO]). Cells were then resuspended in RPMI-1640 supplemented with L-glutamine and buffered with morpholinepropanesulfonic acid (MOPS: Angus Buffers and Chemicals, Niagara Falls, NY) and adjusted to the desired cellular density by counting in a haematocytometer (see below).

Formation of C. albicans biofilms under flow conditions using an improved modified Robbins device (MRD)

We designed and constructed an improved MRD built from an acrylic block containing six individual ports in a linear array along a channel of rectangular cross-section. Each port accepts a press-fit plug holding a disc of the desired material (in this case poly methyl methacrylate - PMMA, casted in Teflon moulds as previously described by our group [19,21], with an approximate diameter of 25 mm whose anterior surface comes in contact with the flushed infusate. The entire MRD containing the PMMA disks was placed inside a laboratory incubator to maintain a constant temperature of 37 °C in the MRD. The discs, tubing, valves and MRD were washed, assembled and sterilized (disinfected with 70% ethanol and exposed to UV light) prior to each run. Assembly was performed in a Laminar Air Flow cabinet to prevent contamination. Masterflex® Tygon® laboratory tubing (Cole-Parmer®) was connected to a peristaltic pump and flasks with growth media, as depicted in figure 1. A "seed and feed" model of biofilm formation was then implemented for which the apparatus was initially seeded with a standard cellular density of *C. albicans* (10^6 cells/ml) in RPMI for 4 h at 37 ºC to allow initial adhesion of fungal cells to the biomaterial. Following this initial step, sterile RPMI at 37 ºC was then pumped through the MRD at a constant flow rate (150 ml/h) using a variable peristaltic pump (Masterflex L/S® Easy-Load®) II (Cole-Parmer®) for 24 or 48 hours. Biofilms formed on the surface of the PMMA discs were recovered and visualized by scanning electron microscopy (SEM) and confocal scanning laser microscopy (CSLM) (see below)

Scanning Electron Microscopy

For scanning electron microscopy (SEM), *C. albicans* biofilms were formed using the MRD as described above. After washings, 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 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 5 min) with hexamethyldisilizane (HMDS: Polysciences Inc., Warrington, PA), 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, CA).

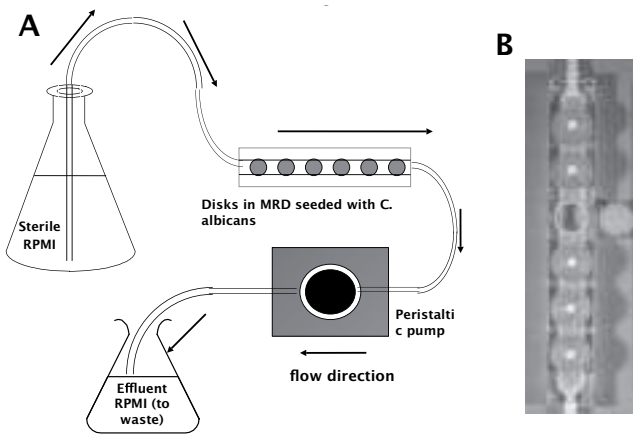

Figure 1. Panel A, schematic representation of the improved MRD. The system was placed inside a laboratory incubator to maintain a constant temperature of 37 °C. Panel B shows a photograph of the actual device.

Confocal Scanning Laser Microscopy (CSLM)

C. albicans biofilms were formed using the MRD as described above, washed with PBS and stained using the LIVE/DEAD fluorescent staining (Molecular Probes, Eugene, OR). 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 μm 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., Mountain View, CA).

Results and discussion

A seed and feed model of in vitro formation of C. albicans biofilms under flow conditions using an improved MRD

Most information on *C. albicans* biofilm development and architecture comes from in vitro experiments in which different biofilm models and microscopic techniques were implemented by different groups of investigators (reviewed in [18]). These model systems include catheter disks and sheets and tubing from different materials, glass slides, a perfused biofilm fermentor, cylindrical cellulose filters, acrylic strips and discs, germanium substratum, microtitre plates, tissue culture flasks, etc. Although the majority of these models utilize static incubation conditions, several models have also been described in the literature that use flow-through conditions to try to mimic the environment encountered within the host [1,8,11,12,15,16]. The results and observations from these investigations have given an excellent insight into *C. albicans* biofilm development and some important associated characteristics. Importantly, comparisons with in vivo formed biofilms seem to indicate that most biofilms studied in vitro closely resemble those formed in vivo using both animal models of biofilm formation and samples retrieved directly from patients [2,19,22,23].

Here we have designed and manufactured an improved MRD that can hold up to six disks of the desired material in a linear array as substrates for the formation of microbial biofilms. Compared to conventional MRDs, the design in this improved MRD utilizes larger disks to allow the recovery of enough quantity of biological material (*i.e.* RNA, protein) necessary for future analyses of gene and protein expression in biofilms formed under flow conditions. Importantly, crystal violet staining and XTT-readings of the resulting biofilms used as semi-quantitative measurements of the biofilm biomass (not shown) validated the equivalency between each biofilm formed in each multiple independent port. Thus, this system allows the formation of multiple (up to six) independent biofilms under flow conditions and with replenishing nutrients in a highly reproducible manner. Some of the limitations of this model are that it is not as amenable to high throughput screening as the 96 well microtiter plate model previously described by our group [20], and that it requires the use of specialized equipment not generally available in a regular laboratory.

SEM and CSLM visualization of C. albicans biofilms formed using the MRD

The biofilms formed in the MRD were retrieved and visualized using SEM (Figure 2). Despite its destructive nature, SEM observations provided useful information

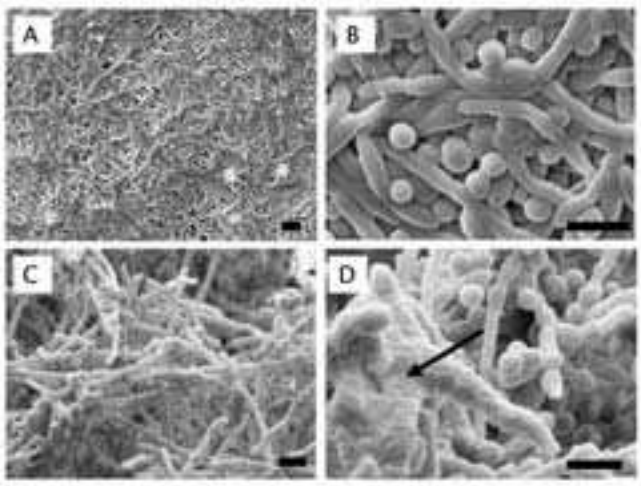

Figure 2. SEM analysis of 24 h *C. albicans* biofilms formed using the improved MRD under flow conditions, unfixed (panels A and B) and fixed (panels C and D). Biofilms that were unfixed clearly shows yeast cells and filaments embedded within an exopolymeric matrix. Fixed biofilms show complex 3-D architecture that is spatially interspersed with water channels. Arrow points to the remains of large quantities of exoplymeric material that can be observed surrounding the cells. Bar is 10 μm for all panels.

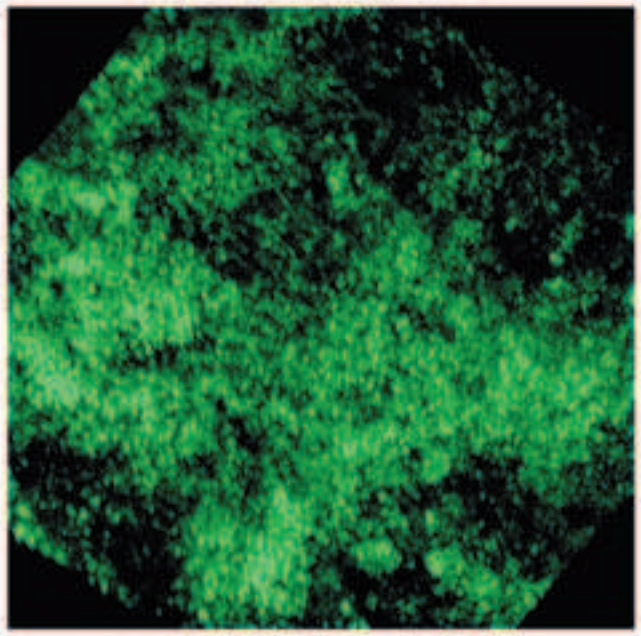

Figure 3. Three-dimensional reconstruction by CSLM of a *C. albicans* 24h biofilm formed using the improved MRD under flow conditions. Note the highly undulating 3-D architecture (approximately 90 - 240 μm in depth). Areas of increased fluorescence represent dense networks of filamentous and yeast elements. Darker areas represent ramifying water channels interspersed between the sessile cells.

on the different cellular morphologies present in the developed biofilms. As in biofilms formed under static conditions [21], mature biofilms formed under shear stress and with a flow of replenishing nutrients consisted of a dense network of yeast cells, pseudohyphae and true hyphae. These fungal elements were deeply embedded within a matrix of exopolymeric substance (EPS) that was better preserved when the biofilms were not fixed (compare panels A and B versus C and D). In fixed biofilms, most of the EPS was lost during processing; although the remains of the EPS material were visible and, in agreement with previous observations [1,3,13], it would seem that the

amount of EPS is increased in biofilms formed under flow as compared to biofilms formed using similar conditions but under static incubation [21].

As opposed to the destructive nature of SEM, the non-invasive CSLM technique enabled imaging of intact biofilms and visualization of the three dimensional structured associated with biofilms formed using the improved MRD. Fig. 3 shows a three dimensional reconstruction of a 24 h old *C. albicans* biofilm formed using the improved MRD under flow conditions. Overall results indicate that *C. albicans* biofilms form under flow conditions display significant channelling and porosity and a complex architecture with extensive spatial heterogeneity and highly variable undulating topography. Also, *C. albicans* biofilms formed under flow conditions with replenishing nutrients are thicker than biofilms formed under static conditions [21].

Overall, we have developed an in vitro "seed and feed" model of *C. albicans* biofilms formed using an improved MRD designed by our group that permits reproducible formation of biofilms on different biomaterial discs. Using this method, biofilms are formed under shear forces with a flow of replenishing nutrients, thus emulating the physiological conditions encountered by microorganisms in the host. Characterization of the resulting biofilms using SEM and CSLM indicated an increased architectural complexity and an increased production of EPS. This may have implications on antifungal treatment of biofilms, and should be used in conjunction with current static screening methods to evaluate the effectiveness of novel antifungal therapies. In addition, this model permits the collection of large quantities of cells which can be used for proteomic and genomic studies of *C. albicans* biofilms.

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