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Impact of infectious *Candida albicans* biofilm on biomaterials

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In the present investigation, biofilm formation by *Candida albicans* was studied on different polymeric surfaces, viz., polypropylene (PP), polystyrene (PS), polyvinylchloride (PVC), and silicone rubber (SR). Amongst these polymeric surfaces, the maximum biofilm formation was recorded to be 64.19, 50.31, and 45.09% for PS, PP, SR, respectively in comparison to PVC after 48 h using XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)2H-tetrazolium-carboxanilide] tetrazolium reduction assay. Exopolysaccharides (EPS) production during biofilm formation, when assessed using acetone precipitation technique, was found to be 11.45, 9.41, 8.65 and 6.95 μg/cm^2 for PVC, PS, PP and SR, respectively. Atomic force microscopic and goniometric analysis showed maximum roughness (134 nm) and hydrophobicity (97°) for PVC. Confocal laser scanning microscopy (CLSM) studies revealed maximum biofilm thickness (117.5 μm) on PVC surface when analyzed by z-sectioning. Further, the data were confirmed by scanning electron microscopy (SEM) for biofilm growth on these biomaterials. It was observed that PVC as biomaterial is most susceptible for *C. albicans* biofilm formation, while material surface properties like roughness and hydrophobicity promotes *C. albicans* adhesion and biofilm development.

Keywords: Biofilm, biomaterials, *Candida albicans*, CLSM, XTT

Introduction

The medical advances associated with invasive procedures in combination with the widespread use of broad-spectrum antibiotics are likely to be responsible for escalating the occurrence of infectious complications related to medical devices. The medical consequence of these device-related infections can be life threatening and may lead to device removal. In such a situation, the management of these devices is a difficult and costly affair[1]. An increasing proportion of these device-related infections, particularly those involving the central and peripheral venous catheters, urinary catheters, enditracheal tubes, neurosurgical shunts, voice prostheses, dentures, and intrauterine devices are being caused by *Candida* spp., and most notably *Candida albicans*[2,3]. This opportunistic dimorphic fungus can populate and penetrate implanted surfaces to form biofilm, a structured community composed of a mixture of cell types (yeast, pseudohyphal, hyphal cells) in a extracellular polymeric matrix (EPM) comprising of polysaccharides and proteins[4]. These implanted devices provide the necessary platform for the biofilm architecture to build up, starting from surface colonization, growth and proliferation leading to EPM formation.

Mature *Candida* biofilm exhibits a complex 3-D structures and extensive heterogeneity with typical micro-colony/water channels encased in exopolymeric materials[5,6]. From the clinical perspective, the sessile cells in biofilm display phenotypic trends that are dramatically different from their planktonic counter parts and also show high level of resistance to conventional antifungal therapy[7,8]. Hence, the current consensus to get rid of *C. albicans* biofilm infection requires the removal of the implanted devices which would have profound affect on the patient's discomfort and on the cost of the medical cure[9]. Although much work has been done on morphological and physiological stages of *Candida* biofilm formation on different biomaterial surfaces, the molecular and physical interactions that govern adhesion to biomaterials has yet to be deciphered[10]. In the present investigation, we have focused on the impact of *C. albicans* biofilm on different biomaterial surfaces, and tried to establish its correlation with its surface properties.

Materials and Methods

Microorganism and Culture Conditions

C. albicans isolated from clinical samples earlier[11] was grown on yeast extract peptone dextrose broth

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(YEPD) medium containing 20 g/L peptone, 10 g/L yeast extract and 20 g/L dextrose and incubated for 48 h at 30°C with agitation (120 rpm). The strain was routinely maintained in YEPD media with 50% glycerol at −70°C.

Biomaterials

Commercially available biopolymers, namely, silicone rubber (SR), polyvinyl chloride (PVC), polypropylene (PP) and polystyrene (PS), were used to study analysis of *C. albicans* biofilm. Squares of 1.0 cm × 1.0 cm were cut from these materials for experimental usage. Before use, the biomaterials were decontaminated by 2% Decon (SD Fine Chemicals, India) with mild agitation (30 rpm) and finally soaked in Milli-Q water. These samples were then dried in a laminar airflow hood, and sterilized using 100% ethylene oxide.

Surface Properties of Biomaterials

Surface characteristics were analyzed using atomic force microscopy and contact angle measurements[12].

Atomic Force Microscopy (AFM)

Dimension 3000 Scanning Probe Microscope with tapping mode AFM was used to analyze roughness of biomaterial samples (5 × 5 μm^2). The average roughness was then calculated by Nanoscope software and represented as the arithmetic average of the deviation from the center plane. Three points were analyzed on each square and results were expressed as mean±standard deviation.

Contact Angle Measurement

Hydrophobicity of biomaterial surface was determined by contact angle measurement using Rame-Hart contact angle goniometer (Mountain Lakes, NJ, USA; Model 100-00). Distilled water was used as contact angle liquid. A drop of distilled water was placed on the surface of biomaterial and image was captured by a camera equipped with the Rame-Hart 2001 imaging software. The angle made by the drop with respect to biomaterials was calculated and results were expressed as mean±standard deviation for contact angle of 5 drops.

Quantification of Biofilm

Biofilm quantification was performed using modified XTT reduction assay on capped polypropylene tubes[13]. Briefly, 1 cm^2 pieces of different biomaterials were dipped in 1 mL of culture with 5×10^8 cfu/mL of 48 h grown *C. albicans* and placed for 90 min of adhesion phase at 37°C. The biomaterial pieces were then washed with sterilized PBS (0.1 *M*; *p*H 7.2) to remove loosely adherent cells. To the washed pieces, 1 mL of sterilized YEPD broth was added and incubated at 37°C for 48 h.

XTT Reduction Assay

XTT (Sigma, St. Louis, Mo.) solution (1 mg/mL in PBS) was prepared, then filter sterilized through a 0.22 µm pore size filter and stored at −70°C. Menadione (Sigma) solution (0.4 m*M*) was prepared and filter sterilized immediately before each assay. Prior to each assay, XTT solution was thawed and mixed with the menadione solution at a ratio of 5 to 1 by volume. The biofilms were washed 5 times with 1 mL of PBS, and then 1 mL of PBS with 60 µL of the XTT-menadione solution was added to each of the pre-washed and control tubes having different biomaterial samples. The tubes were then incubated in the dark for 2 h at 37°C. Following the incubation, brown coloured, water-soluble tetrazolium formazan product was formed in the solution, which was measured spectrophotometrically at 492 nm (Varian, USA).

EPS Production

EPS production was assessed from culture supernatant of *C. albicans* on different biomaterials (PVC, PP, PS & SR) using the acetone precipitation technique as described earlier[11,14]. Briefly, 48 h grown culture supernatant was treated with 1.5 volume of chilled acetone and centrifuged at 17000 g for 15 min. The pellet so obtained was redissolved in a small volume of distilled water and centrifuged to remove any insoluble material. Water soluble polymer was reprecipitated with two volumes of chilled acetone for 2 h at 4°C. The precipitate was finally washed with ether and evaporated to dryness in warm air current before being weighed.

Confocal Laser Scanning Microscopy (CLSM)

C. albicans biofilm on the different biomaterials was fixed with 2.5% glutaraldehyde in 0.1 *M* phosphate buffered saline (PBS; *p*H 7.2) for 1.5 h. CLSM (Radiance 2100, BioRad) was performed under a Nikon microscope (objective Plan Apo 60X/1.4 oil, Japan).

Scanning Electron Microscopy (SEM)

C. albicans biofilm formed after 48 h on different biomaterials surfaces were fixed with 2.5% (v/v) glutaraldehyde in PBS for 2 h at room temperature. They were then treated with 1% (w/v) uranyl acetate for 1 h and washed with distilled water. The samples were dehydrated with ethanol series (30, 50, 70, 90 & 100%). All the samples were dried to critical point by

Polaron critical point drier, coated with gold and viewed under SEM (Leo 435, England).

Statistical Analysis

Experimental data were analyzed with paired t-test. *P* levels of less than 0.05 were considered significant. All the experiments were done in triplicate.

Results

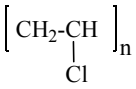
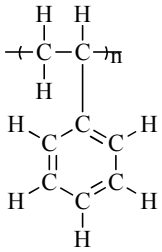
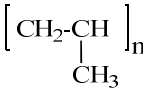
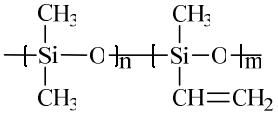
Surface Properties of Biomaterials

The roughness and hydrophobicity of biomaterial samples were measured in order to determine surface characteristics, which account for adhesion of *Candida* cells. Among the different biomaterial samples tested, the order of roughness recorded was PS<SR<PP<PVC (Table 1). AFM of different biomaterials depicted maximum number of grooves and crusts in case of PVC, while no such deformities were reported in the case of PS. PVC with maximum contact angle (97°) indicated higher hydrophobic nature in comparison to other biomaterials tested (Table 1).

Quantification of Biofilm

Biofilm quantification on different biomaterial samples was performed using XTT reduction assay to measure variation in metabolic activity of biofilm residing *C. albicans* cells. Amongst these polymeric surfaces, the biofilm formation was recorded to be 64.19, 50.31, and 45.09% for PS, PP, SR, respectively in comparison to PVC, used as a control, after 48 h. Thus, maximum biofilm formation was measured in the case of PVC.

Table 1—Average contact angle (°), roughness (nm) and amount of exopolysaccharides (µg /cm^2 area of polymer) produced by *C. albicans* biofilm on different biomaterial surfaces

Biomaterials	Structures	Contact angle ± SD (°)	Rough-ness (nm)	EPS (µg /cm^2)
Polyvinyl chloride (PVC)		97± 1.20	134± 0.8	11.45± 1.2
Polystyrene (PS)		91± 0.07	24± 2.2	9.41± 0.9
Polypropylene (PP)		84± 1.2	43± 1.6	8.65± 0.6
Silicon rubber (SR)		77± 1.3	27± 0.6	6.95± 0.7

Data are result of three individual experiments plotted in triplicates and shown as ± standard deviation.

EPS Production

EPS production on different biomaterial samples was performed using acetone precipitation technique. Maximum EPS was obtained in the case of PVC, followed by PS and PP; while production was least in the case of SR (Table 1).

CLSM Studies

C. albicans biofilm on biomaterial samples was observed using CLSM. The z-sectioning for biofilm thickness, intensity measurements and EPS production after staining with PI, and FITC-ConA illustrated that PVC had maximum fluorescent intensities, followed by PS, PP and SR (Fig. 1). When

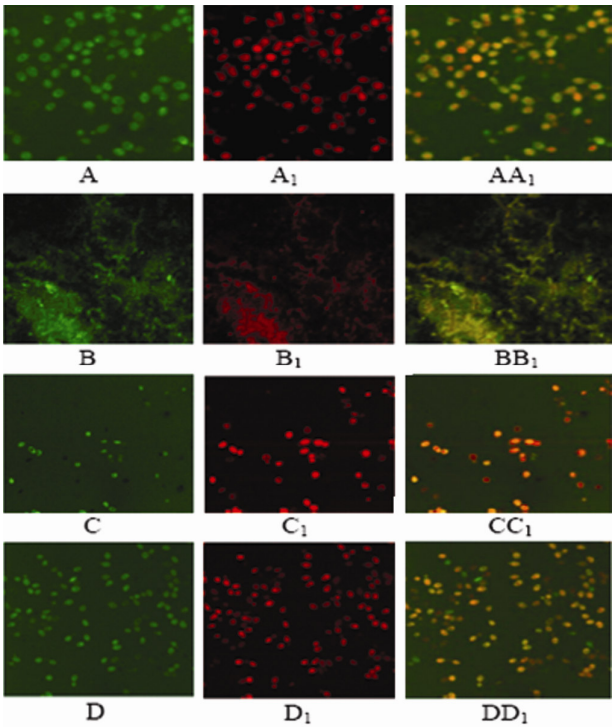


Fig. 1—CLSM images of *Candida* biofilm formed on biomaterial surfaces: (A) Polyvinylchloride, (B) Polystyrene, (C) Polypropylene, & (D) Silicon rubber. Green images show exopolysaccharides by FITC-ConA staining (A-D). Biofilm forming PI stained sessile cells in red (A_1-D_1), resulting image (AA_1-DD_1) produced by overlap of green and red images showing production of exopolysaccharide as capsular component in yellow. CLSM images taken with Nikon microscope (objective Plan Apo 60X/1.4 oil, Japan).

CLSM images with red and green fluorescent intensities were superimposed, yellow colour (green+red) revealed that the extracellular FITC-ConA-reactive polysaccharide (green) was produced in the intracellular spaces (red), indicating thereby that extracellular polysaccharides were produced as a capsular component in biofilm. Interestingly green intensity was recorded to be more in the case of SR than to PP surface, while red intensity was found to be more in the case of PP surface.

SEM Analysis

SEM analysis on different biomaterial surfaces showed maximum colonization of *C. albicans* biofilm in the case of PVC, followed by PS, PP and SR (Fig. 2).

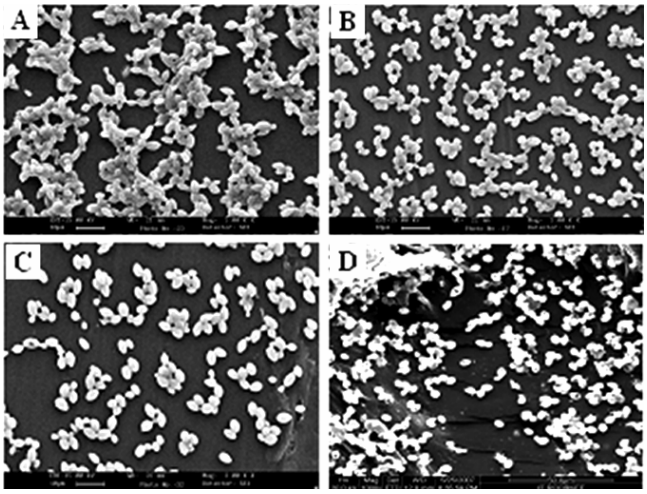


Fig. 2—SEM of *C. albicans* biofilm formed on different biomaterial surfaces: (A) Polyvinylchloride, (B) Polystyrene, (C) Silicon rubber, (D) Polypropylene.

Discussion

The most important aspect in the pathogenesis of foreign body-associated infections is the ability of microbes to colonize the foreign body (polymer) surface by the formation of a thick multilayered biofilm[15]. Depending on the kind of device, its insertion side, duration of insertion and the nature of infecting microorganism, biofilm constitutes a major reason for infections to occur and persists at various sites in the human body[16]. It is evident that biofilm forming organisms attribute their properties towards pathogenesis more successfully than other organisms, with more toxins and tissue damaging exoenzymes[17]. During our investigation biofilm quantification was made using the tetrazolium salt, 2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]2H-tetrazolium-carboxanilide (XTT) to monitor the biofilm formation by *C. albicans*. The salt reduced by mitochondrial dehydrogenase to a brown coloured, water-soluble tetrazolium formazan product, which depicted a linear relationship with metabolic activity of *C. albicans* biofilm as reported earlier[11]. The heterogenic complexity of biofilm, which is initiated with the adhesion phase, provides a protective shield to the colonizers of various surfaces. In fact, EPM is composed of a wide variety of organic materials, including polysaccharides, proteins, nucleic acids, phospholipids, uronic acid and humic substances, which provide protection against phagocytosis, interference with the cellular immune response and resistance against antibiotic effects[17]. The EPM composition may be the result of active secretion, shedding of cell surface material, cell lysis, and adsorption from the environment. Recently, due importance has been given to the functions of EPM in microbial communities, which may include cell adhesion and aggregation, granulation, organic chemicals degradation, biofouling, cell-to-cell communication, resistance of biofilm structure to heavy metal toxicity, etc[4,11]. However, little information is available on the chemical constituents, characteristics and functions of EPM content of the *C. albicans*[4,18]. Our studies showed that maximum EPS produced by *C. albicans* biofilm was recorded on PVC surface, followed by PS, PP and SR. This suggests more pathogenic and resistant nature of *Candida* biofilm formed on PVC in contrast to other biomaterial surfaces. Earlier, researchers have shown that when the microorganisms reach the proximity of a surface, attachment is determined by physical and chemical interactions, which may be attractive or repulsive in nature, depending upon the complex interplay of the chemistry of the microorganisms and substrate surfaces, and the aqueous phase[19]. The differential susceptibility of biomaterials towards *Candida* biofilm may involve many material surface properties. The biofilm formation was found to be the maximum with PVC, followed by PS, PP and SR, depicting decrease in hydrophobicity along with reduction in contact angles. Researchers have earlier shown that absorption of protein on hydrophobic surface was found to increase surface hydrophilicity, which resulted in the reduction of adhesion[20]. Since the cell surface hydrophobicity of *Candida* cells is known to be an important factor in its adherence to acrylic surface, this factor may also play a key role in the biofilm development on these biomaterials[21]. Our observations showed that *C. albicans* biofilm

formation decreased with contact angles of biomaterials samples used. PVC with contact angle of 97° formed maximum biofilm, while least development of biofilm was recorded on SR surface. These results are in agreement with the previous studies showing that the correlation between contact angle and biofilm formation is dependent on surface properties[22,23]. AFM analysis used to quantify the biomaterial surface roughness showed high surface area in the case of PVC, which may account for greater surface area and the depressions as the roughened surfaces provide more favourable sites for the colonization *of C. albicans*[24]. It has also been reported that surface materials with different porosity, groove and braid have higher infection rates than flat ones, probably due to the increased surface area[25]. Reports also suggest that minute grooves or scratches on biomaterial surfaces increase the contact area and hence the binding potential[26]. In fact, *C. albicans* biofilm formation is a complex process dependant upon multiple variables, including proteinaceous preconditioning of films during early adhesion events. Following initial adhesion, the structural integrity and developmental characteristics of *C. albicans* biofilms on different biomaterials was monitored using SEM and CLSM techniques. The increased magnification and resolution power associated with the SEM technique permitted a more detailed examination of biofilms. *C. albicans* biofilms consist of a mixture of yeast and filamentous forms embedded within exopolymeric material. The application of CLSM has provided a powerful non-destructive method for biofilm analysis[27]. CLSM made it possible to observe biofilm without destruction of the structures and revealed that biofilm developed in both horizontal and vertical direction[27]. CLSM has been adopted for biofilm analysis using fluorescent dye specific for nucleic acids (PI) and glycoconjugate fraction (FITC-ConA) of biofilm. PI binds to DNA by intercalating between the bases with little or no sequence preference and with a stochiometry of one dye per 4-5 base pairs of DNA. This dye is commonly used for identifying dead cells in a population and as a counter stain in multicolour fluorescent techniques. CLSM, coupled with fluorescence methods and image analysis, provides images with better resolution, thus giving more information pertaining to biofilm. *C. albicans* biofilm on biomaterial samples were observed using CLSM. Hence, from the current work it can be inferred that these staining techniques could be used simultaneously to probe viable and non-viable *Candida* cells. The combined effects of individual stained images showed EPS produced in cell in bound as well as secreted form. The present scheme can also be made applicable to other disaggregate systems, such as, biofilm in wastewater sludge floccules[28].

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