Influence of serum and polystyrene plate type on stability of *Candida albicans* biofilms

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Abstract

Adhesion is a crucial initial step in microbial biofilm formation. Firm attachment to a target surface subsequently ensures successful colonization and survival despite turbulent conditions. In the laboratory, polystyrene plates are commonly used in biofilm experiments and the ‘washing/rinse steps’ before staining are critical for assaying biofilm viability. However, these rinse steps risk the removal (partially or entirely) of the formed biofilm, resulting in inconsistent results. The aim of the present study was to optimize conditions for firmer biofilms, less prone to disruption and thus significantly reducing well-to-well variability. *Candida albicans* SC5314 was used in five different polystyrene 96-well plates from four different manufacturers. Irrespective of how gently we performed the rinse, biofilms came off certain polystyrene plates more easily compared to others. Importantly, preconditioning the polystyrene surfaces with foetal bovine serum (FBS) had a negative impact on firm biofilm attachment. Costar® plates provided the most suitable surface for firm biofilm attachment, both in the presence and absence of FBS. Substratum properties even among seemingly identical synthetic materials may influence biofilm attachment and its subsequent sturdiness, affecting experimental results.

Key words: Biofilm stability, *C. albicans*, Adhesion, Polystyrene multiwell plates, Foetal bovine serum

1. Introduction

The term ‘biofilm’ refers to the attachment onto surfaces of microbial communities of cells enclosed in self-produced extracellular polymeric matrices (Cegelski *et al* 2008). In *in vitro* systems, different types of synthetic materials have been used as substrate for biofilm attachment and formation. They include plastics, silicones, ceramics, glass and also metals (Coad *et al.*, 2014; Nwaugo *et al* 2007; Chandra *et al.*, 2001; Webb *et al.*, 1999). Attachment to these substrates is greatly influenced by physical and chemical interactions between the cell surface and the substratum. In bacteria and *C. albicans*, mainly hydrophobic interactions and to some extent electrostatic forces are responsible for the initial attachment to these surfaces (Varghese *et al.*, 2013; Donlan, 2002; Matz and Jürgens, 2001; Gristina *et al.*, 1987). Additionally, substrate surface roughness has been shown to play a critical role in cell adhesion (Biazar *et al.*, 2011, Radford *et al.*, 1999).

Adhesion of *C. albicans* to substrates is multifactorial and has been associated with key factors such as formation of an extracellular matrix (Chandra *et al.*, 2001), adhesins (De Groot *et al.*, 2013), yeast-hyphae transition (Beaussart *et al.*, 2012) and type of growth media (Hoyer *et al.*, 1995). Additionally, in order to closely mimic *in vivo* conditions, some authors have preconditioned substrates with biological substances such as saliva, human serum, FBS, urine, etc. (Kuchari'kova' *et al.*, 2015; Silva *et al.*, 2009; Cannon and Chaffin., 1999; Thein *et al.*, 2007).

Standard biofilm assays to determine the fraction of surviving cells after treatment with antimicrobials, typically use multi-well 96 plates as this provides an inexpensive, rapid, and simple format (O'Toole, 2011 ; Pierce *et al.*, 2010; Ramage *et al.*, 2001b). Moreover, in these assays, gentle rinse/washing steps typically precede a colorimetric analysis (Delattin *et al.*, 2014; Peeters *et al.*, 2008; Pettit *et al.*, 2005). This ensures removal of non-adherent cells, enabling accurate estimation of surviving biofilm cells and hence effectiveness of an antimicrobial compound. This rinsing, however, can also loosen and subsequently remove attached biofilms, depending on their degree of attachment to a substrate (Pitt *et al.*, 1993), resulting in inaccurate results. While great care should be taken during the washes (Stepanovic' *et al.*, 2007), biofilms will inevitably detach (partly or completely) if cell-substrate adhesion is weak. Though not widely discussed, biofilm assays in polystyrene plates often yield variable results (Mizan *et al.*, 2016; Nett *et al.*, 2011) and a lack of firm adhesion to polystyrene surfaces is likely to play an important role in this.

Thus the aim of this study was to optimize conditions for firmer (more robust) biofilms, so as to reduce well-to-well variability significantly. To ameliorate the variations in our experiments due to loss (partial or complete) of biofilms during rinse steps, we compared the adhesion and retention patterns of *C. albicans* in five brands of polystyrene plates in the presence and absence of FBS to select optimal conditions for firm biofilms.

2. Materials and Methods

C. albicans strain SC5314 was a gift from Prof. Patrick van Dijck (KU Leuven, Belgium).

Stocks were stored in yeast-extract peptone dextrose (YPD) broth and 20% glycerol at -80°C in cryovials. Roswell Park Memorial Institute (RPMI) powder with L-glutamine without sodium

bicarbonate, 4-morpholinepropanesulfonic acid (MOPS) and FBS were purchased from Sigma (St. Louis, MO, USA). Resazurin dye was purchased from Acros Organics (New Jersey, USA). Five flat (F)-bottom polystyrene 96-well plates were compared: F-bottom, black (ref 655090) and F-bottom, clear (ref 655101) from Greiner Bio-one, F-bottom (ref 3370) from Costar®, F-bottom (ref 10062-900) from VWR International, Belgium, and tissue culture plates, F-bottom (ref 92096) from TPP Techno Plastic Products AG, Switzerland.

2.1 Biofilm formation, Rinsing and Visualization

Material from the frozen stock was streaked out on YPD agar plates, which were incubated overnight at 37°C. A few colonies were inoculated in YPD broth and incubated overnight at 37°C in a shaking incubator. This overnight culture was then centrifuged at 164 rcf for 3 minutes and the supernatant was discarded. The pellet was then resuspended in RPMI-MOPS, pH 7 and used in the experiments. Different seeding cell densities were used in the presence and absence of FBS using a standard biofilm protocol (De Brucker *et al.*, 2016), with some adjustments. To this end, 100 µL of FBS was added to each well (for the FBS-coated plates) and incubated at 37°C for 24 hours; the FBS was then removed and the wells were washed once with 100 µL phosphate-buffered saline (PBS). The optical density (OD) of the resuspended overnight culture in RPMI-MOPS was measured in a Perkin Elmer spectrophotometer (MA, USA), and the OD₆₀₀ was adjusted to 0.1, 0.2, 0.5, 1.0 or 2.0 in RPMI-MOPS medium before dispensing 100 µL of the cell suspension per well. After 90 minutes incubation at 37°C, the medium was gently aspirated and the wells were rinsed once with 100 µL PBS. Thereafter, 200 µL of RPMI-MOPS medium was gently added, and plates were incubated at 37°C for 24 hours. Following this incubation period, the medium was aspirated and the wells were washed twice with 200 µL PBS. Hundred

μL of freshly-prepared resazurin dye (0.01% w/v) in PBS was added to each well and the plates were incubated for 1 hour at 37°C. Accurate quantification by fluorescence spectroscopy was not possible as masses of biofilms, peeled off during the washes, could not be completely aspirated and remained lingering on the sides of the wells. When the stain was added, these floating films of cells resulted in false positives as there were no attached biofilms in these wells. We thus opted for visible light microscopy. Biofilms were visualized and photos taken using a fluorescence stereomicroscope (Leica M165FC) connected to a Leica CLS 150 XD light source and a 3iCube - USB3.0 camera from New Electronic Technology (NET) GmbH. The experiment was repeated on three different occasions with at least 16 biofilms for each cell density tested.

2.2 Statistical analysis

A scoring system with five levels was used to evaluate the state of the biofilms after the washes: 1: no attached biofilm, 2: few patches of attached and severely distorted biofilm, 3: more patches of attached and distorted biofilm, 4: almost fully attached and slightly distorted biofilm and 5: fully attached and intact biofilm (see photographs below for representative states of biofilms). Scores of the biofilms originating from the five seeding cell densities were compiled for every plate type in the absence or presence of FBS to determine the most suitable plate for sturdy biofilm formation. Error bars represent standard deviation of the mean scores from three independent experiments.

3. Results and Discussion

To counter the challenge of biofilms either peeling off entirely or breaking apart irregularly during washing steps, we set out to identify suitable polystyrene microplates that offer firm attachment substrates for *C. albicans* biofilms. For five polystyrene plates, in the presence and

absence of FBS and seeded at different cell densities, we determined how firmly attached the biofilms were after the washing steps.

3.1 Effect of FBS

Prior to washing, we observed that biofilms in 4 of the 5 FBS-coated plates were thicker than in the non FBS-coated plates. Similar observations of FBS enhancing *C. albicans* biofilm formation have been reported in the literature (Ramage *et al.*, 2001a; Samaranayake *et al.*; 1980; Frade and Arthington-Skaggs, 2011; Řiřicová *et al.*, 2010). This may be attributed to the presence of serum proteins in FBS, such as albumin, that *C. albicans* cells efficiently bind to, increasing adhesion (Ramage *et al.*, 2001a). However, in the FBS-coated clear Greiner plates, we observed masses of floating cells. Biofilms in the other plates were visibly attached.

Upon performing the first rinse, it became apparent that biofilms in the FBS-coated plates were typically more susceptible to peeling off than their counterparts in non FBS-coated plates, with the exception of Costar plates (Figure 1 and 2). We want to emphasise that the washing was performed as gently as possible, always pipetting PBS slowly against the side of the wells, and letting it flow down to the bottom of the wells covered with biofilms; this eliminated high fluid shear forces which have been shown to cause detachment of bacterial biofilms (Sharma *et al.*, 2005) in dynamic flow chambers. This step was followed by gently aspirating the fluid overlying the biofilm with tips held against the side of the wells, avoiding direct suction perpendicular to the bottom of the wells that may detach biofilms (Busscher and van der Mei, 2006). In this way, a thin layer of liquid always covered the biofilm, even at the end of aspiration. Thus, liquid-air interfaces such as air bubbles or simply air was not allowed to flow over our biofilms since these

have been shown to disrupt biofilms in flow chambers (Gómez-Suárez *et al.*, 2001; Pitt *et al.*, 1993).

The FBS-coated black Greiner plates had severely distorted biofilms after the washes. The FBS-coated TPP plates were also affected particularly at the low cell densities which are commonly used (De Brucker *et al.*, 2016; Ramage *et al.*, 2001b). Despite gentle washes and slow fluid aspirations from the wells, biofilms in these plates consistently displayed poor attachment in the presence of FBS. Interestingly, in the absence of FBS, biofilms in the black Greiner and TPP plates exhibited significantly improved adherence (Figure 2). It is thus evident that a negative interaction exists between FBS and some polystyrene plates resulting in weaker adhering biofilms, and that this is quickly reversed in the absence of FBS. Schneider (1996) pointed out that conditioning films may not only modify the surface free energies of the substratum and attaching molecules, but also other physicochemical parameters of the substratum, affecting adhesion. Moreover, both the TPP and black Greiner plates are specially treated to enhance attachment of mainly mammalian cells and thus are more hydrophilic. Several studies have demonstrated that serum proteins adsorb well on hydrophobic surfaces compared to hydrophilic surfaces (Parhi *et al.*, 2010; Perry *et al.*, 2006; Samorjai and Kim, 2003). This may additionally explain the loss of biofilms from these hydrophilic surfaces upon washing, as the interaction between the FBS and the plate surfaces is unstable. Similarly, since Costar plates are untreated and hence maintain a more hydrophobic surface (Kennedy and O’Gara, 2004), the remarkable sturdiness of biofilms on these FBS-coated plates may be attributed to the favourable interaction between FBS and these hydrophobic surfaces.

Figure 1: Effect of FBS on *C. albicans* biofilm stability. The error bars throughout represent the standard deviation of mean scores from three independent experiments.

Figure 2: Biofilm stability of *C. albicans* on non FBS-coated polystyrene plates

3.2 Effect of plate properties

While all the plates used in this study are made of polystyrene, the treatments they undergo during production result in surface modifications that may play a role in cell attachment and influence their sturdiness on these surfaces. Such surface treatments are known to alter the surface energies, affecting cell surface hydrophobicity and influencing adhesion patterns. Additionally, Biazar *et al.* (2011) in their experiments on polystyrene surface modifications showed that, plasma treatments with inert and reactive gases resulted in variable surface roughness that resulted in improved cellular adhesion as surface roughness increased. Thus, while a complex interplay of factors is responsible for firm adhesion (Krasowska and Sigler,

2014), we hypothesize that physico-chemical properties of these modified substrata may also be responsible for the observed differential attachment.

With the exception of clear Greiner plates which are marketed as non-sterile, the rest of the plates are sold as sterile plates, commonly sterilized by irradiation. Additionally, the surfaces of both the clear Greiner and Costar plates are not specially treated to enhance attachment, hence they remain largely hydrophobic (Sigma Aldrich a.). The manufacturer additionally states that Costar plates are medium-binding (Sigma Aldrich b). The remaining plates (TPP, black Greiner and VWR) have their surfaces treated (opto-mechanically, physical surface treatment and via plasma, respectively) generating hydrophilic surfaces. It is therefore interesting to note that the main differences between the best (Costar) and the poorest (clear Greiner) plates for firm biofilm attachment seem to be the irradiation process and the medium-binding ability. Sterilization by irradiation of untreated plates modifies the surfaces and affects cell adhesion, as attachment proteins bind through hydrophobic interactions (Sigma Aldrich c). It is, however, not immediately clear to us what the role of ‘medium-binding’ property in Costar plates could be in promoting sturdy *C. albicans* adhesion. Additionally, while it is conceivable that the hydrophobicity of Costar plates contributes to the remarkable attachment by *C. albicans* via hydrophobic interactions with cell surface structures (Beaussart *et al.*, 2012; Krasowska and Sigler, 2014, Fukazawa and Kagaya, 1997; Klotz *et al.*, 1985), a link between sturdy adhesion of *C. albicans* and the rather hydrophilic surfaces of non-FBS coated TPP and black Greiner plates is not a direct one and several explanations can be given. The surface treatments of the latter two plates could have increased their surface roughness, which is known to increase the surface area for attachment and provide ‘pockets’ where particles (like microbes) are protected from detaching easily (Radford *et al.*, 1999). Additionally, a review by Krasowska and Sigler, 2014

suggests that “though the primary adhesion of a hydrophobic particle to the hydrophilic surface is weak, the bridging polymers give rise to long-range attraction which will effectively anchor the particle to the surface”. Experimental analysis of these polystyrene plastics may provide more insight into these observations. Nevertheless, our results already provide useful guidance to choose the most suitable plates for *C. albicans* biofilm experiments.

3.3 Effect of cell seeding density

In general, sturdiness of biofilms in TPP and VWR plates coated by FBS improved with higher cell densities. In the absence of FBS, the same trend was seen for VWR plates but an opposite trend for TPP plates. It is conceivable that with higher cell densities, there was increased chance of more cells attaching to these surfaces, and more cells remaining after the initial wash at initial adhesion (90 minutes), leading to denser biofilms that (together with the other discussed factors), better weathered the rinsing steps. While the Costar plates provide the best attachment surfaces for firm biofilms, the clear Greiner plates seem to be generally unfavourable for biofilm adhesion and retention, and their material could maybe find application in situations where biofilm formation is undesirable, like in implantable medical devices.

In summary, we have shown that plates made of seemingly similar synthetic material (polystyrene) display highly different biofilm retention during washing procedures whether they were pre-treated with FBS or not. Though the same *C. albicans* strain was used throughout, and the growth medium or temperature were not varied either, we cannot exclude that the *Candida* reacted differently to the different surfaces, e.g. by expressing different cell surface adhesion proteins. Differential expression of genes responsible for surface adhesion was indeed seen by Harapanahalli and colleagues (2015) for *S. aureus* on different biomaterials. Analysis of plate

properties and corresponding gene expression may shed more light on this. In any case, optimized conditions favouring firm biofilm attachment that resists peeling or breakage during washing steps should greatly reduce variability in *C. albicans* biofilm viability experiments, enabling more accurate inferences.

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Competing interests

The authors declare no competing interest.

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