Early adhesion of *Candida albicans* onto dental acrylic surfaces

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Abstract:

Denture-associated stomatitis is a common candidal infection that may give rise to painful oral symptoms, as well as be a reservoir for infection at other sites of the body. As poly (methyl methacrylate) (PMMA) remains the main material employed in the fabrication of dentures, the aim of this research was to evaluate the adhesion of *C. albicans* cells onto PMMA surfaces, by employing an atomic force microscopy (AFM) single-cell force spectroscopy (SCFS) technique. For experiments, tipless AFM cantilevers were functionalised with PMMA microspheres and probed against *C. albicans* cells immobilised onto biopolymer-coated substrates. Both a laboratory strain and a clinical isolate of *C. albicans* were utilised for SCFS experiments. SEM and AFM imaging of *C. albicans* confirmed the polymorphic behaviour of both strains, which was dependent on growth culture conditions. AFM force-spectroscopy results showed that the adhesion of *C. albicans* to PMMA is morphology dependent, as hyphal tubes had increased adhesion compared to yeast cells ($p<0.05$). *C. albicans* budding mother cells were found to be non-adherent, which contrasts with the increased adhesion observed in the tube region. Comparison between strains demonstrated increased adhesion forces for a clinical isolate compared to the lab strain. The clinical isolate also had increased survival in blood and reduced sensitivity to complement opsonisation, providing additional evidence of strain-dependent differences in candida / host interactions that may affect virulence. In conclusion, PMMA-modified AFM probes have shown to be a reliable technique to characterise the adhesion of *C. albicans* to acrylic surfaces.

Introduction:

Candida albicans is a commensal yeast found living on skin and mucosal surfaces; however, it also has the capacity of causing opportunistic surface or deep tissue infections in immunocompromised patients (Kabir et al. 2012; Salvatori et al. 2016), and it has further been associated to infection of medical devices in diverse parts of the body (Nett et al. 2010). Typically, *C. albicans* exists as a polymorphic fungus, which means it has the capacity to grow in either yeast or filamentous form (Veses and Gow 2009). Filamentous forms, also known as hyphae, are tubular growths of *C. albicans* cells associated with tissue penetration and invasion (Thomson et al. 2016). Overall, *C. albicans* transition towards hyphae forms is considered one of the most important virulence factors of this particular strain (Jackson et al. 2014).

Despite the introduction of novel biomaterials and techniques in dentistry, such as titanium based implants, the use of non-implant retained acrylic dentures remains one of the most commonly used methods to rehabilitate partially or fully edentulous patients (Pan et al. 2015). Most denture bases are constructed using poly (methyl methacrylate) (PMMA) as a main structural component (Uzunoglu et al. 2014). Nevertheless, denture surface infection with *C. albicans* biofilm continues to be a major problem, particularly when patients fail to maintain good denture hygiene (Barbeau et al. 2003). Adhesion of *C. albicans* to denture surfaces is necessary in order to initiate the process of infection and biofilm formation (Park et al. 2008). Once established, this candida biofilm is associated

to the onset of denture associated stomatitis, a chronic inflammatory form of oral candidiasis that can affect up to 70% of denture wearers (Cao et al. 2010; Izumida et al. 2014; Nett et al. 2010). Clinically, this disorder can sometimes give rise to pain or irritation of the oral mucosa (Yarborough et al. 2016). Also, it can be a potential source of systemic candida infection in immunocompromised individuals, and together with any accompanying bacterial colonisation, a possible reservoir for respiratory infection (O'Donnell et al. 2016; Wu et al. 2015). The presence of high counts of hyphae in the biofilm has been related to the severity of the disease (Park et al. 2008). There remains no specific therapy for treating this disorder, although typical strategies are physical cleaning of the dentures and topical use of antimicrobials such as chlorhexidine and/or antifungals such as miconazole (Yarborough et al. 2016).

In the past decade, atomic force microscopy (AFM) has been increasingly used to image and study the nanomechanic behaviour of a vast range of microorganisms at the nano-Newton level (Aguayo et al. 2015b; Taubenberger et al. 2013). Furthermore, as no sample preparation is required for AFM, it is possible to immobilise fungi under buffer conditions and obtain high-resolution imaging of viable cells (Braga and Ricci 2011). Recently, the AFM has also proven to be an effective tool to characterise the adhesion of cells to substrates at the single-cell and single-molecule levels (Beaussart et al. 2014). Techniques such as single-cell force spectroscopy (SCFS) allow researchers to probe nanoadhesion between single cells and surfaces of interest. In the case of *Candida*, several studies have analysed its attachment onto both bacterial cells

and abiotic surfaces (Alsteens et al. 2009; Formosa et al. 2015a; Hwang et al. 2015; Ovchinnikova et al. 2013). Although many surface adhesins are believed to play part in attachment, mannoproteins have been shown to be important in mediating *Candida* adhesion towards substrates (Alsteens et al. 2012; Beaussart et al. 2013). However, there are currently no studies evaluating the adhesion of *C. albicans* onto clinically relevant biomaterial surfaces at the single-cell level. As forces driving *C. albicans* towards PMMA surfaces remain unknown, the aim of this research is to investigate the adhesion between *C. albicans* yeast cells and hyphae and PMMA surfaces at the single-cell level, in hopes of understanding the crucial initial yeast-denture interaction and thereby provide insight into effective methods or preventing and/or treating this common disorder.

Materials and Methods

1.1. *C. albicans* strains

C. albicans ATCC 10231 and a clinical isolate of *C. albicans* (C1, isolated from the Royal Free Hospital, London) were utilised throughout the study. From frozen stocks, *C. albicans* were grown in Sabouraud broth (Oxoid Ltd, UK) for 16 hrs at 37°C and aeration to obtain the yeast cell phenotype. For hyphal differentiation, *C. albicans* were grown in BHI broth (Oxoid Ltd, UK) for 3 hrs at 37°C and aeration. Subsequently and for both cases, 100 µl of fungal suspension was diluted into 1 ml final concentration of phosphate-buffer saline (PBS 1x, Lonza, Belgium) and harvested at 5000 rpm for 1 min (2655 rcf, Eppendorf 5417R, UK). Resulting pellets were re-suspended in 1 mL PBS and transferred immediately to the AFM for experiments.

1.2. Non-destructive fungal immobilisation for AFM imaging and nanomechanics in buffer

To allow effective immobilisation of *C. albicans* yeast and hyphae for AFM experimentation, glass cover slides were coated with either a 100 µl droplet of poly-L-lysine (PLL, P4707, Sigma-Aldrich) or a 100 µl droplet of 4 mg/ml dopamine hydrochloride in 10 mM TRIS buffer, pH 8.0 (poly-DOPA, Sigma-Aldrich). After drying for 2 hrs, surfaces were washed with deionised water (dH_2O), dried with N_2 airflow and stored at 4°C until experimentation. PLL-coated slides were utilised to immobilise *C. albicans* yeast cells, while poly-DOPA was the preferred method to attach hyphal forms of the strain. Scanning electron

microscopy (SEM, Philips XL30 FEG SEM, FEI, Eindhoven, Netherlands) was utilised to confirm successful immobilisation of *C. albicans* onto coated surfaces.

1.3. Fabrication of PMMA-functionalised AFM probes

For single-cell force spectroscopy (SCFS), customised AFM probes were constructed in order to explore the adhesion of PMMA to surface immobilised *C. albicans*. Probe functionalisation was obtained by utilising a JPK Nanowizard system (JPK Instruments, Germany) mounted on an inverted optical microscope (Olympus IX71, Olympus, Japan). Tipless AFM cantilevers (NP-O10, Bruker, USA) were brought into contact with a thin layer of UV-curable glue (AA 350, Loctite, UK) spread out on a glass slide for 10 s. Each cantilever was then approached to a 10 µm PMMA microsphere (Cospheric, USA) for 1 min to allow attachment to the glue-covered surface. Subsequently, functionalised cantilevers were UV-cured for 10 mins, and correct placing of the microsphere was assessed by optical and SEM imaging **(Supplementary Figure 1)**. Finally, each PMMA-functionalised cantilever was calibrated utilising the JPK proprietary tuning software (~0.3 N/m spring constants) before experimentation.

1.4. AFM imaging and nanomechanics setup

All AFM imaging and nanomechanic experiments were carried out with a JPK Nanowizard system. Images were obtained with intermittent contact mode in fluid; by employing MSNL-10 cantilevers (Bruker, USA) with a spring constant of 0.1 N/m. Gain parameters were adjusted during imaging to allow for optimum image acquisition. Images of *C. albicans* cells and hyphae were acquired at different magnifications with 256x256 pixels and a scan rate of 0.3 Hz. SCFS

experiments were carried out with a loading force of 1 nN, a constant speed of 2 μm/s, and surface delay times of 0, 1, 5, 10 and 30 s. For yeast cells and the budding cells, force curves were obtained on the centre of the cell as observed with the optical microscope **(Figure 1)**. For hyphal tubes, force curves were performed in the middle portion of the tube **(Figure 1)**. All SCFS experiments were carried out in PBS buffer, maintaining probes and *C. albicans*-coated surfaces fully hydrated throughout the whole process. Force curves obtained over a total of 7 independent yeast cells and hyphae were utilised for data analysis.

1.5. Ex-vivo growth in blood

Human blood was obtained with written consent from healthy human volunteers under ethical approval granted by the local University College London ethics committee (application 3076/001). Blood clotting was prevented by the addition of 300IU/50ml of heparin sodium solution. Growth in blood was investigated using an inoculum of approximately $5x10^6$ CFU/ml. Samples were then incubated at 37°C for 4 hrs before serial dilution and plating onto Sabouraud agar plates.

1.6. Complement C3 binding assay

Pooled human serum was obtained from normal human volunteers and stored in single use aliquots at -70°C. Binding to *C. albicans* after incubation in human serum were measured by means of a well described flow cytometry assay (Brown et al. 2002). Briefly, $1x10^6$ CFU of *C. albicans* were incubated in 100 μl of 10% human serum for 30min, before addition of 1 in 300 of fluorescein

isothiocyanate (FITC)-conjugated polyclonal anti-human C3 antibody (MP Biomedicals), fixing in 3% formalin and analysis by flow cytometry. Markers for identifying positive cells were set using *C. albicans* incubated in PBS and then secondary antibody. Results of the assay are presented as a fluorescence index (FI, percentage of positive bacteria multiplied by the geometric mean MFI of C3 binding) in arbitrary units.

1.7. AFM data analysis:

Images and resulting force curves were analysed using the JPK Data Processing Software v.5.1.8 (JPK Instruments, Germany). 3D reconstruction images were obtained from corresponding AFM height scans. Maximum adhesion force, expressed in nN, was determined as the lowest negative value during the retraction phase; and energy of adhesion, expressed in aJ, was obtained by integrating the area under the retraction curve. Data was graphed as mean±SE and significance was determined with the Mann-Whitney test ($p<0.05$).

Results and Discussion

- **Imaging of *C. albicans* immobilised to biopolymer surfaces**

Initial characterisation of surface-bound *C. albicans* was carried out in order to check the effectiveness of PLL and poly-DOPA as immobilisation agents for SCFS experiments. Overall, PLL was only found to be effective for immobilising yeast cells for liquid imaging, as hyphae had a tendency to detach from the surface during scanning. Poly-DOPA was the selected agent for the attachment of hyphae as it was able to maintain stability by resisting the lateral shear forces generated by the AFM tip during scanning. SEM images were also obtained for strain characterisation and to confirm hyphal differentiation, which was effectively obtained by incubating *C. albicans* for 3 hrs in BHI media **(Supplementary Figure 2.a and 2.d)**. 3 hrs was found to be the optimal incubation time to obtain hyphae for AFM experiments, as increased incubation periods lead to clustering and difficulty of obtaining single-hyphae.

AFM intermittent contact imaging allowed for high-resolution imaging of *C. albicans* yeast cells, pseudohyphae and hyphae in buffer **(Supplementary Figure 2.b and 2.e)**. Thus, it was possible to obtain images with no previous sample preparation (i.e. fixation, dehydration), and therefore minimise sample alteration. It is also possible to obtain high-resolution images of budding scars at higher magnifications, in which a central elevation of the cell wall can be observed surrounded by a ring-like structure **(Supplementary Figure 2.c)**. Budding scars were only observed on the surface of *C. albicans* yeast cells and pseudohyphae, and absent in hyphae. All morphologies of *C. albicans* observed

with both SEM and AFM were consistent with previous observations reported in literature (Formosa and Dague 2015; Salvatori et al. 2016). Finally, it is important to mention that the AFM/optical microscopy setup was efficient in allowing correct positioning of the imaging probe atop cells and hyphae of interest.

- **PMMA-*C. albicans* interaction is morphology dependent**

SCFS of living *C. albicans* in buffer was possible by immobilisation to PLL and poly-DOPA surfaces **(Figure 1)**. Cells and hyphae remained immobilised throughout measurements, and detachment from surfaces was rarely observed. Furthermore, our approach allowed for controlled positioning of the PMMA microsphere atop yeast cells and hyphae (both budding cell and tube). This positioning was consistent for different cells, and therefore reproducible results were observed across independent cells and hyphae (n=7).

In the case of yeast cells, the mean adhesion forces between *C. albicans* C1 and PMMA were found to be 0.12±0.0 nN, 0.24±0.0 nN, 0.43±0.0 nN, 0.45±0.0 nN and 0.29±0.0 nN for 0, 1, 5, 10 and 30 s respectively. A similar time-dependent increase was also found for adhesion energy. Interestingly, adhesion forces between PMMA and hyphal tubes were found to be increased at every time point compared to yeast cells, with values of 0.34±0.0 nN, 1.68±0.1 nN, 2.47±0.1 nN, 3.71±0.2 nN and 6.09±0.4 nN for 0, 1, 5, 10 and 30 s respectively. Thus, PMMA-hyphae adhesion was found to be increased by an order of magnitude compared to the PMMA-yeast cell forces. At a maximum contact time of 30 s, adhesion forces between PMMA-hyphae are nearly twenty times higher than those of PMMA-yeast cell. However, the hyphal head was found to have

adhesion forces of ≤0.05±0.0 nN (50 pN). As these force values are in the range of the system detection limit (20-40 pN), adhesion between PMMA and this portion of the hyphae can be considered non-existent. This is similar to what was described previously in the case of mother budding yeast cells, which were also found to be non-adherent (Formosa et al. 2015b), and demonstrates the plasticity of *C. albicans* adhesin expression. The same morphology-dependent adhesion between PMMA and *C. albicans* was also found to be present in the ATCC 10231 strain; however, forces between PMMA-hyphae were only four times higher than between PMMA-yeast cells at 30 s contact times (**Figure 2**). Also, increasing the contact time was found to have a greater influence in the case of hyphae, which suggests the presence of increased numbers of surface adhesins available for interacting with biomaterial surfaces (Beaussart et al. 2012; de Groot et al. 2013).

Candidal hyphal differentiation is a key factor in pathogenicity as it promotes adhesion and tissue penetration (Naglik et al. 2014). Our results support these previous observations, as it was observed that adhesion forces between PMMA and the tube portions of *C. albicans* hyphae were significantly increased compared to yeast cells (**Figure 2**). This supports the notion that polarised growth observed in *C. albicans* hyphae is indeed a mechanism to increase adhesion and penetration to host tissues (Brand 2012), and suggests that hyphae formation plays an important role in the attachment of this strain onto acrylic surfaces. It also appears that adhesion within the hyphae is morphology-selective, as the budding cell portion is non-adhesive, displaying attachment

forces that are even lower than the ones observed for yeast cells. This observation suggests that, although adhesion on the hyphal tube is significantly increased, adhesin expression is 'shut off' in the hyphal head. Thus, it remains possible that the hyphal head plays a minor role in surface adhesion and tissue invasion, and mostly serves as a starting point for the hyphae to begin its growth. Also, similar to what has been observed in bacterial SCFS experiments (Aguayo et al. 2015a), adhesion was increased at higher contact times between *C. albicans* and PMMA. This effect was much more pronounced in the clinical strain, where increasing contact time from 0 s to 30 s raised adhesion to PMMA by 20-fold **(Figure 3)**. This effect could be due to either a time-dependent cell wall-surface contact area, or adhesin bond strengthening after the initial surface-receptor coupling has occurred (Ovchinnikova et al. 2012). These results also demonstrate the ability of *C. albicans* to rapidly attach to PMMA surfaces after only a few seconds of contact.

- **Adhesion of *C. albicans* to PMMA is strain specific and suggests correlation to strain virulence**

To compare the adhesion forces between strains displaying varying degrees of virulence, SCFS experiments were carried out by using *C. albicans* ATCC 10231 and *C. albicans* C1 strains. Interestingly, adhesion forces and energy were increased in the clinical strain for both yeast cells and hyphae **(Figure 3)**. In the case of hyphal tubes, adhesion between PMMA and the clinical strain was found to be ~10 times higher compared to *C. albicans* ATCC 10231. To establish

infection, pathogens including *C. albicans* need to be able to replicate under physiological conditions found within the host and evade local innate immune mechanisms, which may include opsonisation with complement. Compatible with their differences in virulence we found that *C. albicans* C1 demonstrated increased survival in blood and reduced sensitivity to opsonisation with complement compared to *C. albicans* ATCC 10231 **(Figure 4)**. Hence increased adhesion observed for C1 compared to ATCC 10231 was associated with additional evidence of strain-dependent differences in candida / host interactions that may affect virulence. These results suggest that *C. albicans* pathogenicity could be linked to its ability to adhere to and colonise biomaterial surfaces at the single-cell level (Mayer et al. 2013). In the case of candida infection, increased adhesion capabilities at early time points could favour the formation of biofilm on the surface of biomaterials, and the capacity to successfully grow and evade complement mediated immunity could be additional crucial factors for creating chronic low grade infection and inflammation as observed in patients with denture-related stomatitis (Shirley et al. 2015).

The present AFM-based approach to obtain PMMA-modified probes allowed correct positioning of the bead on the end of the tipless cantilever in a highly reproducible manner **(Supplementary figure 1)**. Therefore, it was possible to approach the microsphere onto the *C. albicans* surface with great precision **(Figure 1)**. In the case of hyphae, this approach allowed specific positioning of PMMA onto each morphological region (hyphal head and tube) to obtain adhesion forces in the nano- and pico- newton range. It is important to address

that experiments were performed in the absence of saliva; and therefore the effect of salivary pellicle on the adhesion of *C. albicans* to acrylics has not been considered in this study. Although this work is centred on PMMA as an important surface colonised by *C. albicans*, further studies should focus on unravelling the interactions between this pathogen and other materials and biological surfaces of interest in the oral cavity, as well as aiding in the search for novel anti-adhesive and antifungal molecules.

Conclusion:

The use of PMMA microspheres to selectively probe the adhesion of polymorphic *C. albicans* has been employed here for the first time to characterise fungal attachment to biomaterial surfaces at the nano-Newton level. Specific positioning of the probe was crucial to understand the adhesion forces driving attachment of PMMA to each region of *C. albicans.* Overall, PMMA adhesion was found to be higher for the tube region of hyphae, compared to yeast cells where forces were decreased by an order of magnitude. Also, the budding cell region of hyphae was found to be non-adherent at all studied contact times. Furthermore, adhesion forces between PMMA and the clinical strain of *C. albicans* were significantly increased compared to the lab strain, suggesting that adhesion may indicate differences in surface/host interactions that are perhaps more likely to result in increased pathogenicity and virulence. Overall, we believe this customised AFM cantilever approach is an effective method to study attachment between *C. albicans* and biomaterial surfaces with high morphological precision, and could potentially be utilised with different materials and fungal strains to support the development of strategies that can prevent or treat this common clinically relevant disorder.

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Figure Legends

Figure 1: Overview of the single-cell force spectroscopy (SCFS) setup for studying adhesion between a poly (methyl methacrylate) (PMMA) functionalised AFM probe and living *C. albicans* cells. (A) Diagrammatic representation of the setup used to study adhesion between a PMMA-functionalised cantilever and *C. albicans* yeast cells, germinating yeast and hyphal tubes in buffer. Cells were immobilised onto glass substrates by covalent binding to poly-L-lysine (PLL) in the case of yeast cells or poly-dopamine (DOPA) for hyphae. (B), (C) and (D) are optical images depicting probe positioning atop *C. albicans* yeast cells, germinating yeast and hyphal tubes, respectively (scale bar 10μm).

Figure 2: Nanoadhesion of *C. albicans* C1 to PMMA is morphology dependent. (A) Adhesion forces and (B) energy of adhesion between PMMA-functionalised AFM probes and yeast cells, hyphal head and hyphal tubes (n=7 yeast cells and hyphae). Increased adhesion was observed in hyphal tubes compared to yeast cells, whilst the germinating portion of the hyphae was non-adherent. Significant differences were observed for both parameters (mean ± SEM; $p<0.05$, Kruskall-wallis).

Figure 3: Comparison between the adhesion of *C. albicans* 10231 and *C. albicans* C1 to PMMA-functionalised probes at increasing contact times (0-30s). Overall, the clinical *C. albicans* strain demonstrated increased adhesion forces to PMMA compared to 10231 ($p<0.05$, Kruskall-wallis). The hyphal head was also non-adherent for *C. albicans* 10231.

Figure 4: *C. albicans* growth in blood and C3 complement binding assay demonstrates phenotype likely to be associated with increased virulence for the clinical strain. (A) Incubation in human blood for 4hrs showed increased survival of *C. albicans* C1 compared to 10231. Bars represent percent change in the number of CFU/ml^{-1} in relation to the inoculum and error bars shown represent SEM. Survival of less than 100 represents bacterial killing, whereas % survival is greater than 100, this represents bacterial growth (B) A decreased degree of opsonisation was found in *C. albicans* C1, suggesting improved immune evasion properties compared to 10231. (C) Corresponding FIT-C histograms for both *C. albicans* C1 and 10231, respectively. PBS control curves are shown in red (*$p<0.05$, t-test).