Nanoimprinting of biomedical polymers reduces candidal physical adhesion

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Running title: Nanoimprinting reduces yeast adhesion

Abstract word count: 80 words

Manuscript words: 1512

References: 25

Number of Figures: 4

Number of Tables: 0

Supplementary: 0

Disclosures: Gordon Ramage has received research funding and participated in advisory boards for GlaxoSmithKline Oral Healthcare, and has acted as a consultant for Gilead Sciences.

Conflicts: There are no conflicts of interest

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Abstract

Management of fungal biofilms represents a significant challenge to healthcare. As a preventive approach, minimising adhesion between indwelling medical devices and microorganisms would be an important step forward. This study investigated the anti-fouling capacity of engineered nanoscale topographies to the pathogenic yeast *Candida albicans*. Highly ordered arrays of nano-pit topographies were shown to significantly reduce the physical adherence capacity of *C. albicans*. This study shows a potential of nanoscale patterns to inhibit and prevent pathogenic biofilm formation on biomedical substrates.

Key words: Nanotopography, *Candida albicans,* adhesion, polymethyl-methacrylate, polycarbonate.

Introduction

Fungal infections affect more than a billion people, resulting in approximately 11.5 million life-threatening infections,[1] and while there has been significant strides made in tackling these infections, the worldwide impact of these measures has yet to be realized. One of the most important fungi worth considering is the opportunistically pathogenic yeast *Candida albicans*, which displays an arsenal of virulence factors.[2] Adhesion of *C. albicans* to surfaces and its capacity for morphological transition from yeast cells into filamentous biofilms is arguably the most virulent attribute.[3] This represents a highly resistant and pathogenic state that is associated with enhanced mortality in systemic catheter-related bloodstream infection and severe inflammation of the mucosa.[4,5] Many biomedical substrates often contain surface topography that beneficially enhances microbial adhesion, due to macro- and microscopic surface properties and irregularities.[6] The translational possibilities obtained from our understanding of the anti-fouling and self-cleaning natural systems reveal the intrinsic importance of surface topography at micro-/nanoscale and their interaction with microorganisms.[7] Therefore, preventing initial adhesion through nanoscale modification of the physical substrate is an elegant strategy realised both through these natural observations and innovative studies showing the impact of nanotopographical surfaces on cellular adhesion properties.[8, 9] Micro/nano patterned topographies have been used in a biomedical context to effectively minimise bacterial surface fouling,[7, 10-13] though to date only one single study has actively reported adhesion of *C. albicans* to irregularly spaced micro-topography, with mixed outcomes.[14] Here

we report for the first time the successful application of nano-topography as an anti-adhesion surface against *C. albicans*

Material and Methods

Fabrication of substrates

Nanopatterned master substrates were prepared by electron beam lithography.[15] In brief, silicon substrates were coated with PMMA and exposed in an electron beam lithography tool (Vistec VB6 UHRWF). After development, the substrates were electroplated to form nickel shims.[16] These shims were used for injection moulding of polycarbonate (Makrolon OD2015) substrates.[17] Alternatively, heat cure polymethylmethacrylate (PMMA) denture material (Chaperlin and Jacobs Ltd, UK) nano-pit topographies were replicated using the dental compression moulding technique. In polycarbonate substrates, three different arrangement forms of nano-pit arrays were fabricated. These arrays were square (SQ), near square (NSQ50) and hexagonal (HEX). The pits were of 120 nm diameter, 100 nm depth and 300 nm pitch (pit centre to pit centre) with an offset of ±50 nm for the NSQ50 topography. For PMMA, only the SQ arrangement form was fabricated. For both materials, flat surfaces were used as controls.

Candida albicans adhesion

C. albicans SC5314 was propagated in yeast-peptone-dextrose medium (Sigma-Aldrich) for 18 h at 30˚C in an orbital shaker at 150 rpm. The cells were washed in sterile phosphate buffered saline (PBS, Sigma-Aldrich, UK) and standardised to an inoculum density of 1×10^6 CFU/ml in RPMI-1640 medium

(Sigma-Aldrich, UK). The nano-imprinted sections were distributed in the appropriate wells plates (Costar, Corning Incorporated, USA), and inoculated with cells allowed to adhere for 30 and 90 min at 37ºC. These sections were then washed with PBS and retained cells removed through sonication at 35 kHz for 10 min (Ultrasonic bath, Fisher scientific, UK), followed by 15 sec vortexing. DNA and RNA was extracted using a combination of mechanical (disruption with 0.5mm glass beads) and chemical methods (TRIzol™, Invitrogen, Paisley, UK).[20] The adherent cells were quantified using qPCR through amplification of the *Candida*-specific 18S DNA.[5] The expression of specific *C. albicans* adhesion genes (*ALS1, ALS3* and *EAP1*) was also investigated at each exprimental parameter using real-time qPCR.[20]

Surface properties

Non-contact 3D optical profiling was performed for the flat sections of both materials using ContourGT-X 3D optical profiler (Bruker, UK). The images were corrected to a line-wise plane and the average of the surface roughness (R_a) was calculated from 180×180 µm acquired images via Vision64™ software. Static water contact angle (WCA) were obtained for the patterned and flat sections using a Theta optical tensiometer (Biolin Scientific, Sweden).

Statistical analysis

Unpaired t-test was used to compare the data of the flat and patterned sections. Normal distribution analysis was taken into consideration and log transformation was undertaken in need. Statistical strength is represented: *= $p<0.05$, **=$p<0.01$ and ***= $p<0.001$.

Results and discussion

Fungal adhesion and subsequent biofilm formation is a significant clinical problem.[4] Discovering new strategies to manipulate these adhesive properties are of immense significance. This study focussed on the influence of nanotopographies on adhesion of yeast (Y) and germlings (G) cells (Figure 1A). Assessment of these two morphologies adhering to SQ, NSQ and HEX nanopits on polycarbonate was quantified (Figure 1B), where it was shown that the highly ordered SQ arrangement significantly reduced adhesion of both Y (Figure 1C) and G cells (Figure 1D). Conversely, NSQ and HEX had no significant anti-adhesion properties for either cellular morphology. In parallel, expression profiles of key adhesins were assessed, where it was shown that all three adhesion-related genes (*ALS1, ALS3* and *EAP1)* were down-regulated in the nanotopographies in comparison to the flat topographies in both Y and G cells, but notably for the SQ arrangement *ALS1* was significantly ($p<0.05$) down-regulated in both cell types (Figure 1E). Analysis of gene expression profiles yielded interesting observations in that the substrate differentially impacted the expression of key adhesins, suggesting that the biophysical properties of the nanopits were sufficient to overcome molecular interactions between *C. albicans* morphological types, their adhesins, and the surface.

Based on these data, we developed nanoimprinted (SQ form arrangement) denture acrylic resin material (PMMA) using the dental compression moulding. The validity of this approach was investigated by scanning electron microscopy

(SEM) (Figure 2A), where the presence of imprinted nanopits was observed regularly spaced. As with polycarbonate surfaces, we demonstrated a significant reduction of adherence of both Y and G cells ($p<0.05$) on PMMA (Figure 2B & C). Analysis of the adhesion expression profiles showed no difference between the control and SQ surface (Figure 2D). The difference in the gene expression profile of the materials tested could be attributed to their surface properties. We propose that reduction of adhesion was primarily driven by physical mechanisms, i.e. lack of available surface area for adhesins to attach, which is permitted through these regular nanopits. Indeed, physicochemical interactions are triggered by the attachment of fungi to surfaces, and this contact sensing interaction can be attributed to specific mechanisms allowing *C. albicans* to discriminatively respond to different topographies.[21] Furthermore, "attachment point" theory supports the physical impact of the nanopit topography on the capacity of *C. albicans* adhesion.[22, 23] This theory supposes that the microorganism has a stronger attachment if it is smaller than the topography feature and vice versa.

Finally, we explored the physical properties of the two materials with respect to surface roughness and wettability. A significant difference was observed between the R_a of the flat PMMA denture material (R_a 1549 nm) and that of the flat polycarbonate (R_a 4.1 nm) ($p<0.001$) (Figure 3A), while nanopits R_a was not assessed as we already know these have a 100nm depth. Translationally, using nanopit topography with PMMA has its benefits over flat surfaces, as its associated large R_a becomes negligible when nanoimprinted.

When the WCA was assessed for the nanopit SQ imprinted materials a significant difference was observed in the patterned topographies relative to the flat ($p<0.05$). A higher WCA was also observed in the polycarbonate material relative to the PMMA denture material in both flat and patterned topographies ($p<0.01$). These data may explain the observed differences in gene expression between polycarbonate and PMMA, especially when know that Y and G are hydrophilic and hydrophobic, respectively.[24] These physicochemical interactions may selectively induce stress and differential induction of adhesins.[3] Indeed, hydrophobic microbes preferentially adhere to hydrophobic surfaces and the hydrophilic microbes adhere to hydrophilic surfaces.[25] Figure 4 integrates these concepts, illustrating the interaction between yeast cells and materials that may influence the expression profile of control surfaces.

Here we show for the first time that regularly spaced nanopits with SQ form arrangement is capable of significantly affecting the adhesive capacity of the pathogenic yeast *C. albicans* in both Y and G morphologies. This is in contrast to previous work on titanium micro-pits that showed no quantitative differences for *C. albicans*.[14] In the production of dentures this has immense potential value where the denture-induced stomatitis affecting millions of denture wearing individuals globally, a disease of candidal aetiology, though overcoming the obstacles to manufacturer these will prove challenging.

Figure 1. Quantification of adhered *Candida albicans* to polycarbonate nanotopographies. SEM micrograph of *C. albicans* Y and G morphological forms, scale bar is 2 and 5µm, respectively **(A)**. SEM micrograph of nanotopographical arrangement forms **(B)**. Quantification of adherent colony forming equivalents (CFEs) of yeast **(C)** and germlings **(D)**, and adhesion profiles **(E)**. *p<0.05.

Figure 2. Quantification of adhered *Candida albicans* to PMMA nanotopographies. SEM micrograph of replicated SQ nano-pit topography (scale bar is 200 nm) **(A)**. Quantification of adherent colony forming equivalents (CFEs) of yeast **(B)** and germlings **(C)**, and adhesion profiles **(D)**. *p<0.05.

Figure 3. Surface properties of polycarbonate and PMMA topographies. **(A)** Surface roughness of flat surfaces with ContourGT-X 3D microscopic images. **(B)** Static WCA of all investigated materials.

Figure 4. Schematic representation of adhesion of Y and H cells on hydrophobic and hydrophilic surfaces. Yellow circles represent adhesins.

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