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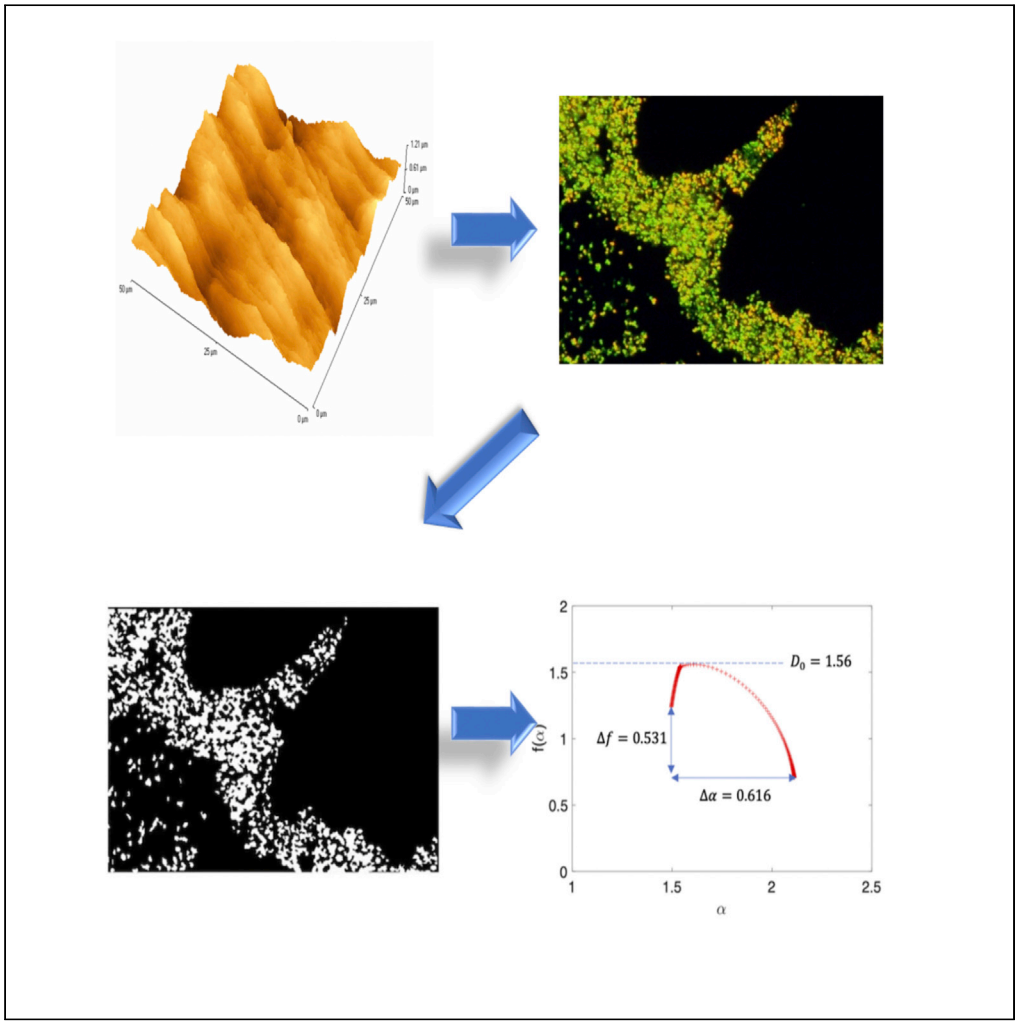
Diverse surface properties reveal that substratum roughness affects fungal spore binding



Kathryn A. Whitehead, Christopher M. Liauw, Stephen Lynch, ..., Andrea Preuss, Ted Deisenroth, Joanna Verran

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Highlights

Multifractal analysis gave measures of density, dispersion, and clustering of spores

A. niger 1957 attached in higher numbers to PTFE

A. niger 1988 and *A. pullulans* bound in highest numbers to glass

Substratum surface roughness influenced spore binding

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Diverse surface properties reveal that substratum roughness affects fungal spore binding

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SUMMARY

Binding to surfaces by fungal spores is a prerequisite to biofilm formation. The interactions of polytetrafluoroethylene (PTFE), glass, and silicon with three fungal spores, of differing shapes and sizes (*Aspergillus niger* 1957, *Aspergillus niger* 1988, and *Aureobasidium pullulans*), were investigated. A multifractal analysis was conducted to provide quantitative measures of density, dispersion, and clustering of spores on the surfaces. The PTFE, glass, and silicon surfaces presented a range of surface topographies and wettabilities. PTFE was the roughest and most non-wettable surface, whereas silicon was the opposite in terms of both these aspects. The *A. niger* species were more non-wettable than *A. pullulans*. Overall, *A. niger* 1957 attached in higher numbers to PTFE, whereas *A. niger* 1988 and *A. pullulans* bound in highest numbers to glass. The results of this work demonstrated that the overall substratum surface roughness influenced spore binding rather than the physicochemical or chemical properties of surfaces or spores.

INTRODUCTION

Surface biodeterioration is a common issue affecting diverse sectors, from domestic bath sealants and shower curtains to industrial cooling and food processing systems. A number of surfaces are particularly vulnerable to the undesirable effects of fungal spore colonization (Whitehead et al., 2011). The adhesion of fungal spores to surfaces occurs readily and is a pre-requisite to biofilm formation, which can result in undesirable effects on surface properties, including discoloration and/or biodeterioration (Liauw et al., 2020). The biodeterioration of surfaces is a serious problem that occurs in many environments, and is particularly prevalent on polymeric materials; on interior and exterior surfaces, including paint surfaces (Whitehead et al., 2011); as well as on books and paper (Magaudda, 2004), stone (Warscheid and Braams, 2000), textiles (Purwar and Joshi, 2004), window glass (Greenberg and Steffek, 2005), stone monuments (Scheerer et al., 2009), and synthetic polymers (Barratt et al., 2003; Cosgrove et al., 2007; Cappitelli and Sorlini, 2008; Whitehead et al., 2011). In addition, based on their native properties and due to fungal spore attachment, coatings developed with the intention of decreasing biofouling of surfaces often fail to perform as expected (Ma et al., 2008).

In the environment, biofilms are known to be composed of multispecies microorganisms, and these may include algal, fungal, and bacterial species. However, fungi are known to be instrumental in biofilm formation and polymer biodeterioration. Among the microbial species of biofilms associated with polymer degradation, one prevalent fungal species was shown to be *Aspergillus niger* (Pathak and Navneet, 2017). *Aureobasidium pullulans* is another fungal species that has been found to be the principal colonizing fungus on polyvinyl chloride surfaces (Webb et al., 2000). Hence the focus of this article was on the key microorganisms involved in polymer biodeterioration and biofilm formation, that is, fungal spore binding.

Spores are propagative microbial vectors that can survive, multiply, and persist in extreme thermal, chemical, and mechanically disruptive conditions and in physically tough environments (Bowen et al., 2000). The transmission of fungal spores can have detrimental consequences in the medicine, agriculture, and food industry (Clement et al., 1993; Whitehead et al., 2011); thus an enhanced understanding of the interactions involved in spore attachment could lead to the development of novel approaches to reduce spore adherence.

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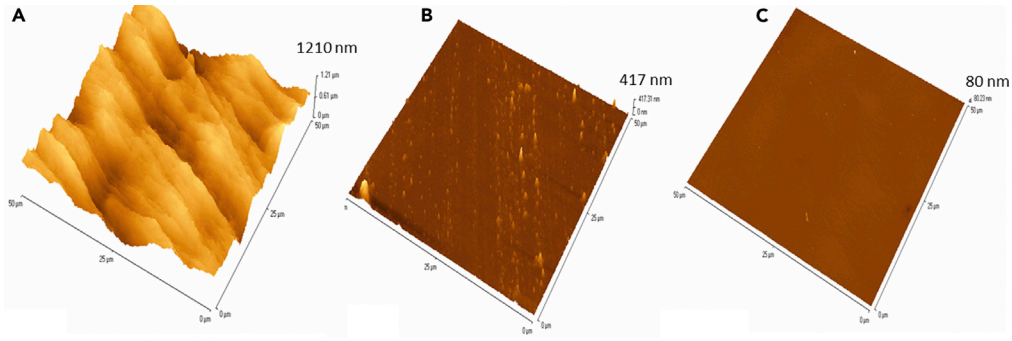

Figure 1. AFM images illustrating surface topography of the three surfaces used in this study.
(A–C) (A) PTFE, (B) glass, and (C) silicon (n = 9).

A. niger is one of the key microbes used in biotechnology (Whitehead et al., 2011). *A. niger* has been the subject of research and industrial use for several decades and has already been in use to produce extracellular (food) enzymes and citric acid in the industry (Frisvad et al., 2018). *A. niger* is a filamentous fungus that grows under aerobic conditions on organic matter (Anderson and Smith, 1971; Liauw et al., 2020). In nature, it is found in soil and litter, in compost, and on decaying plant material in biofilm formations. *A. niger* spores are unicellular and hydrophobic (non-wettable) (Bowen et al., 2000) but can vary noticeably in shape. *A. pullulans* is a yeast-like fungus that is abundant in the environment and colonizes several habitats, usually in multispecies biofilms (Cooke, 1962, 1987), particularly coated wood surfaces (Eveleigh, 1961; Webb et al., 1999). It was observed to be the main fungus that causes biodeterioration of polyvinyl chloride films during outdoor trials in Florida, USA (Hamilton, 1983). Owing to the abundance of melanin in its cell wall, *A. pullulans* colonies are generally black pigmented (Pouliot et al., 2005). The presence of melanin represents a physical protective barrier to the organism and aids its adhesion to surfaces (Pouliot et al., 2005).

Substratum and microbial surface hydrophobicity is as an important feature that enhances the capacity of microorganisms to attach to surfaces (Rosenberg and Kjelleberg, 1986). The initial adhesion of a microorganism to a surface may be more enhanced by the chemical properties and interactions between the spore and the surface, whereas subsequent retention may be most affected by topographical features, which could augment or reduce microbial retention (Verran and Whitehead, 2006; Whitehead et al., 2011). However, the effect of surface properties on the attachment, adhesion, and retention of fungal spores to surfaces is conflicting and this may be due to, in part, the degree in variance in the surface properties tested.

Difficulty in determining the distribution and pattern of fungal spores across the surfaces is an issue, because the underlying surface properties will inherently affect spore binding. However, one way to overcome such problems is to use multifractal analysis (MFA), which uses a box counting method to calculate fractal dimensions (Yildiz and Yildiz, 2020). This method enables the distribution and agglomeration of the fungal spores to be calculated, and for quantitative analysis to be used to better describe their distribution and dispersion (Wickens et al., 2014).

The present study aimed to investigate the effect of substratum surface properties, including topography and roughness, on the attachment of the fungal spores of *A. niger* and *A. pullulans* to inert substrata. The fungal species used in the study were chosen based on the noticeable differences in their morphology and hydrophobicity, and on their significant use and importance in biofouling and biodegradation of environmental surfaces.

RESULTS

Surface analysis

Atomic force microscopy was used to determine the surface roughness of test substrata (Figure 1) Polytetrafluoroethylene (PTFE) presented a greater Z height (1,210 nm) compared with the glass (417 nm) or silicon surfaces (80 nm). The surface roughness results were represented by R_a (average value of the mean height derived from a center line) (Figure S1), which correlated with the images in terms of degree of roughness. PTFE had an R_a value of 182.9 nm, whereas the glass (117.8 nm) and silicon (0.6 nm) surfaces had much

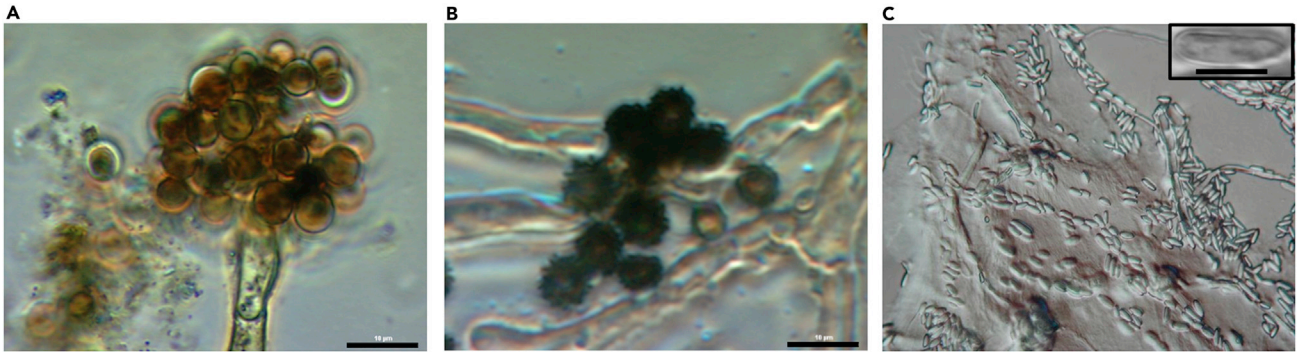

Figure 2. Light microscopic images of fungal spores.
(A–C) (A) *A. niger* 1957, (B) *A. niger* 1988, (C) *A. pullulans*. Scale bars, = 10 μm. Inset in (C) *A. pullulans* spore.

smoother surfaces. There was a significant difference between the roughness of the PTFE surface and the glass or silicon surfaces ($p \leq 0.001$). The surface contact angles followed a trend whereby the rougher PTFE surface demonstrated the highest contact angle (107°) and the least wettable surface, whereas the smoothest surface, the silicon, demonstrated the lowest contact angle (42°) and hence the most wettable surface. There was a significant difference in the wettability between the PTFE and the glass surface and the PTFE and the silicon surface ($p \leq 0.001$) (Figure S2).

Wettability and chemistry of the spores

The morphology of the spores was investigated using light microscopy. The *A. niger* 1957 spore was found to be spherical, smooth, and 4–6 μm in diameter (Figure 2A). *A. niger* 1988 spores were also spherical and of a similar size/diameter to spores of *A. niger* 1957, but they presented a spiky surface texture (spike length ~0.5 μm in length) (Figure 2B). The *A. pullulans* spores were elliptical varying from 5–12 μm in length by 2–3 μm width and were transparent and colorless. They presented a smooth edge, with a smooth, defined outer spore surface (Figure 2C).

The wettability of the spores was determined using the salt aggregation assay. Interestingly, both *A. niger* spp. demonstrated similar wettability profiles (0.06) but were less wettable compared with *A. pullulans* (0.12) (Figure 3). There was only a significant difference in the results between the hydrophobicity of the *A. niger* 1988 and the *A. pullulans* spores ($p < 0.05$).

Diffuse Reflectance for Infrared Fourier Transform Spectroscopy (DRIFTS) spectra for the three fungi demonstrated that all the spectra had the following common features: broad hydrogen bonded OH stretching bands (3,800 cm^{-1}–2,300 cm^{-1}), C-H stretching vibrations (centered at 2,900 cm^{-1}), ester or other carbonyl bands (shoulder at 1,730–1,720 cm^{-1}), amide I and II carbonyl bands (ca. 1,650 cm^{-1} and ca. 1,640 cm^{-1}, respectively), and C-O-C bending vibrations (ca. 1,060 cm^{-1}) (Figure 4). The areas of the OH stretching and CH stretching bands were determined. Owing to the complexity of the fingerprint region, it was not possible to determine the area of the amide I bands; instead the absorbance was measured. The area of the OH stretching band ($A_{O\text{-}H}$) was divided by the absorbance of the amide I band ($Abs_{amide\ I}$) to give $A_{O\text{-}H}/Abs_{amide\ I}$. The area of the O-H stretching band ($A_{O\text{-}H}$) was calculated as follows: $A_{O\text{-}H} = A_{O\text{-}H\ +\ C\text{-}H} - A_{C\text{-}H}$. The area of the CH stretching bands was also divided by the area of the OH stretching bands to give $A_{C\text{-}H}/A_{O\text{-}H}$. These ratios demonstrated that the *A. pullulans* had the most OH groups as it gave the highest $A_{O\text{-}H}/Abs_{amide\ I}$ value and the lowest $A_{C\text{-}H}/A_{O\text{-}H}$ value (Table 1).

Binding of the spores to the surfaces

Attachment, adhesion, and retention assays were carried out to determine the number of spores that bound to the surfaces following the different assays. The images obtained from the assays revealed that the nature of the interaction between the spores and the surfaces altered the pattern of binding following retention assays (Figure 5). For example, *A. niger* 1957, non-wettable spores, were distributed relatively homogeneously across the less wettable surfaces (Figures 5A and 5B respectively) whereas they became grouped in clumps (aggregated) when applied to more wettable surfaces (Figure 5C). However, the spiky *A. niger* 1988, although they had the same surface wettability as the *A. niger* 1957 spores, presented a

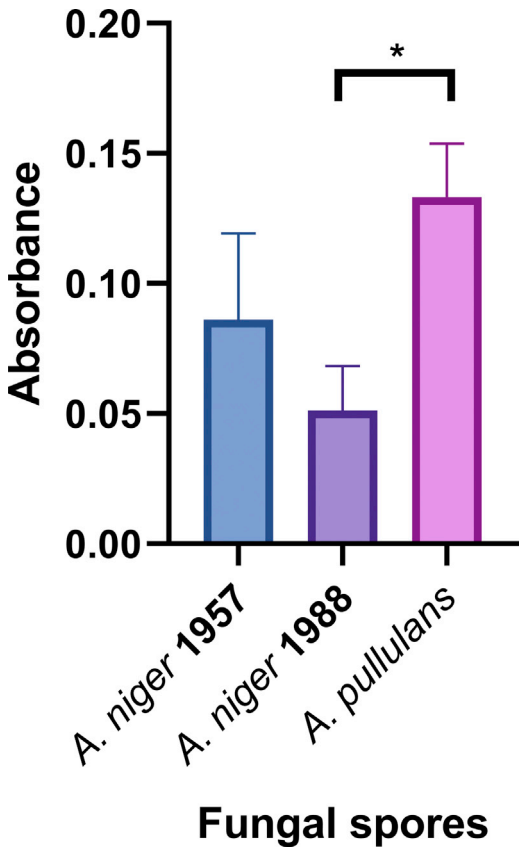

Figure 3. Wettability of fungal spores determined using salt aggregation assay demonstrating a significant difference between the surface wettabilities (n = 15)

Asterisks denote significance, *p ≤ 0.05,.

different pattern of adhesion on the surfaces. The non-wettable *A. niger* 1988 spores bound to the non-wettable surfaces in clumps on the most non-wettable surface (PTFE) (Figure 5D), and less so on the more wettable glass surface (Figure 5E), whereas on the most wettable surface, silicon, the *A. niger* 1988 spores were well distributed across the surface (Figure 5F). The larger and most wettable spores, *A. pullulans*, were arranged into clumps on all three surface types, although larger clumps of spores were observed on the most non-wettable PTFE surfaces (Figure 5G) when compared with the more wettable glass (Figure 5H) and silicon (Figure 5I) surfaces.

Binding of the spores using attachment, adhesion, or retention assays showed that the methodology used to bind the spores to the surfaces also affected the numbers remaining on the surfaces. The lowest numbers of spores were bound overall following the adhesion assay, then the retention assay, when compared with the numbers of spores bound following the attachment assays, presumably due to lack of washing (Figure 6).

Following the attachment assays, it was demonstrated that *A. niger* 1957 was attached in the highest numbers to the PTFE (4.3×10^5 spores cm^2). There was a significant difference in the number of *A. niger* 1957 spores attached to the PTFE compared with the silicon surface ($p \leq 0.0001$) and the number of spores attached to the glass compared with the silicon surface ($p \leq 0.01$) (Figure 6A).

A. niger 1988 was attached to the PTFE and glass surfaces in similar numbers (1.5×10^5 spores cm^2 and 1.7×10^5 spores cm^2, respectively) and there was no significance in the results, although a significant difference was demonstrated for the number of *A. niger* 1988 spores attached on the PTFE and silicon surfaces ($p \leq 0.05$) and the glass and silicon surfaces ($p \leq 0.0001$). *A. pullulans* was attached in the highest numbers to the glass surface (2.5×10^5 spores cm^2), and there was a significant difference between the numbers of spores retained on the PTFE and glass ($p \leq 0.0001$) and the PTFE and silicon

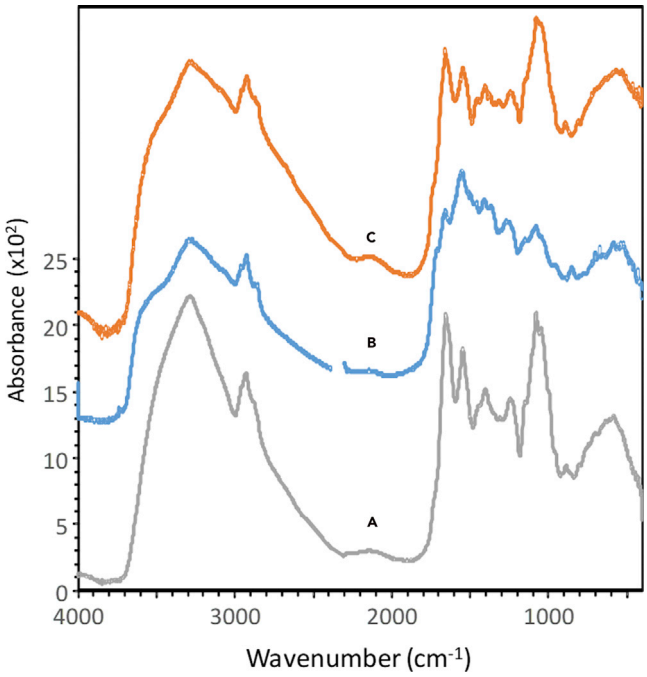

Figure 4. DRIFTS spectra of (a) *A. niger* 1957, (b) *A. niger* 1988, and (c) *A. pullulans* showing the marginally different fingerprint region for *A. niger* 1988.

A. niger 1957 and *A. pullulans* gave similar spectra (n = 5).

surfaces ($p \leq 0.001$). *A. niger* 1957 and *A. niger* 1988 were attached in the lowest numbers to silicon (6.6×10^4 spores cm^2 and 3.1×10^4 spores cm^2, respectively), whereas *A. pullulans* was attached in the lowest numbers to the PFTE surface (5.1×10^4 spores cm^2) (Figure 5A).

Following the adhesion assays, *A. niger* 1957 was adhered in the highest numbers onto the PTFE and glass surfaces (2.3×10^3 spores cm^2 and 2.8×10^3 spores cm^2, respectively), whereas *A. niger* 1988 and *A. pullulans* were adhered in the highest numbers to the glass surface (1.5×10^3 spores cm^2 and 1.2×10^4 spores cm^2, respectively) (Figure 6B). *A. niger* 1957 was adhered in the lowest numbers to the silicon surfaces (1.0×10^3 spores cm^2), whereas *A. niger* 1988 was adhered in the lowest numbers to the PTFE surface (1.9×10^2 spores cm^2). *A. pullulans* was adhered in the lowest numbers to the silicon surface (9.3×10^1 spores cm^2) (Figure 6B). There was a significant difference in the numbers of adhered *A. pullulans* spores retained between the glass surface and the PTFE or silicon surface ($p \leq 0.001$).

A. niger 1957 was retained in the lowest numbers to the glass surface (2.7×10^3 spores cm^2), and there were significantly more *A. niger* 1957 spores retained on the PTFE surface than the glass surface ($p \leq 0.0001$) and the silicon surface compared with the glass surface ($p \leq 0.05$) (Figure 6C). *A. niger* 1988 was retained in the highest numbers to the glass surface (3.3×10^4 spores cm^2) (significant difference PTFE and silicon [$p \leq 0.0001$], glass and silicon [$p \leq 0.05$]). *A. pullulans* were retained in the highest numbers to the PTFE surface (1.9×10^4 spores cm^2), and there was a significant difference between the numbers of spores retained on the PTFE and silicon surface ($p \leq 0.01$) and the glass and silicon surface ($p \leq 0.05$). Both *A. niger* 1988 and *A. pullulans* were retained in the lowest numbers to the silicon surfaces (2.8×10^2 spores cm^2 and 1.9×10^2 spores cm^2, respectively) (Figure 6C).

Table 1. AO-H/absamide I and AC-H/AO-H values obtained from DRIFTS spectra of the conidia (n = 5)

Conidia type	$A_{O\text{-}H}/Abs_{amide\ I}$	$A_{(C\text{-}H)}/A_{(OH)}$ (x 10^2)
A. niger 1957	750	3.00
A. niger 1988	826	3.03
Aureobasidium pullulans	1,028	2.17

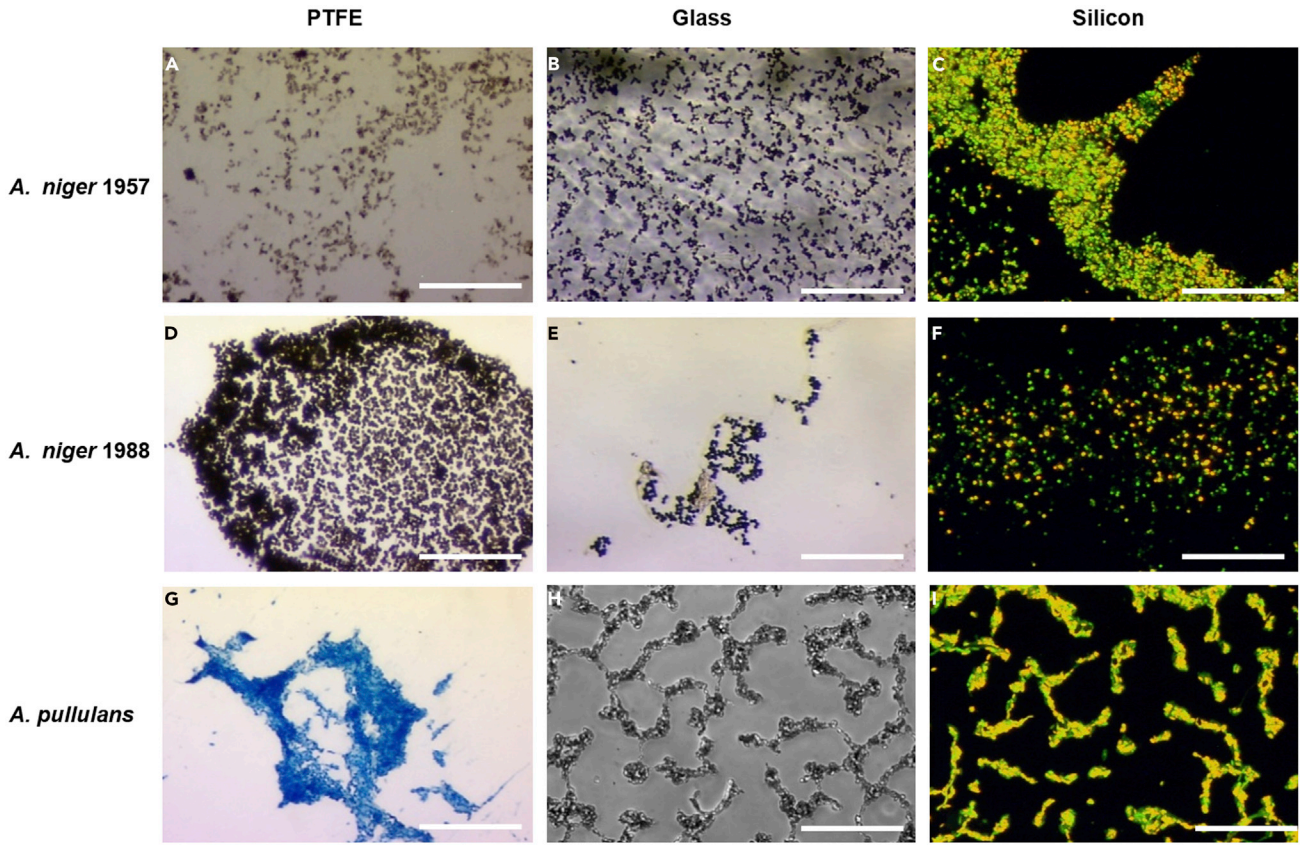

Figure 5. Examples of the different patterns of spore retention on surfaces

A. niger 1957, *A. niger* 1988, and *A. pullulans* on (G) PTFE, (H) glass, (I) silicon. Scale bar, 100 μm.

Multifractal analysis

An MFA was carried out in a manner similar to that adopted in our earlier articles (Wickens et al., 2014; Slate et al., 2020). MFA is typically used to measure the density, dispersion, and clustering of objects on a surface. The theory and applications of MFA can be found in Lynch, (2014, 2018) and Salat et al., 2017. Typical multifractal f-α curves are plotted as in Figure 7. The multifractal objects are created with packages such as MATLAB and Python. The first column shows the motifs for the multifractals, and the second column displays the multifractal images after 8 iterations of the motifs. The motifs are 2×2 matrices; after the first iteration a 4×4 matrix is obtained, and after 8 iterations a 512 × 512 matrix is generated. The images were produced with MATLAB and Python using image processing techniques, where the pixels are on a gray scale—0 would give a black pixel and 1 would give a white pixel on this scale. The contrast feature is used so that a representative image is visible. The final column shows the computed multifractal f-α curves. In each case, the width of the f-α curve is given by $\Delta\alpha$ and gives a measure of dispersion. The asymmetry of the f-α curve is given by Δf; if Δf is negative (as in case (a)), then this implies clustering of dark pixels, and if Δf is positive (as in case (c)), then this implies clustering of bright pixels. In all cases, the pixels cover the whole surface, so in each case $D_0 = 2$ (the dimension of a plane). The multifractal generated using motif (a) is the most dispersed (heterogeneous), and the multifractal generated using motif (b) is the least dispersed, or most homogeneous.

Figure 8 shows binary images of the fungal spores of (a) *A. niger* 1957 on silicon and (b) *A. niger* 1957 on glass. The second column displays the corresponding multifractal f-α curves. Reading the quantitative values, it can be seen that the density (fractal dimension D_0) was higher in case (b) than in case (a). The dispersion ($\Delta\alpha$) was also higher in case (b) than in case (a). Finally, in case (a), $\Delta f > 0$ implies the clustering of fungal spores, whereas in case (b), $\Delta f < 0$ implies the clustering of gaps. The results for all the images where a MFA could be applied are presented in Figure 9. When the images displayed no adhesion or very little adhesion, the MFA was not applied to those images. Although the measures of percentage

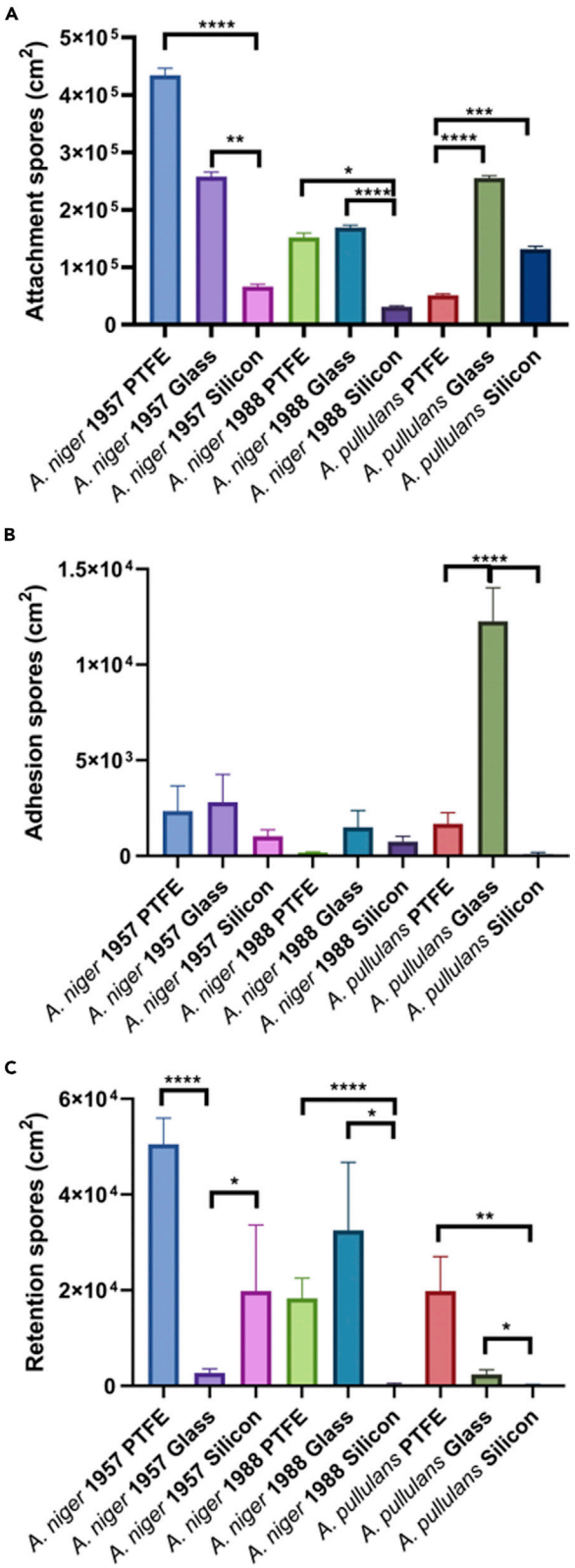
A
Attachment spores (cm²)
5×10⁵
4×10⁵
3×10⁵
2×10⁵
1×10⁵
0
****
**
*
****
***
****
A. niger 1957 PTFE
A. niger 1957 Glass
A. niger 1957 Silicon
A. niger 1988 PTFE
A. niger 1988 Glass
A. niger 1988 Silicon
A. pullulans PTFE
A. pullulans Glass
A. pullulans Silicon
B
Adhesion spores (cm²)
1.5×10⁴
1×10⁴
5×10³
0
****
A. niger 1957 PTFE
A. niger 1957 Glass
A. niger 1957 Silicon
A. niger 1988 PTFE
A. niger 1988 Glass
A. niger 1988 Silicon
A. pullulans PTFE
A. pullulans Glass
A. pullulans Silicon
C
Retention spores (cm²)
6×10⁴
4×10⁴
2×10⁴
0
****
*
****
*
**
*
A. niger 1957 PTFE
A. niger 1957 Glass
A. niger 1957 Silicon
A. niger 1988 PTFE
A. niger 1988 Glass
A. niger 1988 Silicon
A. pullulans PTFE
A. pullulans Glass
A. pullulans Silicon

Figure 6. Number of spores retained following attachment, adhesion, and retention assays

Number of spores retained following attachment, adhesion, and retention assays on PTFE, glass, and silicon surfaces using (A) *A. niger* 1957, (B) *A. niger* 1988, and (C) *A. pullulans*. Asterisks denote significance, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, and ****p ≤ 0.0001. (n = 45)

coverage demonstrate spore adhesion to the surfaces, there was little or no dispersion and the clustering was very small.

To quantify the density, dispersion, and clustering of the spores across the surfaces MFA was carried out on the images (Figure 9). However, only analysis on the surfaces following the attachment and retention assays could be carried out because following the adhesion assays the spores were too widely dispersed across the surfaces. Analysis of the spore density, dispersion, and clustering across the surfaces following the attachment and retention assays demonstrated that the retention assays gave a more diverse set of results.

Following the attachment assays, there was little difference in the density (range *A. niger* 1957 1.56–1.66; *A. niger* 1988 1.44–1.72; *A. pullulans* 1.35–1.71) (Figure 9A) or dispersion (range *A. niger* 1957 0.62–1.01; *A. niger* 1988 0.71–1.02; *A. pullulans* 0.80–1.07) (Figure 9B) of the spores across the surfaces. However, clustering of the spores gave a more diverse effect with the *A. niger* 1988 on the glass surface (−0.43) and the *A. pullulans* (−0.37) on the silicon surface being much less clustered than the spores on the other surfaces (Figure 9C).

The results from the retention assays showed that the density of the *A. niger* 1988 spores on the silicon surface (0.49) and the *A. pullulans* spores on the silicon surface (0.43) demonstrated much lower spore densities across the surfaces than the other spore-surface combinations tested (Figure 9D). The dispersion results were also diverse with the *A. niger* 1957 on the PTFE (0.73), *A. niger* 1988 on the silicon (0.50), and the *A. pullulans* on the glass surface (0.71) demonstrating lower levels of dispersion across the surfaces (Figure 9E). In contrast to the attachment results, the results for the clustering of the spores across the surfaces demonstrated that the clustering of the *A. niger* 1957 spores on the PTFE surface (0.84) and the *A. pullulans* spores on the glass surfaces (1.10) were much higher than the other combinations tested (Figure 9F).

DISCUSSION

It is known that the interplay between the properties of a surface and a fungal spore can influence their binding to a substratum (Whitehead et al., 2011). Furthermore, it has also been demonstrated that the methodology used in such assays can affect the interplay of spore binding, and hence the factors affecting the results (Liauw et al., 2020; Whitehead et al., 2020).

Both the *A. niger* 1957 and *A. pullulans* had smooth surfaces, whereas the *A. niger* 1988 had a spiky surface. Furthermore, both the *A. niger* species demonstrated non-wettable properties, whereas the *A. pullulans* was more wettable. This was confirmed by the DRIFTS results, which demonstrated that the OH groups may be more predominant on the surface, leading to confirmation that *A. pullulans* was the most hydrophilic of the three fungal conidia examined. Aside from the exopolymeric components and biochemical enzymes involved in fungal spore binding, (Epstein and Nicholson, 1997), surface properties also provide a multifactorial aspect of spore binding to surfaces, which is the subject of this article.

In this work, three surfaces with different physicochemical and topographical properties were used in three binding assays to determine the effect of the properties of the fungal spore and the surface on spore attachment. PTFE was the roughest and least wettable surface, whereas silicon was the smoothest and the most wettable surface. However, it was clear that the differences in the surface and spore properties affected the pattern of distribution of the spores across the surfaces.

The type of assay used affected the numbers of spores that bound to the surfaces, with the attachment assays (no wash, therefore maximum opportunity for spores to remain on the surface) demonstrating the least difference between the greatest and the lowest numbers of fungal spores retained on the surfaces (~0.5 logs). However, the greatest difference between the highest and the lowest numbers of spores bound to the surfaces was demonstrated for the retention assays (spores remaining on the surface after immersion following a washing step) (1–2 logs), with the difference in the numbers of spores for the adhesion assays (spores remaining on the surface after an attachment assay) falling between the two (0.5–2 logs).

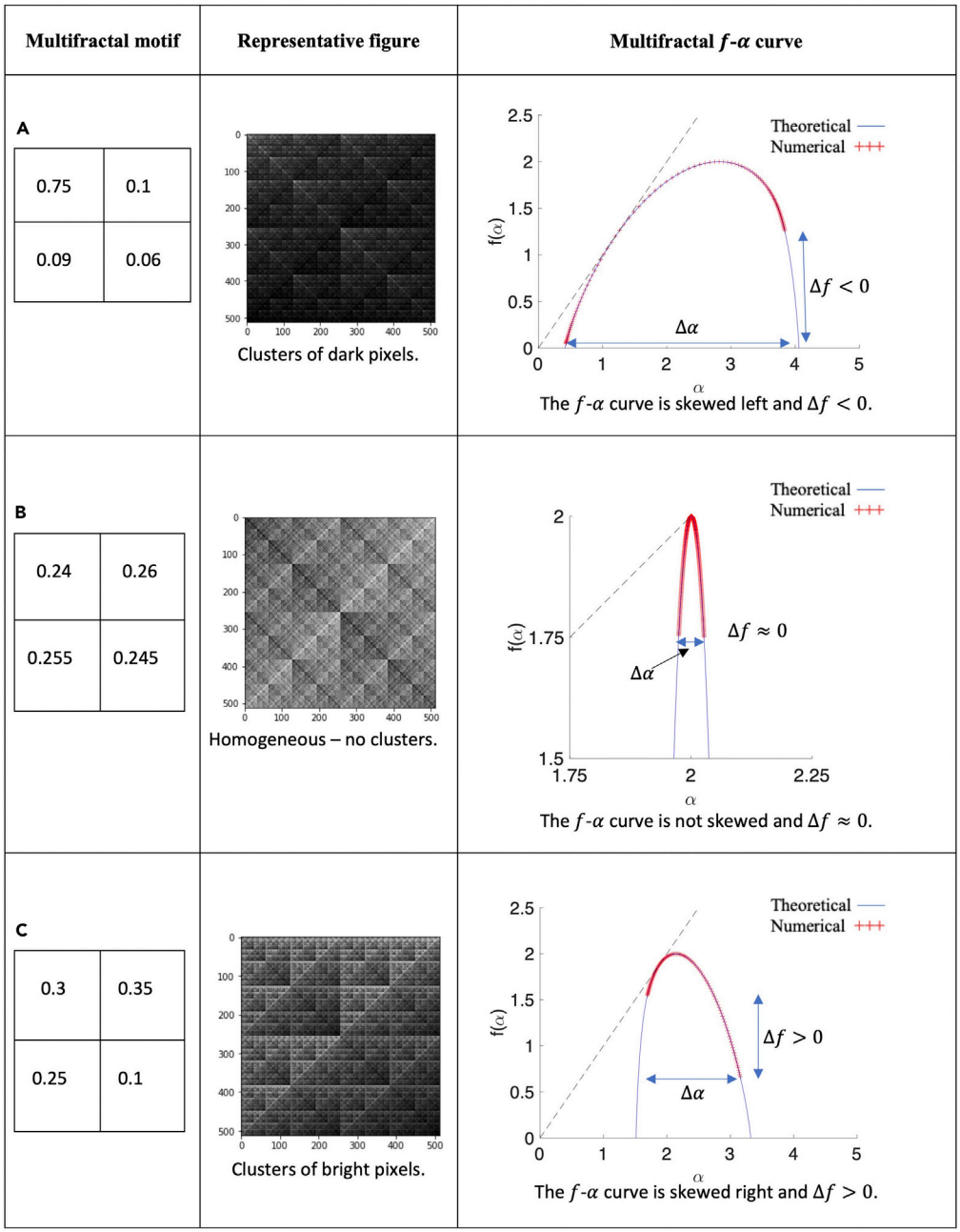

Figure 7. Theoretical and numerical f-α curves for multifractals generated with MATLAB and Python

The theoretical multifractal spectra are plotted as blue curves, and the corresponding numerical f-α curves are plotted using red + signs. Note that in each case the fractal dimension D0 is 2 as the pixels cover the whole plane. (A) Clusters of dark pixels. (B) Homogeneous dispersion. (C) Heterogeneous dispersion.

Following the binding assays, it was clear that different parameters particularly affected the highest and lowest spore binding to the surfaces—perhaps because more extreme surface/spore properties are apparent. The attachment, adhesion, and retention assays demonstrated that all three types of fungal spores bound in the greatest numbers to the glass or PTFE surfaces. This suggests that the surface properties, particularly surface roughness, influenced spore binding to the surfaces rather than the physicochemical or chemical effects of the surfaces or the spores. However, the results for the factors that resulted in the least numbers of spores bound to the surfaces were less obvious. Overall, regardless of the assay or spore type used, the fungal spores attached in the lowest numbers to the smoothest surface, the silicon

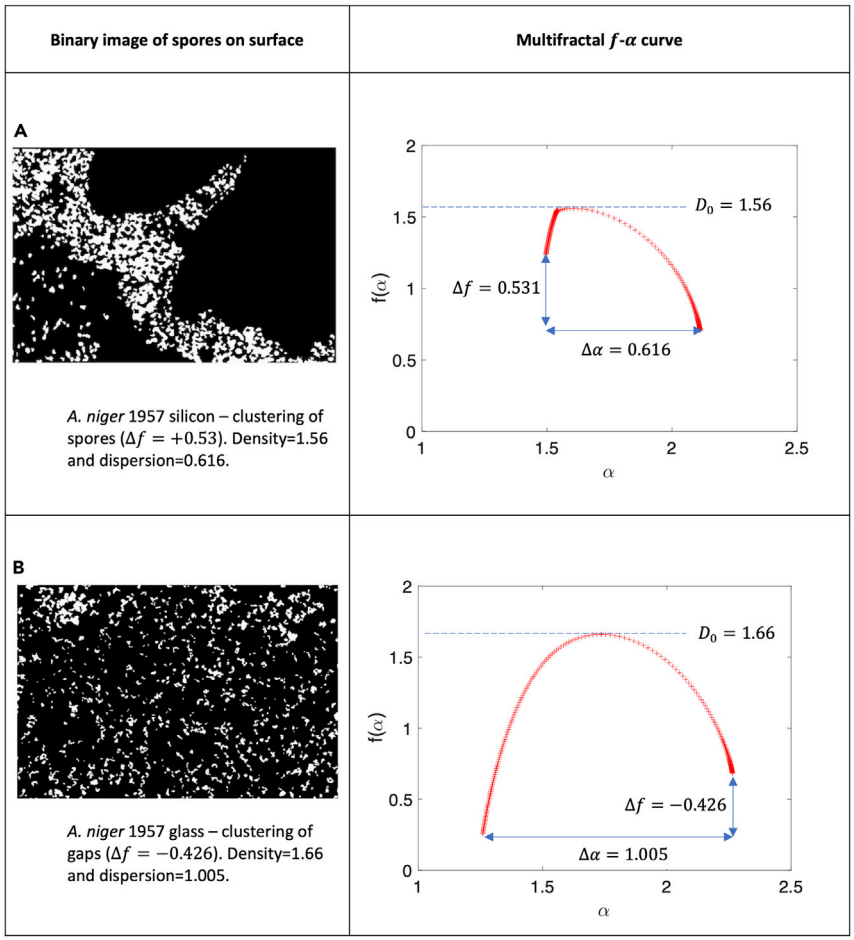

Figure 8. Binary images following multifractal analysis of spores on surfaces

Binary images of (a) *A. niger* 1957 on silicon and (b) *A. niger* 1957 on glass. The second column shows the corresponding multifractal f-α curves for these binary images.

surface, with three exceptions: *A. pullulans* attached in lowest numbers to PTFE, *A. niger* 1988 adhered in lowest numbers to PTFE, and *A. niger* 1957 was retained in lowest numbers to the glass surfaces. This may be explained in part by examining the data in greater detail. For the lowest numbers of bound spores, the attachment assays demonstrated the phenomenon whereby the opposite wettabilities between the spore and the surfaces were attractive, therefore, it might be suggested that the interplay between both the surface and the spore had an effect, in the absence of a washing step. However, following the adhesion assays, the lowest numbers of the *A. niger* 1988 on the PTFE surface may have been influenced by the spiny features on the surfaces of the spore resulting in the lowest adherence to the PTFE surface, maybe due to the spines causing reduced contact area between the spore and the surface. The reason for the lowest spore numbers binding of the *A. niger* 1957 spore to the surface of the glass following the retention assay is unclear. Conflicting results have also been found by others. When the adhesion of the conidia of *Bipolaris sorokiniana* to surfaces was determined, it was demonstrated that spore adhesion occurred with 1 h of hydration (Apoga and Jansson, 2000). However, the attachment of the *Cochliobolus heterostrophus* to the surfaces demonstrated no specific relationship to surface hydrophobicity (Braun and Howard, 1994).

Before biofilm formation occurs, the microorganisms will bind to the surface in a series of steps (Tetlow et al., 2017). The initial step, which is governed by physicochemical forces, is the attachment stage, and microorganisms may be easily removed from the surface at this point. The next step, the adhesion phase, occurs once the microorganisms have gained some tenure to the surface, and they may not be as easily removed from the surface using gentle physical forces. Adhesion may be defined as a specific attachment between a surface receptor and a microbial ligand (Epstein and Nicholson, 1994). Finally, retained

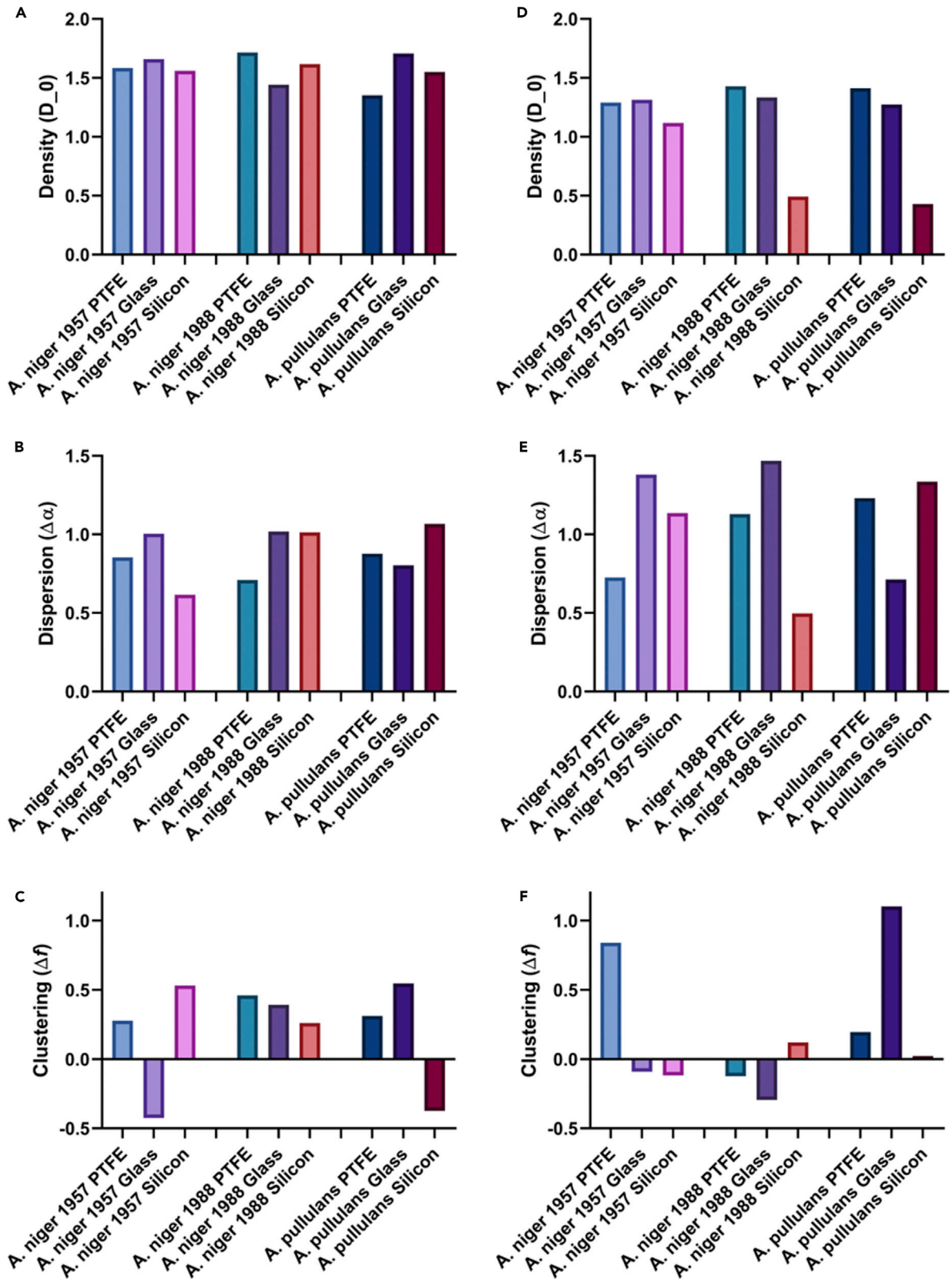

Figure 9. Density, dispersion, and clustering of fungal spores across the surfaces following multifractal analysis evaluation

(A–F) Density, dispersion, and clustering of fungal spores across the surfaces following (A–C) attachment assays and (D–F) retention assays.

microorganisms have had more time to bind to the surface (in this case, 1 h) and will not be removed when a greater physical force is applied, such as a more robust washing step.

MFA can be used to quantify surface features or cells across surfaces (Wickens et al., 2014; Moreira et al., 2017; Yildiz and Yildiz, 2020). Interestingly, analysis using the MFA demonstrated that the inclusion of the washing step immediately after the spores were applied to the surfaces (adhesion assays) resulted in the spores being widely distributed across the surfaces, so that it was not possible mathematically to quantify their density, distribution, or clustering. The results following the retention assays were also more diverse than following the attachment assays, further demonstrating how the methodology can affect the pattern of cell binding across different surfaces. The largest difference in the results was demonstrated by the clustering results whereby, following the attachment assays, the fungal spores were generally more clustered across the surfaces, whereas following the retention assays, the spores were less clustered across the surfaces. This could be an effect of the surface-spore interactions because the retention assays are carried out under a spore suspension in liquid for an hour. The interactions of the water molecules may alter the binding of the spores to the surfaces, resulting in less clustering across the surfaces in many cases. To the authors' knowledge, this is the first time that MFA has been used to quantify fungal spore binding across surfaces. However, previous work using bacteria has demonstrated that different bacterial species bound to surfaces in different patterns. For example, *Staphylococcus aureus* displayed a more heterogeneous cell dispersion than *Staphylococcus epidermidis* (Wickens et al., 2014). This may explain the differences in the results suggesting that when considering microbial binding to a surface each species needs to be considered on an individual basis.

Previous work using MFA to determine the pattern of bacterial binding to metal surfaces demonstrated that the surface properties affected the spread and clustering, but not the density of the bacteria, which may suggest that the surface properties also influence the pattern of spore binding to the surfaces. In agreement with our work, when three very similar surfaces based on a Polymethyl methacrylate (PMMA) moiety were used in fungal binding assays, it was demonstrated that following attachment assays, the most wettable surfaces attached the greatest numbers of conidia (Whitehead et al., 2020). When binding assays were carried out on Polyvinyl acetate (PVAc) and Polyviynl alcohol (PVOH) surfaces that had wider ranging surface topographies, chemistries, and wettabilities than the PMMA surfaces, it was determined that fungal spore binding to the surfaces was influenced by both the physicochemistry and chemistry of the spores and surfaces (Liauw et al., 2020). The results from this work, using three surfaces with distinct properties, demonstrated that surface roughness had the dominating influence on spore binding, rather than the physicochemical or chemical properties of the surfaces or the spores. Thus, it may be concluded that the effect of spore binding is an interplay influenced by the degree of variation in the properties of the surfaces tested and the differences in the microbial species.

Conclusion

The three substrata used in this work presented different surface topographies and wettabilities. The three types of fungal spores used in these assays presented different wettabilities and shapes. This work showed that all spore types bound in the lowest numbers to the smoothest surface, although the results were assay dependent to some extent. MFA enables quantitative measurements of spore binding following attachment and retention assays and demonstrated that following the attachment assays, the fungal spores were generally more clustered across the surfaces, whereas following the retention assays, the spores were less clustered across the surfaces. The leading factor that influenced spore binding seemed to be substratum surface rather than the physicochemical or chemical effects of the surfaces or spores.

Limitations of the study

The findings of this work were limited by using only a limited number of spore types. By using a wider range of spores and investigating their surface properties in more detail, their binding properties may be better understood, and this aspect will be incorporated into our future work.

Resource availability

Lead contact

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Materials availability

This study did not generate new unique reagents.

Data and code availability

Original/source data for the figures and data in the paper is available from the lead contact.

METHODS

All methods can be found in the accompanying transparent methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102333.

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AUTHOR CONTRIBUTIONS

The concept behind the work was developed by A.P., T.D., C.M.L., J.V., and K.A.W. The experimental methodology was developed and carried out by K.A.W. and C.M.L., and the MFA was conducted by S.L. The manuscript was written by M.E.M., S.L., and K.A.W. All the authors were involved in the final proofing of the manuscript.

DECLARATION OF INTERESTS

The authors declare no conflicts of interest.

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Supplemental information

Diverse surface properties reveal that substratum roughness affects fungal spore binding

Kathryn A. Whitehead, Christopher M. Liauw, Stephen Lynch, Mohamed El Mohtadi, Mohsin Amin, Andrea Preuss, Ted Deisenroth, and Joanna Verran

Transparent methods

Surfaces

Polytetrafluoroethylene (PTFE) was purchased from Direct Plastics (Yorkshire, UK) Laboratory Supplies (UK). Glassware was pre-washed with 69% fuming nitric acid prior to a solvent wash, which consisted of soaking the glassware in distilled water, acetone, methanol, ethanol and sterile distilled water for 5 min each. Nitric acid and solvents were purchased from BDH (Basingstoke, UK). Silicon wafers were obtained from Montco Technologies (PA, USA).

Determination of surface roughness and topography

An Atomic Force Microscope (AFM) (Explorer, Veeco, UK), was used to carry out the surface imaging and roughness measurements in air at ambient room temperature. Surface roughness analysis was conducted with a triangular cantilever tip (Veeco, UK) in contact mode with a spring constant of 0.12 N m^{-1} (glass and silicon surfaces) or using a non-contact cantilever in non-contact mode with a spring constant of 50 N m^{-1} (PTFE surfaces). All imaging and data acquisition were collated using NanoScope Software 6.13. The topographical features of the surfaces were determined from three areas taken from three different replicate surfaces (n = 9).

Surface wettability

The sessile drop technique was used to determine the contact angle measurements of surfaces using 5 µL volumes of HPLC grade water (BDH, UK) at room temperature (KRÜSS GMBH, Hamburg, Germany). The wettabilities of the surfaces were determined from five areas taken from three different replicate surfaces (n = 15).

Fungal cultivation

Fungal cultures were re-suspended from freeze dried lyophilizates into 3 mL NaCl– Triton X-100 (*A. niger* spp.) or Sabouraud broth (*A. pullulans*) (BDH, UK) and rigorously vortexed for 5 min. The fungal suspensions were dispensed onto Sabouraud agar plates (BDH, UK) prior to incubation for 3–21 days at 29 °C. Following growth of the fungi, "working" cultures were prepared by transferring fungi into fresh agar plates. Cultures for short-term storage were prepared on agar slopes and for long-term storage were covered in a layer of sterile mineral oil (Sigma, UK) and stored at 4 °C for up to 12 months. Following fungal growth from short-term or long-term agar slopes, 5 mL of sterile diluent was added the fungal culture and the spores were transferred using a sterile glass Pasteur pipette into a sterile beaker. The suspension was stirred for 30 min and filtered through glass wool (VWR, UK), then centrifuged at 3000 × g for 10 min, washed three times in sterile distilled water and re-suspended to achieve an optical density of 1.0 at 610 nm, which equated to, *A. niger* 1957 $5.26 \pm 0.31 \times 10^6$ spores cm^2 , *A. niger* 1988 $5.33 \pm 0.59 \times 10^6$ spores cm^2 and *A. pullulans* $4.97 \pm 1.71 \times 10^6$ spores cm^2 . Fresh spore suspensions were stored at 4 °C and used within two weeks. Spore quality and purity were checked prior to each experiment using a light microscope.

Imaging of fungal species

Following the attachment, adhesion or retention assays, spores on PTFE or glassware were covered with 1 % methylene blue (Sigma, UK) for 2 min. Spores on silicon wafers were incubated with 0.03 % acridine orange (Sigma, UK) for 2 min. All samples were washed with sterile distilled water and dried at room temperature. Light microscopy (Nikon Eclipse E600, UK) was used to visualise the PTFE and glass surfaces, whilst epifluorescence microscopy was used to visualise the spores on the silicon surfaces. The Nikon Eclipse E600 microscope

was mounted with a Hitachi HV-D37P mounted colour camera which has a resolution of 800 and a pixel size of 768 x 494 (Nikon, UK) and the number of spores on the surfaces were counted individually following imaging using Lucia Image Analysis software (Nikon, UK). All imaging was carried out using oil immersion at x1000 magnification.

Salt mediated aggregation and sedimentation assay (SAS)

All buffers and stock solutions were filtered using 0.4 µm acrodisc filters prior to use. Washed spore suspensions were re-suspended in 2.0 mM sodium phosphate buffer (pH 6.8) to an optical density of 0.6 at 610 nm. The spore suspension was diluted at a 1:10 ratio in 2.0 mM sodium phosphate buffer (BDH, UK) (pH 6.8) inside a glass culture tube. A volume of 1 mL of 0.2 ammonium sulphate (BDH, UK) was added to the spore/sodium phosphate buffer suspension. The mixture was vortexed vigorously optical density readings were taken (Jeffs and Khachatourians, 1997). The wettabilities of the fungal spores were determined from three different spore suspension carried out from fresh spore solutions on different days (n = 15).

Diffuse reflectance Fourier transform infrared spectroscopy (DRIFTS)

Ten milliliters of washed spore suspension was put into a sterile, new, clean glass Petri dish. The diluent was allowed to evaporate off in a class 2 flow hood. The spores were dried in a phosphorous pentoxide desiccator for 14 days then blended (ca. 5% w/w) with finely ground potassium bromide (KBr). This was carried out whilst wearing high grade 0.2 µm particulate masks, and gloves whilst working in a restricted area. The resulting spore/KBr mixture was then placed in a Spectra-Tech DRIFTS cell fitted to the same FTIR as described for the ATR method. Spectra were made up of 164 scans with resolution set to 4 cm^{-1} (n = 5).

Attachment assay

Replicate substrata (1 cm x 1 cm) were attached to a stainless steel tray using adhesive gum (Impega, Malaysia). The tray and attached substrata were placed vertically in a class 2 flow hood. The spore suspension (OD610 1.0) was sprayed at a distance of 10 cm ten times from left to right at a speed of 50 mm × sec^{-1} over the substrata at a flow rate of 0.2 mL × sec^{-1} per (Badger Airbrush, UK with a gas Canister, Esselte Letraset Ltd, UK). Immediately following spraying, the substrata were laid horizontally and dried without rinsing (n = 45).

Adhesion assay

The surfaces were treated as above, except that immediately following spraying, substrata were held vertically and rinsed once, to gently remove loosely attached spores using 5 mL distilled water was dispensed at a 45° angle, with a 3 mm nozzle. The substrata with the retained spores were laid horizontally and aseptically air-dried for 1 hour (n = 45).

Retention assay

Spores were harvested by centrifugation (3600 g for 12 min) and washed 3 times in sterile distilled water and adjusted to an OD 1.0 at 610 nm. Three replicate substrata were placed horizontally in a glass Petri dish, and 20 mL of standardised spore suspension was added and incubated at room temperature for 1 hour without agitation. The test pieces were removed and rinsed once gently with 5 cm^3 distilled using a 3 mm nozzle water, with the distilled water bottle at a 45º angle. Substrata were air-dried in a Class 2 flow hood. *A. pullulans* spores were stained for 2 min using 1 % crystal violet at room temperature in the light (Sigma, UK) and rinsed. *A. niger* spp. spores were not stained (n = 45).

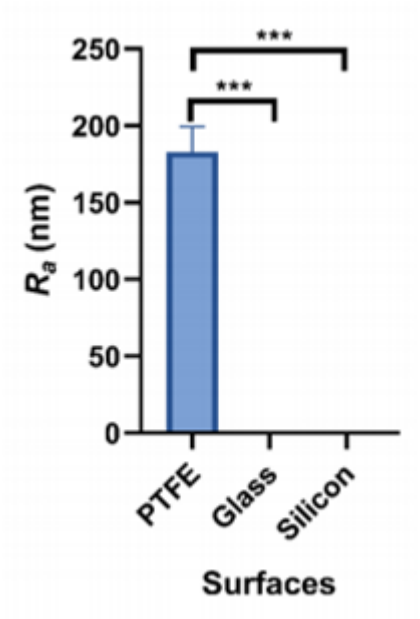
Multifractal Analysis (MFA)

MFA was carried out using MATLAB® R2020b, the MathWorks Image Processing Toolbox® and Python. The MFA was used to give quantitative measures of density (fractal dimension D0), dispersion (heterogeneity) and clustering (clusters of spores/gaps were quantified by positive/negative values of Δf, respectively), of spores on the surfaces.

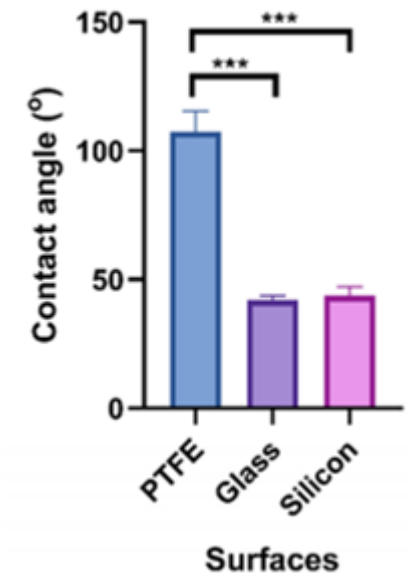
Statistical Analysis

Statistical analysis was conducted by performing two-way ANOVA coupled with Tukey's multiple comparison tests for post hoc analysis using GraphPad Prism (version 8.4.2; GraphPad Software, USA) to determine significant differences at a confidence level of 95 % ($p < 0.05$). Error bars represent the standard error of the mean. Asterisks denote significance, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$.

Supplementary information



Supplementary Figure 1. R_a values for surfaces used in the assays demonstrating the topographies. Asterisks denote significance, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$ (n = 3) Related to Figure 1.



Supplementary Figure 2. Water contact angles for surfaces used in the assays demonstrating the wettabilities. Asterisks denote significance, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001 (n = 3) Related to Figure 1