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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 shape and size (Aspergillus niger 1957, Aspergillus niger 1988 and Aureobasidium pullulans), were investigated. A multifractal analysis was conducted to give 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, whilst silicon was the opposite in terms of both latter aspects. The A. niger species were more non-wettable than A. pullulans. Overall A. niger 1957 attached in higher numbers to PTFE, whilst A. niger 1988 and A. pullulans bound in highest numbers to glass. The results from this work, demonstrated that overall substratum surface roughness influenced spore binding rather than the physicochemical or chemical properties of surfaces or spores.

Keywords: Fungal spore; Surface Roughness; Attachment; Adhesion; Retention; Multifractal analysis (MFA).

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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 colonisation (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 discolouration and/or biodeterioration (Liauw et al., 2020). The biodeterioration of surfaces is a serious problem that occurs in many environments, and biodeterioration is particularly prevalent on polymeric materials, interior and exterior surfaces, including paint surfaces (Whitehead et al., 2011), 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 polyvinyl chloride surfaces (Webb et al., 2000).

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Hence the focus of this paper was one of 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 medicine, agriculture and food industry (Clement et al., 1993; Whitehead et al., 2011), thus an enhanced understanding of the interactions involved in spores attachment could lead to the development of novel approaches to reduce spore adherence.

*Aspergillus 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 (Frisvd 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. *Aureobasidium pullulans* is a yeast-like fungus that is abundant in the environment, and colonises several habitats, usually in multispecies biofilms, (Cooke, 1962; Cooke, 1987), particularly coated wood surfaces (Eveleigh, 1961; Webb et al., 1999). It was observed to be the main fungus that causes biodeterioration of PVC films during outdoor trials in Florida, USA (Hamilton, 1983). Due 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).

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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, whilst 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.

Difficulties in determining the distribution and pattern of fungal spores across the surfaces is an issue, since the underlying surface properties will inherently affect spore binding. However, one way in which to thod enables the distribution and agglomeration of the uses a box countinovercome such problems is to use multifractal analysis, which 2020). This meg method to calculate fractal dimensions (Yildiz and Yildiz, 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*

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Atomic force microscopy was used to determine the surface roughness of test substrata (Supplementary Figure 1). PTFE presented a greater Z height (1210 nm) compared with the glass (417 nm) or silicon surfaces (80 nm). The surface roughness results were represented by the $R_a$ (average value of the mean height derived from a centre line) (Supplementary Figure 1) which correlated with the images in terms of degree of roughness. PTFE had a $R_a$ value of 182.9 nm, whilst the glass (117.8 nm) and silicon (0.6 nm) surfaces had much 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 º) and least wettable surface, whilst the smoothest surface, the silicon, demonstrated the lowest contact angle (42 º) 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 ($p \leq 0.001$) (Supplementary Figure 2).

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 µm – 6 µm in diameter (Figure 2a). *A. niger* 1988 spores were also spherical and of a similar size/diameter to *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 in shape varying from 5 µm – 12 µm in length by 2 µm – 3 µm width and were transparent and colourless. 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

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were more less wettable compared to *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).

DRIFTS spectra for the three fungi demonstrated that all the spectra had the following common features: broad hydrogen bonded OH stretching bands (3800 cm\(^{-1}\) – 2300 cm\(^{-1}\)), C-H stretching vibrations (centred at 2900 cm\(^{-1}\)), ester (or other carbonyl bands (shoulder at 1730 – 1720 cm\(^{-1}\)), amide I and II carbonyl bands (ca. 1650 cm\(^{-1}\) and ca. 1640 cm\(^{-1}\), respectively) and C-O-C bending vibrations (ca. 1060 cm\(^{-1}\)) (Figure 4). The area of the OH stretching and CH stretching bands were determined. Due 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\(_{\text{O-H}}\)) was divided by the absorbance of the amide I band (Abs\(_{\text{amide I}}\)) to give A\(_{\text{O-H}}\)/Abs\(_{\text{amide I}}\). The area of the O-H stretching band (A\(_{\text{O-H}}\)) was calculated as follows: A\(_{\text{O-H}}\) = A\(_{\text{O-H}}\) + C-H – A\(_{\text{C-H}}\). The area of the CH stretching bands was also divided by the area of the OH stretching bands to give A\(_{\text{C-H}}\)/A\(_{\text{O-H}}\). These ratios that the *A. pullulans* had the most OH groups as it gave the highest A\(_{\text{O-H}}\)/Abs\(_{\text{amide I}}\) value and lowest A\(_{\text{C-H}}\)/A\(_{\text{O-H}}\) 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 (Figure 5a and b respectively) whilst they became grouped in clumps (aggregated) when applied to

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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 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), whilst on the most wettable surface, silicon, the *A. niger* 1988 spores were well distributed across the surface (Figure 5f). The larger and most wettable spore, *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 number 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 ($4.3 \times 10^5$ spores cm$^2$). There was a significant difference in the number of *A. niger* 1957 spores attached to the PTFE compared to the silicon surface ($p \leq 0.0001$) and the number of spores attached to the glass compared to the silicon surface ($p \leq 0.01$) (Figure 6a).

*A. niger* 1988 was attached to the PTFE and glass surfaces in similar numbers ($1.5 \times 10^5$ spores cm$^2$ and $1.7 \times 10^5$ 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 ($p \leq 0.05$)

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and the glass and silicon surfaces \((p \leq 0.0001)\). \textit{A. pullulans} was attached in the highest numbers to the glass surface \((2.5 \times 10^5 \text{ spores cm}^2)\) 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 surfaces \((p \leq 0.001)\). \textit{A. niger} 1957 and \textit{A. niger} 1988 were attached in the lowest numbers to silicon \((6.6 \times 10^4 \text{ spores cm}^2\) and \(3.1 \times 10^4 \text{ spores cm}^2\) respectively), whilst \textit{A. pullulans} was attached in the lowest numbers to the PTFE surface \((5.1 \times 10^4 \text{ spores cm}^2)\) (Figure 5a).

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

\textit{A. niger} 1957 was retained in the lowest numbers to the glass surface \((2.7 \times 10^3 \text{ spores cm}^2)\) and there was significantly more \textit{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). \textit{A. niger} 1988 was retained in the highest numbers to the glass surface \((3.3 \times 10^4 \text{ spores cm}^2)\) (significant difference PTFE and silicon \((p \leq 0.0001)\), glass and silicon \((p \leq 0.05)\). \textit{A. pullulans} were retained in the highest numbers to the PTFE surface \((1.9 \times 10^4 \text{ spores cm}^2)\) and there was a significant difference between the numbers of spores retained on the PTFE and silicon.

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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 ($2.8 \times 10^2$ spores cm$^2$ and $1.9 \times 10^2$ spores cm$^2$ respectively) (Figure 6c).

**Multifractal Analysis (MFA)**

A MFA was carried out in a similar manner to that adopted in our earlier papers (Wickens et al., 2014; Slate et al., 2020). MFA is typically used to measure density, dispersion and clustering of objects on a surface. The theory and applications of MFA can be found in Lynch, (2014; 2018) and Salat, (2017). Typical multifractal $f$-$a$ 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 $2 \times 2$ matrices, after the first iteration a $4 \times 4$ matrix is obtained and after 8 iterations a $512 \times 512$ matrix is generated. The images were produced with MATLAB and Python using image processing techniques, where the pixels are on a grey 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 $f$-$a$ curves. In each case, the width of the $f$-$a$ curve is given by $\Delta a$, and gives a measure of dispersion. The asymmetry of the $f$-$a$ curve is given by $\Delta f$, if $\Delta f$ is negative (as in case (a)), then this implies clustering of dark pixels, and if $\Delta f$ 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.

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Figure 8 shows binary images of the fungal spores (a) niger 1957 on silicon and (b) niger 1957 on glass. The second column displays the corresponding multifractal f-a curves. Reading the quantitative values, it can be seen that the density (fractal dimension $D_0$) was higher in case (b) than case (a). The dispersion ($\Delta a$), was also higher in case (b) than 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 of the images where a multifractal analysis 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 coverage demonstrate spore adhesion to the surfaces, there was little or no dispersion and the clustering is very small.

To quantify the density, dispersion and clustering of the spores across the surfaces multifractal analysis was carried out on the images (Figure 9). However, only analysis on the surfaces following the attachment and retention assays could be carried out since 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).

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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 spores 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). Further, 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, whilst the *A. niger* 1988 had a spiky surface. Further, both the *A. niger* species demonstrated non-wettable properties, whilst 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 the *Aureobasidium pullulans* was the most hydrophilic of the three fungal conidia examined. Aside from the exopolymeric components and biochemical enzymes involved in fungal spore

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binding, (Epstein and Nicholson, 1994), surface properties also provide a multifactorial aspect of spore binding to surfaces which is the subject of this paper.

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. The PTFE was the roughest, and least wettable surface, whilst the silicon was the smoothest and 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 lowest numbers of fungal spores retained on the surfaces (~0.5 logs), but with the greatest difference between the highest and lowest number of spores bound to the surfaces 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) between the two (0.5 – 2 logs).

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

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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 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 hours of hydration (Apoga et al., 2001). 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. 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

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

Multifractal analysis 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 multifractal analysis 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 since 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 multifractal analysis 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, *S. aureus* displayed a more heterogeneous cell dispersion than *S. epidermidis* (Wickens

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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 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 PVAc and 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 spores types bound in the

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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: Professor Kathryn Whitehead.

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.

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Author Contributions

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The concept behind the work was developed by AP, TD, CL, JV and KW. The experimental methodology was developed and carried out by KW and CL and the MFA was conducted by SL. The manuscript was written by MEM, SL and KW. All the authors were involved in the final proofing of the manuscript.

**Declaration of Interests**

The authors declare no conflicts of interest.

**Figure and Table legends**

Figure 1. AFM images illustrating surface topography of the three surfaces used in this study a) PTFE b) glass and c) silicon (n = 9).

Figure 2. Light microscopy images of fungal spores a) *A. niger* 1957 b) *A. niger* 1988 c) *A. pullulans*. Scale bars = 10 µm. Inset in c) *A. pullulans* spore.

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, **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001.

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).

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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.

Figure 6. 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)

Figure 7. Theoretical and numerical f-a curves for multifractals generated with MATLAB and Python. The theoretical multifractal spectra are plotted as blue curves and the corresponding numerical f-a curves are plotted using red + signs. Note that in each case the fractal dimension D0 is two as the pixels cover the whole plane.

Figure 8. Binary images of a) *A. niger* 1957 on silicon and b) *A. niger* 1957 on glass. The second column shows the corresponding multifractal f-a curves for these binary images.

Figure 9. Density, dispersion and clustering of fungal spores across the surfaces following a/b/c) attachment assays and d/e/f) retention assays.

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

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### Table 1.

| Conidia type       | $A_{O-H}/Abs\text{amide I}$ | $A_{(C-H)/A_{(O-H)}}$ (x 10²) |
|--------------------|-----------------------------|-------------------------------|
| *A. niger* 1957    | 750                         | 3.00                          |
| *A. niger* 1988    | 826                         | 3.03                          |
| *Aureobasidium pullulans* | 1028                     | 2.17                          |
Absorbance

A. niger 1957  A. niger 1988  A. pullulans

Fungal spores
| Multifractal motif | Representative figure | Multifractal $f$-$\alpha$ curve |
|-------------------|------------------------|--------------------------------|
| 0.75 0.1          | ![Clusters of dark pixels.](image) | ![The $f$-$\alpha$ curve is skewed left and $\Delta f < 0$.](image) |
| 0.09 0.06         |                         | ![The $f$-$\alpha$ curve is skewed left and $\Delta f < 0$.](image) |
| 0.24 0.26         | ![Homogeneous – no clusters.](image) | ![The $f$-$\alpha$ curve is not skewed and $\Delta f = 0$.](image) |
| 0.255 0.245       |                         | ![The $f$-$\alpha$ curve is not skewed and $\Delta f = 0$.](image) |
| 0.3 0.35          | ![Clusters of bright pixels.](image) | ![The $f$-$\alpha$ curve is skewed right and $\Delta f > 0$.](image) |
| 0.25 0.1          |                         | ![The $f$-$\alpha$ curve is skewed right and $\Delta f > 0$.](image) |
| Binary image of spores on surface | Multifractal $f$-$\alpha$ curve |
|----------------------------------|--------------------------------|
| ![Spores Image](Image)           | ![Multifractal F-alpha Curve](Image) |
| (a) Niger 1957 silicon – clustering of spores ($\Delta f = +0.53$). Density=1.56 and dispersion=0.616. | $D_0 = 1.56$
|                                  | $\Delta f = 0.531$
|                                  | $\Delta \alpha = 0.616$ |

| (b) Niger 1957 glass – clustering of gaps ($\Delta f = -0.426$). Density=1.66 and dispersion=1.005. | $D_0 = 1.66$
|                                  | $\Delta f = -0.426$
|                                  | $\Delta \alpha = 0.1005$ |
Highlights

- Multifractal analysis gave measures of density, dispersion and clustering of spores
- *A. niger* species were more non-wettable than *A. pullulans*
- *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