**Special Issue: A cracking approach to inventing tough new materials: fracture stranger than friction**

# Lateral force removal of fungal spores to demonstrate how surface properties affect fungal spore retention

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**Abstract**

Microbial biofouling on polymer surfaces can lead to their biodeterioration. This may result in deterioration of the surface leading to cracking and fracturing. Fungal spores from *Aspergillus niger* 1957, *Aspergillus niger* 1988 and *Aureobasidium pullulans* were tested to determine their strength of attachment on three surfaces, p(γ-MPS-co-MMA), p(γ-MPS-co-LMA), and spin coated PMMA (PMMAsc) using lateral force measurements. The results demonstrated that *A. niger* 1957 and *A. niger* 1988 spores were most easily removed from the p(γ-MPS-co-MMA) surface, which was the surface with the highest $R_a$ value. The *A. niger* 1957 and *A. pullulans* spores were most difficult to remove from the PMMAsc surface which was the hardest surface. *A. niger* 1988 spores were the most difficult to remove from p(γ-MPS-co-LMA), the most hydrophobic surface. The results with *A. pullulans* were difficult to elucidate since the spores bound to all three surfaces and were removed with similar rates of force. The lateral force results demonstrated that spore attachment to a surface is a multifactorial process, and independent surface and microbial factors influence spore binding. Thus, each environmental scenario needs to be considered on an individual basis, since a solution to one biofouling issue will probably not translate across to other systems.

**Keywords**: Atomic force microscopy; polymer surface; lateral force; fungal spores; *Aspergillus*; *Aureobasidium*

## 1. Introduction

Biofouling can include fouling of a surface by organic material (sometimes referred to as a 'soil') [1], by macroorganisms such as barnacles, but also by microorganisms including viruses, bacteria and fungi. Biofouling of surfaces is a major issue for many industries, and some examples where biofouling is a major issue includes the food [2], dairy [3] pharmaceutical [4], water [5], wastewater [6], petrochemical [7], oil [8], marine [9], sea water desalination [10], nuclear [10], shipping [12] and aviation [13] industries to name a few. There is a plethora of surface types used within these industries, with a range of surface finishes, chemical heterogeneities, mechanical strengths, and hydrophobicities. Once microbial attachment and subsequent biofouling has occurred, undesirable changes in material properties due to indirect or direct microbial contamination may be referred to as biocorrosion, microbial corrosion or microbially influenced corrosion [14,15]. Microorganisms such as bacteria and fungi are involved in the degradation of both natural and synthetic plastics [16].

Polymers are used in a wide variety of components including wheels, cams, gears, seals, bushings and brakes [17,18]. Polymethylmethacrylate (PMMA) is widely used in marine antifouling coatings, and is easily prone to biofouling by bacteria, diatoms and macrofouling organisms [19-21]. Poly(lauryl methacrylate) (PLMA) is generally used as a viscosity modifier in motor oils or as an oil absorbency agent or drag reducer. It is also used in a number of copolymers for a variety of applications [22,23].

The environmental cracking of polymer surfaces and thus deterioration can occur due to a number of reasons, but these generally include mechanical stress, or stress due to exposure to fluids such as lubricants and cleaning agents [24]. However, polymer

breakdown can also be due to biological factors such as the metabolic activity of microbes [25]. Fungal attachment and retention can be considered a prelude to microbe induced environmental stress cracking. If the latter is to be avoided, then understanding of fungal spore attachment and how to stop it is vital to prolong product lifetime.

Although much work has been carried out on how surface properties affect microbial binding to a surface, there are a number of different factors which confound the understanding of the data. If the metabolic activity of a microbe occurs at a stress concentration, which may be a poorly designed-in feature, or a surface scratch / defect in a polymeric component, there may be increased probability of crack initiation. Newly formed cracks will increase surface area and may lead to increased microbial interaction within the cracks. This, in turn, could lead to increased metabolic activity at the plastic / craze zone as the fungi utilise the substratum to initialise growth, which can ultimately cause microbe initiated environmental stress cracking and failure of the component. The inside surfaces of scratches and cracks may comprise of chemical heterogeneities, differences in wettabilities and the presence of organic material, dependent on the environment and the material in which the cracking has occurred which is important since it is known that these surface properties affect microbe binding to the surface interface [26-29]. In addition, it has also been demonstrated that different environments and surface properties will bind specific microbial species and so understanding how such material properties influence fungal spore attachment is a primary concern.

*Aspergillus niger* is one of the most important microorganisms used in biotechnology to produce extracellular (food) enzymes and citric acid [30]. However, it is also a problematic fungus and a common fungal isolate from oil refineries and distribution

systems [31]. *Aureobasidium pullulans* is found in decaying wood, leaf litter, wood, and other plant life and hence is also found in water and soil [32]. It has also been found to be critical in the establishment of a microbial community on pPVC [33]. In addition, PVC containing the plasticizers dioctyl phthalate (DOP) and dioctyl adipate (DOA) have been shown to increase the adhesion of *A. pullulans* blastospores to pPVC [34]. *A. pullulans* produces and secretes a number of molecules, being influenced by the ambient growth conditions. However, one of the main biomolecules produced is pullulan, which is a high molecular weight polysaccharide [32] evident as extracellular material surrounding the cells [35,36].

The aim of this work was to demonstrate how the properties of three model surfaces, (comprising various acrylic polymer and copolymer spin coatings on silicon wafers) affected the strength of fungal spore attachment. The acrylic polymers investigated were poly(methylmethacrylate) (PMMA), a random copolymer of gamma-methacryloxypropyltrimethoxysilane (γ-MPS) and methyl methacrylate (MMA) (in a ratio of 10:90) (p(γ-MPS-co-MMA)) and a random copolymer of γ-MPS and laurylmethacrylate (LMA) (p(γ-MPS-co-LMA)) (also in a ratio of 10:90). At a molecular level, these structural variations probe the effect of a flexible side group. The surfaces also had differing nanometer surface topographies which also affected the binding of different fungal spores. Lateral force measurements were used rather than perpendicular force measurements since such measurements are more representative of the bacteria being pushed off a surface, and as such are more representative of those forces found in some cleaning regimes. Such work on a controlled level demonstrates the complexities of understanding the surface

interactions that occur between bacteria and surfaces in applied, complex systems such as cracking and corrosion features.

## 2. Methods

*Imaging of naturally occurring biofouled surfaces.*

*Recovery of fouled surfaces*

To visualise actual biofouling and corrosion / leaching on a polymer surface, a component inlet / outlet was recovered from the internal workings of a used washing machine which had been dismantled.

*Scanning electron microscopy of fungal growth on polymer surface*

Visualization of the microorganisms that had biofouled polymer surfaces were immersed in 4 % w/v gluteraldehyde (Agar Ltd., Essex, UK) for 24 h at 4 ºC, then washed gently with sterile distilled water. The samples were taken through an ethanol gradient consisting of 10 %, 30 %, 50 %, 70 %, 90 % and 100 % ethanol. The samples were retained in each solution for 10 min before being transferred to the higher concentration of ethanol. The samples were stored at room temperature, in a silica gel (Sigma, Dorset, UK) desiccator. The samples were fixed onto stubs for gold sputter coating, which was carried out using a Polaron E5100 (Milton Keynes, UK) SEM sputter coater. Samples were sputter coated at a vacuum of 0.09 mbar, for 3 min, at 2500 V, in argon gas at a power of 18-20 mA. Images of substrata were obtained using a JEOL JSM 5600LV scanning electron microscope.

*Surface analysis*

*Surface roughness using Atomic Force Microscopy (AFM)*

Roughness measurements were obtained using an Explorer AFM (Explorer, Veeco, CA, USA). Analysis was carried out in contact mode using a triangular cantilever. The spring constant was determined using the AFM software before each scan was carried out.

*Dynamic Contact Angle of surfaces (Wilhemy Plate Method)*

Dynamic contact angle analysis was carried out using a DCA 322-1 dynamic angle analyser (Cahn Instruments, USA). Contact angle measurements of clean, dry substrata were taken in HiPerSolv HPLC grade $H_2O$ (BDH, Basingstoke, Hampshire, UK).

*Surface hardness*

Nanoindentor results were carried out using a Micro Materials Nanotest nanoindentor (Micro Materials Limited, Wrexham UK). The measurements were taken using a Berkovich diamond with a maximum load of 10 mN and a minimum load of 0.5 mN. The maximum depth of the indentation was 50 nM.

*Mycology*

*Preparation of fungal species*

The fungal species were cultivated from freeze dried cultures and the *A. niger* 1957 and *A. niger* 1988 were resuscitated using NaCl-Triton X100 (both from Sigma, Dorset, Poole, UK), whilst *A. pullulans* was resuspended in Sabouraud broth (Lab M, Bury, UK). To make up the NaCl-Triton X 100, 0.85g sodium chloride (Sigma, Dorset, UK) was dissolved into 100 mL sterile distilled water, into which was added 1 ml of a 1 % Triton X100 solution. The suspension was vortexed for 5 min or until the fungi became re-suspended, and this was dispensed onto Sabouraud agar (Lab M, Bury, UK) which were incubated for 3 to 21 days at 29 ˚C. Following growth of the fungi, a transfer was made to a new agar plate and incubated as above. This was then repeated to complete a third transfer. To complete the

transfers, the fungi were inoculated onto agar using a sterile swab dipped in NaCl-Triton X100 (*A. niger* spp.) or Sabouraud broth (*A. pullulans*) and incubated as above.

Following confluent fungal growth, 5 mL of NaCl-Triton X100 (*A. niger* spp.) or Sabouraud broth (*A. pullulans*) was added to the fungal culture and the spores were removed from the culture by rubbing a sterile, glass Pasteur pipette over the surface of the culture. The spore suspension was decanted and stirred for 30 min the appropriate media and filtered using glass wool (VWR, Poole, Dorset). The filtered spore suspension was centrifuged for 10 min at 1721 *g*. The spores were washed in sterile distilled water three times, and re-suspended to an optical density of 1.0 ± 0.1 at 610 nm, checked for purity using a light microscope and were stored 4 ˚C for 2 weeks. The number of spores / mL was determined using a hemocytometer.

*Lateral force measurements using fungal spores*

One hundred microliters of the prepared spore suspension was pipetted onto the polymer and dried in a class 2 hood for one hour. The AFM with pyramidal probes was used in contact mode and the cantilevers were calibrated before each use. Following positioning of the surface onto the AFM stage, 1 mL of sterile distilled water was added to the sample and the laser was re-aligned. The lateral force assays were carried out at a speed of 1 Hz, at a scan size of 50 μm x 50 μm. To determine the ease of spore removal, after each scan, the number of spores remaining on the scanned surface was counted and calculated as a percentage. The perpendicular force was calculated from the deflection of the cantilever using the values from the zero of the force, the spring constant and the gradient in the constant compliance region of the force [37]. The deflection of the cantilever was

converted into force (F) using Hooke's law where k was the cantilever spring constant, and d was the deflection.

$$F = kd, \quad [1]$$

To convert the perpendicular force to determine the lateral force measurements, calculations were used according to Deupree and Schoenfisch [38]. To determine the applied force normal to the plane of interaction, equation 2 was used whereby Θ and ϕ equate to the probe geometry and cantilever orientation, respectively [38].

$$F_{app} = kd \sin(\Theta + \phi) \quad [2]$$

To determine the lateral force, equation 3 was used [38].

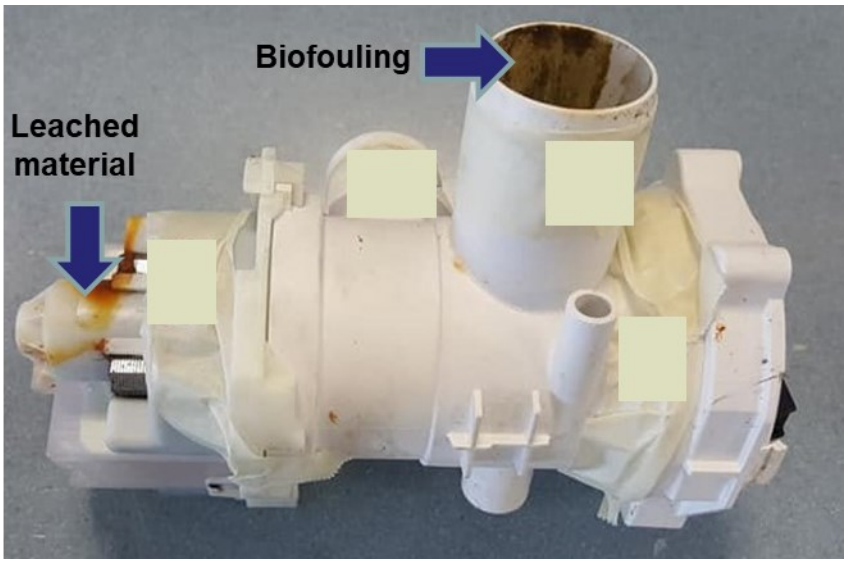
$$F_{lat} = F_{app} \cos(\Theta) \quad [3]$$

*Statistics*

Statistical tests were carried out using a two-tailed distribution t -test with two sample homoscedastic variances. The results were reported as mean ± standard deviation.

## 3. Results

Biofouling and corrosion of surfaces is an important issue in many industrial and domestic settings. Recovery and examination of a polymer component from a used washing machine revealed both biofouling (Figure 1) and an area that has discolored due to the leaching / corrosion of material onto the polymer area (Figure 1).




a) b)

Figure 1. Biofouling and corrosion of a polymer inlet / outlet component of a washing machine.

Due to such biofouling which may lead to subsequent material degradation and potential corrosion issues, it is important to understand how the surface properties of polymers affect the binding of fungal spores. Three polymer surfaces with similar chemical compositions that had been spin coated onto silicon wafers were analysed to determine how their surface properties affected the retention of three different fungal spores that are prevalent in polymer biofouling and deterioration. The PMMAsc surface presented a range of hardness values (0.18 nN – 0.54 nN). The hardness value ranges for the p(γ-MPS-co-MMA) and p(γ-MPS-co-LMA) surfaces were narrower and lower (average 0.26 nN and 0.18 nN respectively). However, there was no significant difference between the hardness values for the three surfaces ($p < 0.05$) (Figure 1).

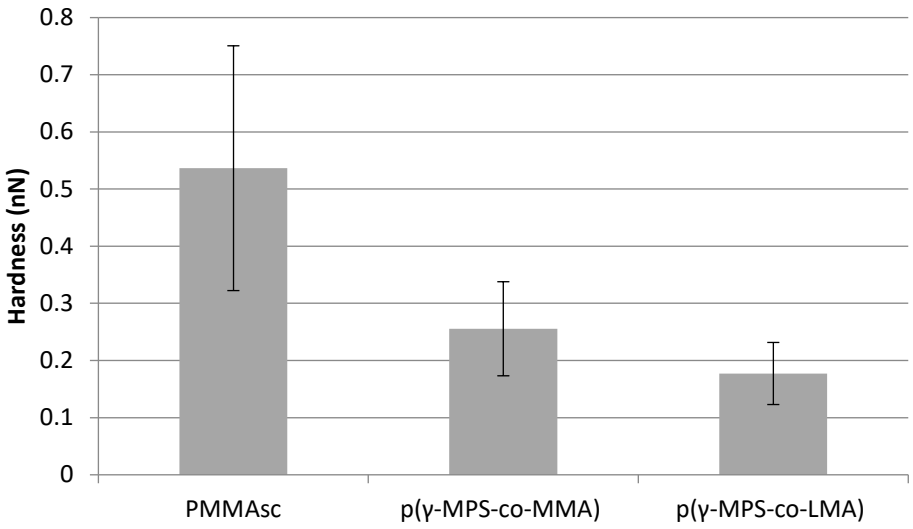



Figure 1. Hardness values of the different polymer based surfaces ($n$ = 3).

The surface roughness and wettability values were determined in previous work and are reported and discussed in the discussion section for completeness. Atomic force microscopy was used to determine the lateral force required to remove the fungal spores from each of the three surfaces. Following each lateral scan, an image was taken to determine the number of fungal spores retained on the surfaces. An exemplar of these images using *A. pullulans* on the PMMAsc surface is presented (Figure 2). It was observed that as the lateral force of the cantilever tip increased (nN), that the number of *A. pullulans* spores decreased.

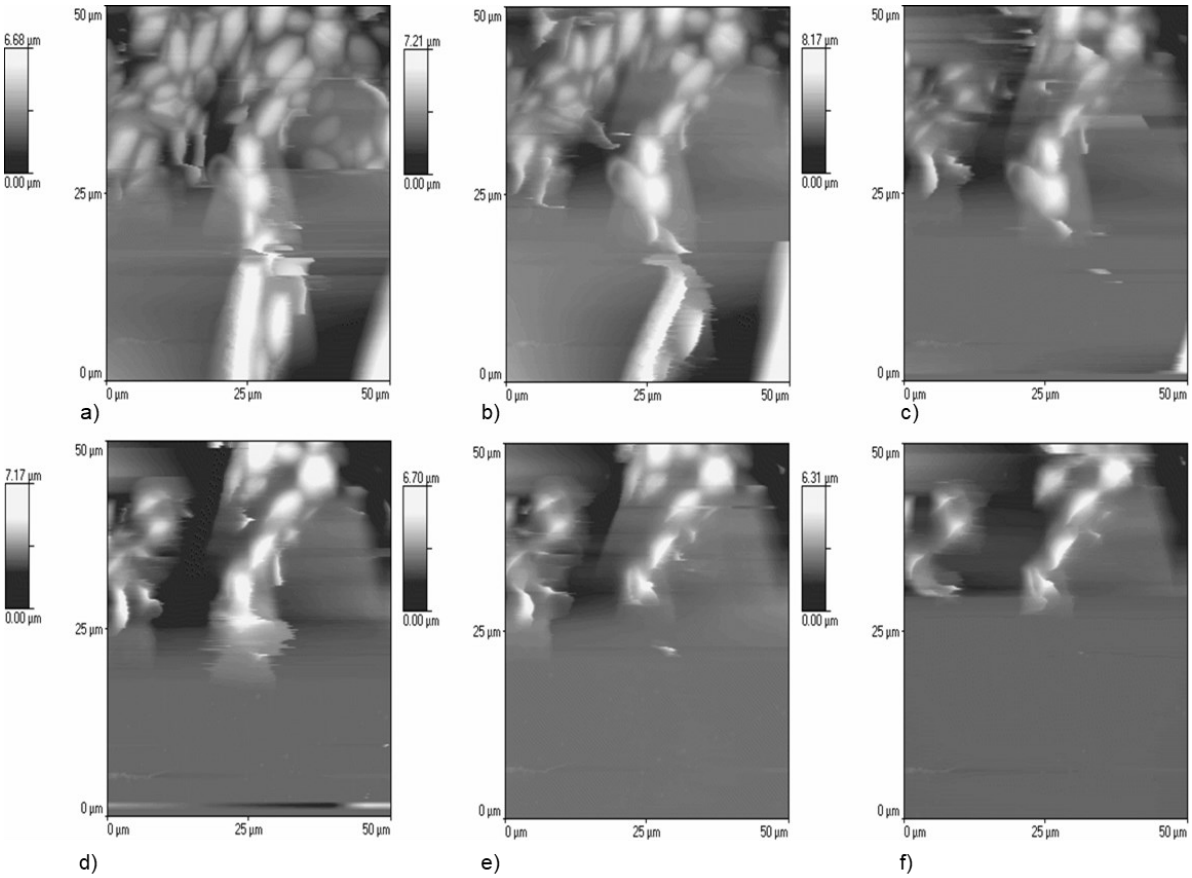



Figure 2. *A. pullulans* removed using lateral force measurements from a PMMAsc surface with increasing force from a) to f).

The removal of the spores from the surface with increased lateral force was demonstrated (Figure 3a-f). For the lateral force measurements, *A. niger* 1957 and *A. niger* 1988 spores were more easily removed from the p(γ-MPS-co-MMA) surface which had the highest $R_a$ value. The *A. niger* 1957 and *A. pullulans* spores were most difficult to remove from the PMMAsc surface which was the hardest surface, whilst the *A. niger* 1988 spores were the most difficult to remove from the p(γ-MPS-co-LMA), the most hydrophobic surface. For all the scan measurements between 2 and 6, the results for *A. niger* 1957 on the p(γ-MPS-co-MMA) surface was significantly different to the two other

surfaces. For *A. niger* 1988, the p(γ-MPS-co-LMA) results were significantly different to the other two surfaces and for *A. pullulans,* there was no significant difference in the results between the three surfaces.

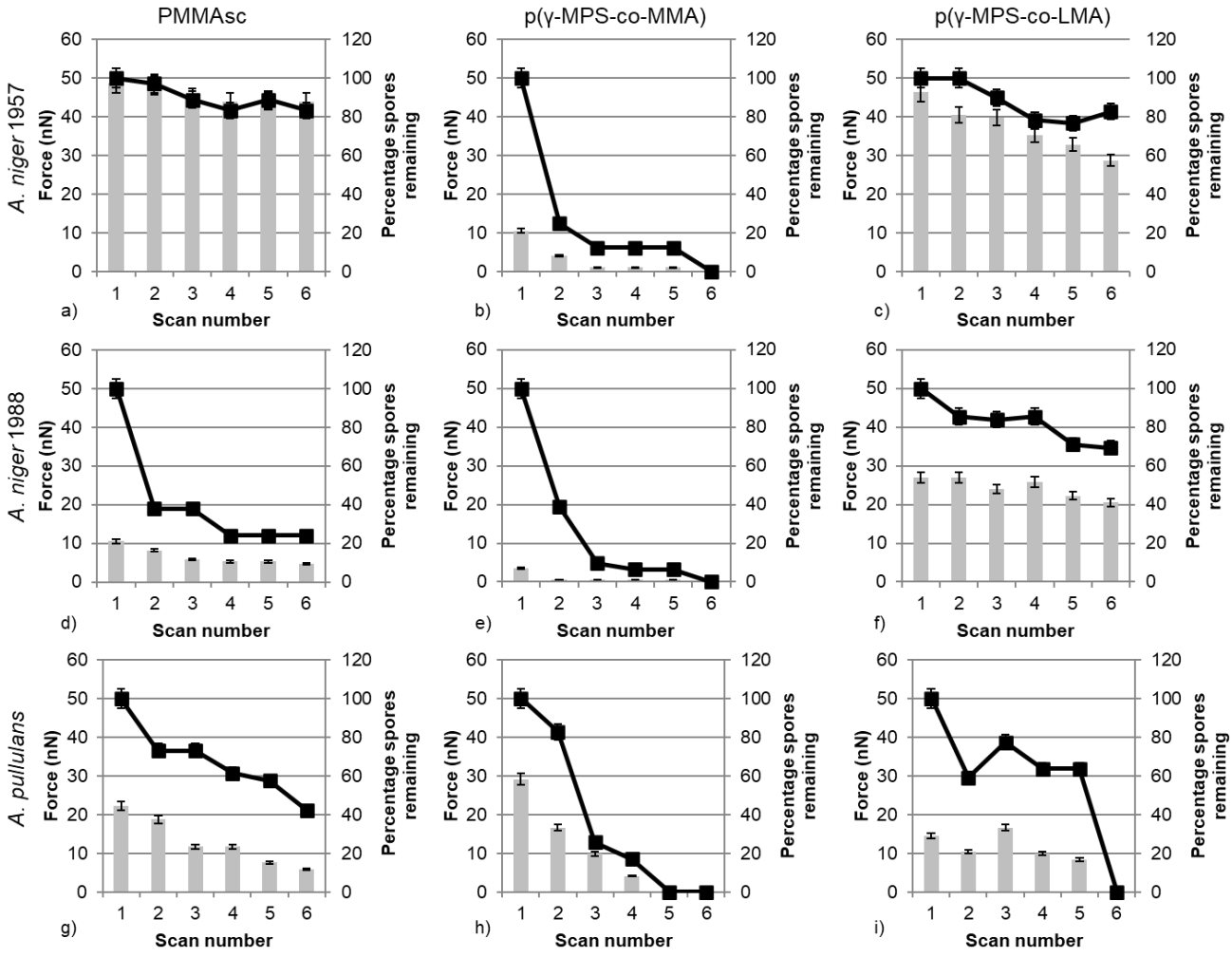



Figure 3. Lateral force removal of spores a-c) *A. niger* 1957, d-f) *A. niger* 1988 and g-i) *Aureo. pullulans* on a, d, g) PMMAsc, b, e, h) p(γ-MPS-co-MMA) and c, f and i) p(γ-MPS-co-LMA)

## 4. **Discussion**

It is well known that many surfaces biodegrade due to the action of fungal deterioration. This breakdown of materials due to microbe induced factors, results in surfaces becoming cracked or stressed which affects their surface properties, potentially resulting in unsightly surfaces and a reduction in their length of use. The results presented in this work, reveal the complexities of understanding how surface properties affect the initial microbial attachment to a surface, in this case using fungal spores as a precursor to polymer degradation. In previous work, both the $R_a$ [35] and wettability [36] of the surfaces presented had been reported and are included for completeness. An average $R_a$ value for each of the p(γ-MPS-co-LMA) and PMMAsc surfaces was found to be below 5 nm, and there was no significant difference between these surfaces [35]. The $R_a$ value for the p(γ-MPS-co-MMA) was over thirty nm and was found to be significantly different to the other two surfaces ($p$ <0.005) [35]. The p(γ-MPS-co-MMA) and PMMAsc surfaces were hydrophilic (61º and 62º respectively) whilst the p(γ-MPS-co-LMA) surface was hydrophobic (108º) [36]. There was only a significant difference between the wettability of the p(γ -MPS-co-LMA) and the other two surfaces (p<0.001) [36].

In this work, the hardness values of the surfaces was determined, and it was found that for the p(γ-MPS-co-MMA) and p(γ-MPS-co-LMA) surfaces no significant difference between the hardness of the three surfaces was demonstrated. The *A. niger* 1957 and *A. pullulans* spores were most difficult to remove from the PMMAsc surface, which was the hardest surface, but it is unclear why this may have occurred and requires further investigation. Other such methodologies that could be used to explain this phenomenon might be such measurements as the elasticity modulus of the surfaces. For the lateral force measurements, *A. niger* 1957 and *A. niger* 1988 spores were most easily removed from the

p(γ-MPS-co-MMA) surface which had the highest $R_a$ value. These spores were the most hydrophobic and were spherical and although they were different shapes (round vs spikey) and they were also smaller (4 μm - 6 μm) in comparison to the larger *A. pullulans* (5 μm – 12 μm x 2 μm – 3 μm wide) [39]. Such results may have occurred for a number of reasons. It may be that the surface with the greatest $R_a$ value resulted in the lowest amount of possible binding points with the spores, resulting in them being more easily removed from the surfaces [40]. Since the size of the surface features was in the nanometer range for the spin coated surfaces, they were small relative to the cell and hence the contact area between the cell and substrate may be reduced and retention therefore reduced. In agreement with the lateral force results described here, work carried out on the fungal spore retention on PTFE, glass, and silicon surfaces found that *A. niger* 1957 attached in higher numbers to PTFE which was the roughest surface [41]. However, it has also been shown that when *Aspergillus niger* spores were removed from a AISI 304 stainless steel surfaces with different surface finished and hence roughness's (shot treated or control), there was no significant effect on cleanability of stainless steel. [42]. Thus, for certain spores on specific surfaces, it would seem that the overall substratum surface roughness influenced spore binding rather than the physicochemical or chemical properties of the surfaces or spores [41]. However, this is clearly not the case for every scenario.

It is well known that the topography of a surface will affect the retention of microorganisms [43]. Previous work from our laboratories has shown that retention was increased when the geometry and dimensions of surface defects matched those of the organism concerned in at least two dimensions [44]. Such compatibility ensures maximized contact area between the cell and the surface and hence an increased number of binding

points [43]. However, there has also been debate over whether surface roughness does affect microbial binding, [44-47]. These conflicting findings may be due in part to the degree of surface roughness on the surfaces with respect to the other surface parameters. One of the factors that adds to the issue of understanding the surface properties that influence microbial binding to a surface, is the scale of the surface topography. Not only do the different scales of the topography affect bacterial binding, but the definitions across the disciplines may vary. For example, in microbiology, a surface with macro surface feature may be (>10 microns), micro (>0.5, <10 microns) and nano (<0.5 microns) [40]. However, in other disciplines, macro topographies may be considered to be much larger than this and be millimeters and above in size [48], thus compounding the difficulty of translation of information across disciplines. For example, when the topographical surface values are of a narrow range, other surface properties, such as the wettability or chemistry may have an overriding effect on bacterial retention [49]. This has been highlighted in work carried out using bacteria, which demonstrated that when *L. monocytogenes* and *S. aureus* retention to a range of metal surfaces was determined, they were mostly affected by surface microtopography, whereas the retention of *E. coli* to the coatings was mostly affected by surface physicochemistry [49].

The hydrophobicity of the surfaces may also affect fungal spore attachment. *A. niger* 1988 spores were the most difficult to remove from the p(γ-MPS-co-LMA), the most hydrophobic surface. *Aspergillus* spp. contain hydrophobins on the spore surface which are small amphipathic proteins [50], and it has been suggested that they have involvement in the binding of fungal spores to surfaces [51-53]. Hydrophobins are amphiphilic proteins able to self-assemble at water-air interphases and are only found in filamentous fungi and

they are characterized by their distinctive pattern of cysteine residues that form four intramolecular disulfide bridges [54].

In agreement with this work, when two surfaces, PVAc and PVOH were used in adhesion and retention assays using fungal spores, it was demonstrated that conidial binding to the surfaces was influenced by the chemistry and physicochemistry of the surfaces and spores [35]. In other work, when fungal spore assays were carried out on three chemically similar surfaces, following attachment, adhesion and retention assays, *A. niger* 1957, *A. niger 1988* and *A. pullulans* spores were retained in highest numbers to the PMMAc surface [36] which was the most hydrophobic but not the roughest surface tested in that experiment.

Results with *A. pullulans* were difficult to elucidate. The spores bound to all three surfaces and were removed with similar rates of force. This could have been due to the larger shape of the *A. pullulans* spore, or due to the EPS surrounding the spore surface. It is well known that *A. pullulans* produces exopolymeric substances (EPS) which are generally composed of the glucan based pullulans and other polysaccharides [55]. It has been suggested that the *A. pullulans* conidia are the most hydrophilic as FTIR spectroscopy has revealed carbonyl bands and high level of OH species [41]. EPS adsorption onto a surface is influenced by the surface properties and will involve rapid monolayer adsorption with an almost flat orientation followed by a slow conformational and orientational rearrangement of previously adsorbed molecules (Vroman effect) [56]. The adsorbed proteins can also form different thicknesses (2 nm – 10,000 nm), which also affects protein conformation. This dynamic change in protein layer composition is particularly evident on negatively charged hydrophilic surfaces. Thus, many physical interactions at the surface

interface influence both initial protein attachment and subsequent conformational rearrangement. The adsorption and orientation of any molecules that adsorb onto the surface will alter the interface properties that the spore become attached to, since it is the layer of organic material on the substrate, not the substrate itself, to which the spores will bind. *Phanerochaete chrysosporium* conidiospores were used to determine their adhesion to surfaces and it was found that the lack of adhesion was not due to transfer of cellular material, but to the hydrophilic nature of the spore surface [57].

The largest force used was to remove the spores in this work was 46 nN. Forces as strong as 2858 ± 1010 pN have been recorded for *Aspergillus fumigatus* adhesion to surfaces [58]. However, there are different forces involved in perpendicular and lateral force measurements of cells on surfaces. Whilst perpendicular force measurements measure the attachment of a cell or spore to a surface, lateral force measurements 'push' the cells from a surface, hence measuring their retention [40,43,39].

## 5. Conclusions

The attachment of fungal spores to a surface precedes their growth and metabolism, leading to the potential degradation of a surface which can lead to material cracking, fracturing and breakdown. *A. niger* 1957 and *A. niger* 1988 spores were most easily removed from the p(γ-MPS-co-MMA) surface which was the surface with the highest $R_a$ value. However, the hydrophobicity of the surfaces may also affect fungal spore attachment, since the *A. niger* 1988 spores were the most difficult to remove from the p(γ-MPS-co-LMA), the most hydrophobic surface. *A. niger* 1957 and *A. pullulans* spores were most difficult to remove from the PMMAsc surface which was the hardest surface. Results with

*A. pullulans* spores were difficult to elucidate. The spores were bound to all three surfaces and were removed with similar rates of force. The lateral force results suggest that spore attachment to a surface is a multifactorial process, and independent surface and microbial factors influence spore binding. This suggests that each environmental scenario needs to be considered on an individual basis, since a solution to one biofouling issue will possibly not translate across to other systems.

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