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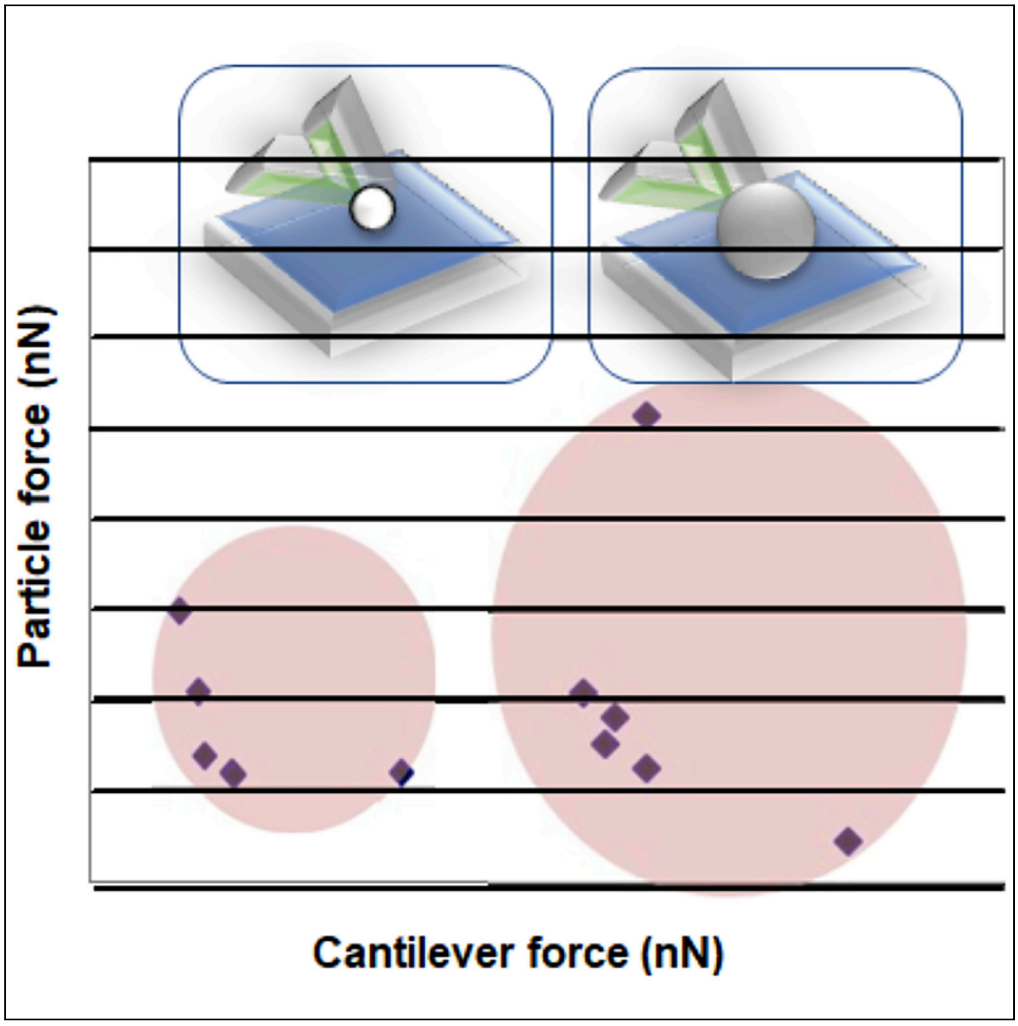

Mohsin Amin, Andrea Preuss, Ted Deisenroth, Christopher M. Liauw, Joanna Verran, Kathryn A. Whitehead

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HIGHLIGHTS

The attachment of fungal spores to surfaces is not well understood

Force measurements of non-biological spheres were similar to those of biological systems

Non-biological systems may be used to represent biological systems

The results were due to the size of the spheres/ spores and their binding energies

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Use of spherical particles to understand conidial attachment to surfaces using atomic force microscopy

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SUMMARY

Binding of particles and spores to surfaces is a natural phenomenon which is a prerequisite for biofilm formation. Perpendicular force measurements were carried out using atomic force microscopy cantilevers modified with a polystyrene or glass sphere. The attachment of the spheres was tested against glass, PVAc, p(γ-MPSco-MMA), p(γ-MPS-co-LMA), PMMAsc, and silicon surfaces. The polystyrene spheres demonstrated less varied force and strength of attachment measurement to the surfaces than the glass spheres. The force of attachment of the polystyrene spheres was also influenced by mobility of the co-polymer surfaces. Surface wettability did not affect the force of polystyrene or glass sphere attachment. The force measurements of the non-biological spheres were similar to those seen in biological systems with fungal conidia, and this was due to their size, shape, and binding energies. The use of non-biological systems may present an insight into understanding the fundamentals of more complex biological processes.

INTRODUCTION

The binding of particles from the environment such as pollen, bacterial cells, or fungal spore to a surface is a natural phenomenon, and understanding such interactions is important to improve many industrial and biological processes. This is of particular importance in controlling the biofouling onto substrata, since the initial attachment of organic material and microorganisms onto surfaces is a prerequisite of biofilm formation (Whitehead and Verran, 2015). However, the complexities of how surface properties affect particle binding to a surface are poorly understood. The use of spherical particles may be useful to carry out investigations to elucidate the fundamental factors that influence surface binding. This is important since the ability of microorganisms to instigate contamination and biodeterioration of surfaces is a major global issue, affecting the marine, food, and water distribution industries (Whitehead and Verran, 2009). For example, once fungal conidia attach, interior and exterior surfaces made from polymers may be degraded and thus understanding the spore: substratum interactions is of importance to enable biofouling to be effectively controlled (Whitehead et al., 2020; Vallieres et al., 2020).

The understanding of how a particle and a surface interact is one of the most important aspects in understanding surface binding. Surface properties have been acknowledged to contribute to the ability of spheres and microorganisms to adhere to a surface (Rosenberg and Kjelleberg, 1986). The initial attachment to surfaces by particles is thought to be due to physicochemical interactions (Seale et al., 2008). The substratum chemistry and its method of production influence both surface wettability and roughness (Stuart et al., 2010; Wassmann et al., 2017). In recent years, surface topography, physiochemistry, and chemistry have been found to significantly influence the interactions between substrata and microorganisms (Whitehead et al., 2005; Nomura et al., 2018; Wu et al., 2018; Akhidime et al., 2019).

Advances in the use of atomic force microscopy (AFM) have enabled the direct measurements of the force of attachment of individual conidia or particles to a surface (Binnig et al., 1986; Bowen et al., 2000; Whitehead et al., 2011). The technique involves the attachment of a single sphere or fungal spore at the end of a cantilever creating a particle probe. The modified cantilever can be used in AFM to enable the direct measurement of the force of adhesion of a single particle in the direction normal to the interacting surfaces

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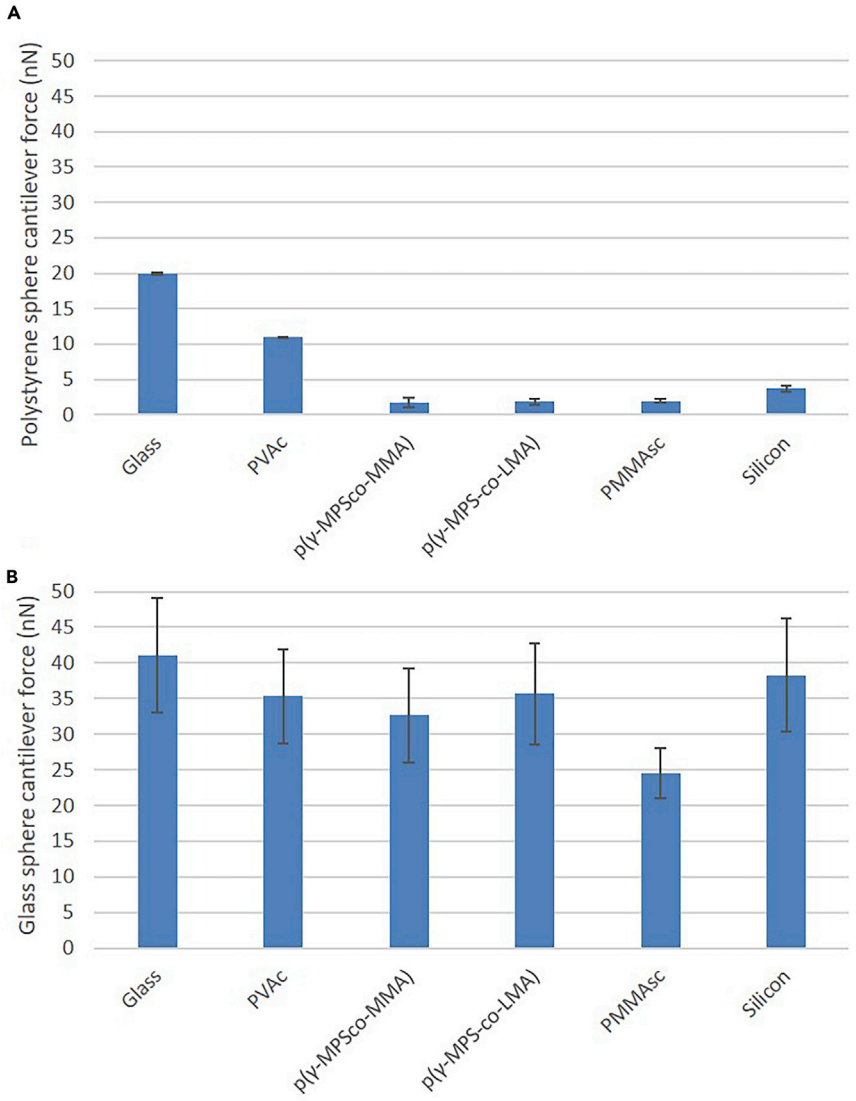

Figure 1. Strength of attachment of the polystyrene and glass spheres onto the surfaces

Strength of attachment of (A) modified cantilever with polystyrene colloid particles and (B) modified cantilever with glass colloid particles to polymer surfaces. Error bars represent the standard error of the mean.

(Binnig et al., 1986; Bowen et al., 2000). However, an understanding of how the use of such non-biological systems relates to biological systems is unclear. In this study, two chemically defined spherical particles (glass and polystyrene) were used to produce particle probes by attachment to the cantilever, and their strength of attachment to surfaces was determined. The results of the non-biological system were compared against attachment results from cantilevers modified with fungal conidia (*Aspergillus niger* 1957, *Aspergillus niger* 1988 and *Aureobasidium pullulans*) to determine if the non-biological system reflected the behavior of a biological system.

RESULTS

Attachment measurements of spheres to surfaces

Tipless cantilevers were modified using polystyrene or glass spheres. The polystyrene spheres were standardized precision-controlled calibration spheres of 6.6-μm diameter, whereas the glass spheres were not uniform, with sizes ranging between 6 μm and 10 μm. The force of attachment of the polystyrene and glass spheres was determined on a range of surfaces which included glass, PVAc, PMMAsc, *p*(γ-MPS-co-MMA),

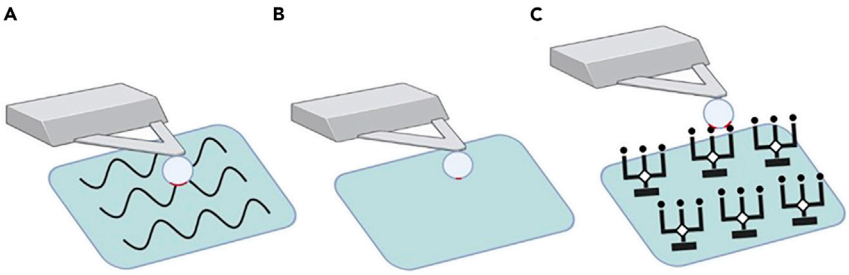

Figure 2. Colloid particles and the interactions of the surface affect the surface area between the spore and the substratum and hence force of attachment

(A) Rough surface, (B) smooth surface, and (C) co-polymerized surface.

p(γ-MPS-co-LMA), and silicon (Figure 1). The polystyrene spheres attached with the greatest force to the glass surface (19.9 nN), followed by the PVAc surface (10.9 nN). The polystyrene spheres demonstrated the lowest attachment to the three PMMA co-polymer surfaces: PMMAsc (1.9 nN), (p(γ-MPS-co-LMA) (1.8 nN), and p(γ-MPS-co-MMA) (1.7 nN) (Figure 1A). The force of attachment of the polystyrene sphere on the silicon surface was the lowest (3.7 nm).

The results obtained using the glass spheres revealed that the attachment measurements were much higher on all the surfaces (range: 24.5 nN–41 nN) than when using the polystyrene spheres (range: 1.7 nN–19.9 nN) (Figure 2B). The highest force of attachment using the glass spheres was demonstrated on the glass surface (41.0 nN), followed by the PVAc surface (35.3 nN). The lowest strength of attachment was demonstrated on the PMMAsc surface (24.5 nN). There was no relationship between the surface roughness or wettability and the force of attachment measurements for the glass spheres.

A schematic was devised to explain these interactions (Figure 2). The polystyrene sphere bound to the rough surface with the greatest number of attachment points (Figure 2A), while on the smooth surface, the number of attachment points between the polystyrene sphere and the smooth surface was much less, hence the reduction in the force of attachment measurements (Figure 2B). The force of attachment of the polystyrene sphere on the surfaces with the dynamic chemistries may have resulted in movement of the polymer chains, resulting in the least binding energies recorded between the polystyrene sphere and the surface (Figure 2C). Such changes may not have been observed for the glass spheres since their irregularity in shape and undefined chemistries may have overridden the detection of such specificities.

Relationship between non-biological results and fungal spores

To determine if the use of spheres could be related to measurements of a biological origin, the data were compared to those of previous attachment measurements using spores from three fungal species (Whitehead et al., 2011). *A. niger* 1957 conidia had a diameter of ~5 μm and they were spherical in shape (Figure 3A), whereas the *A. niger* 1988 conidia (Figure 3B) were slightly larger at ~6 μm–8 μm in diameter and they were round with protrusions across the entire surface. *A. pullulans* conidia ranged from 3 μm to 4 μm in width and 5 μm–12 μm in length (Figure 3C).

To determine the influence of the spheres on the spread of distribution of the force of attachment results, previously determined cantilever force measurements (Whitehead et al., 2011) were plotted against the perpendicular force measurements for the polystyrene and glass spheres. The force measurements were found to be more tightly grouped for the polystyrene spheres (Figure 4A), whereas they were more widely distributed for the glass spheres (Figure 4B). Previous works using the perpendicular force measurements of a biological system with fungal conidia (Whitehead et al., 2011) were also plotted against the cantilever force measurements to demonstrate the similarities between the biological and non-biological systems (Whitehead et al., 2011). The modified *A. niger* 1957 gave a narrowly dispersed range of results (Figure 4C), whereas the force measurements for the *A. niger* 1988 and *A. pullulans* demonstrated a wide distribution of attachment forces (Figures 4D and 4E, respectively). A hypothesis of the results was demonstrated in a schematic form. The hydrophobic polystyrene spheres used to modify the tipless cantilevers were of a consistent shape and diameter (6.6 μm diameter) (Figure 5A). Cantilevers fitted with hydrophilic glass spheres were chemically heterogeneous and had a range of diameters (5 μm–10 μm) and thus had a wider

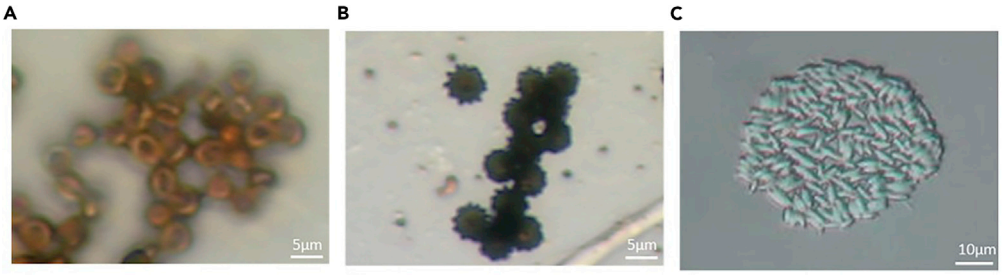

Figure 3. Images of the fungal spores demonstrating their distinct morphologies

Light microscopy images of (A) *A. niger* 1957 (scale bar, 5 μm), (B) *A. niger* 1988 (scale bar, 5 μm), and (C) *A. pullulans* (scale bar, 10 μm).

range of irregularly sized spheres. The hydrophobic *A. niger* 1957 conidia (~5 μm diameter) were of a regular size and spherical in shape, similar to that of a polystyrene sphere (Figure 5C). *A. niger* 1988 conidia had irregular shaped spiny features (6 μm–8 μm), and the hydrophilic *A. pullulans* conidia were of ellipsoidal shape with a range of 5 μm–12 μm (Figures 5D and 5E respectively). Due to the respective size and shape of the spheres/conidia, the results demonstrated similarities between the non-biological and biological systems, as was made clear by the narrow distribution of the regular shaped polystyrene spheres and the *A. niger* 1957 system. However, the glass/*A. niger* 1988 and *A. pullulans* systems demonstrated a more widespread distribution, where data obtained reflected the irregular size and shapes of the spheres/conidia.

DISCUSSION

Although there are a number of caveats regarding using spheres to understand more complex biological interactions, such systems may enable us to understand some of the more fundamental processes of how microorganisms attach to a surface. Tipless cantilevers were modified with non-biological spheres to determine if they could be used to give an insight into more complex biological systems. Regular sized and shaped, hydrophobic polystyrene spheres and hydrophilic glass spheres which had a wider range of diameters were used to modify tipless AFM cantilevers. Polystyrene is a known hydrophobic surface with a contact angle to water of approximately 85° (Li et al., 2007; Thormann et al., 2008 while glass is more hydrophilic, with a contact angle of around 57° (Whitehead et al., 2011). Although fungal spores are chemically complex at the nanoscale, the combined properties of the spore surface will contribute as a whole to their initial attachment to substrata. One reason for using the non-biological spheres was that it has been suggested that the polystyrene spheres have some features that resemble biological surfaces, for example, they are not a smooth surface but have a soft interface consisting of loosely bound and dangling polymers, are slightly charged in aqueous solution, and are charge regulators (Thormann et al., 2008). Glass is a chemically undefined surface, as are microbial cells, and thus, glass may be of use to represent microbial cells in a non-biological system.

In previous studies, it was demonstrated that the surface topographies were within the nanometer range having R_a values (118 nm [glass] and 0.6 nm [silicon]) (Whitehead et al., 2020). The most non-wettable surfaces was p(γ-MPS-co-LMA; 108°) while the most wettable surface was silicon (24°) (Liauw et al., 2020).

The strength of attachment measurements for the polystyrene spheres demonstrated that the force measurements were not greatly varied, and this was in line with the smaller and controlled size range of the spheres. The glass spheres demonstrated a more widespread distribution of the data in line with the broad range of the shape of the spheres. The polystyrene spheres with the defined size also demonstrated lower force of attachment measurements than those taken with the glass spheres. With respect to the results for the polystyrene spheres, the attachment measurements on surfaces with an increased roughness, generally, demonstrated higher attachment results, while smoother surfaces demonstrated lowered attachment of the polystyrene spheres.

With respect to size of surface features, it has been shown that surface roughness will affect the total binding energy between the bacterium and the substratum (Whitehead et al., 2005). Using a 5-μm polymer latex

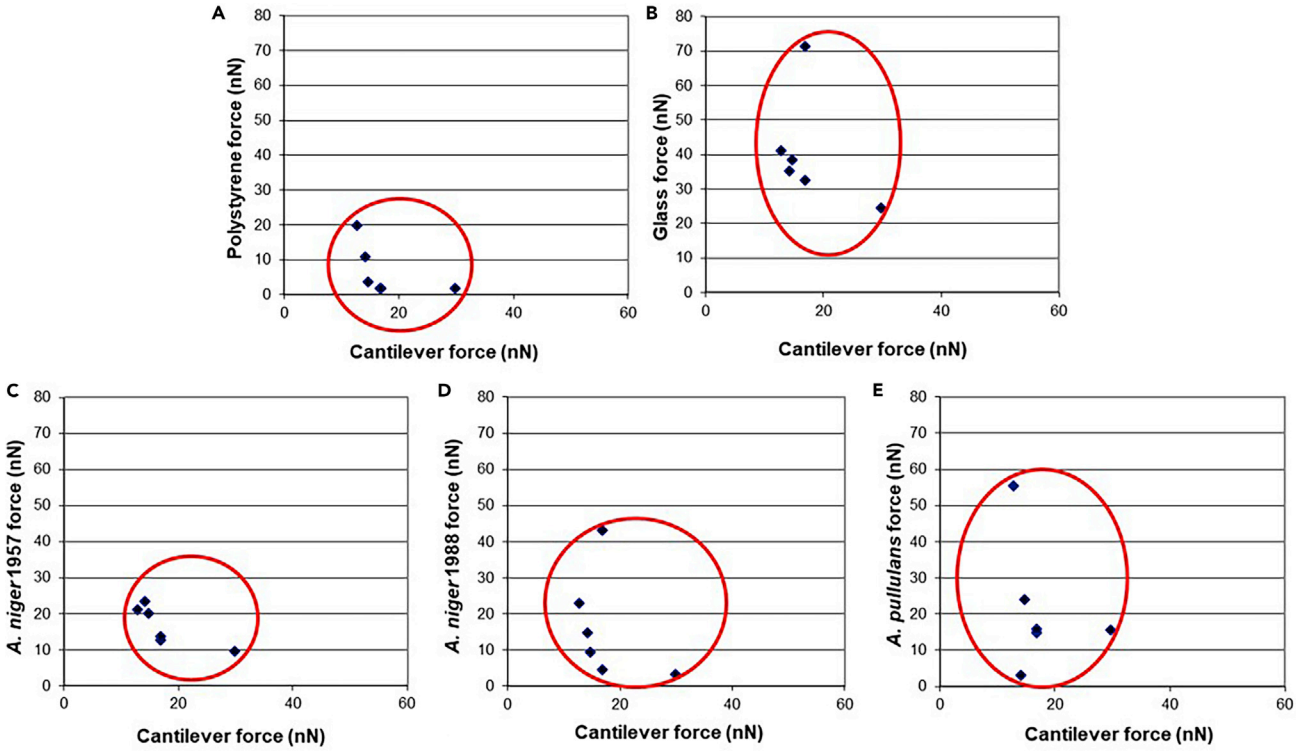

Figure 4. Distribution of non-biological (sphere) and biological (fungal spore) force measurements

Cantilever strength of attachment measurements plotted against (A) polystyrene sphere strength of attachment measurements, (B) glass sphere, (C) *A. niger* 1957, (D) *A. niger* 1988, and (E) *A. pullulans* (Whitehead et al., 2011).

sphere as a particle probe, Bowen et al. (2000) found that probe attachment strength increased with decreasing roughness on stainless steel surfaces except on the smoothest surfaces. This result was in agreement with previous work by Whitehead et al. (2006) in which AFM measurements carried out on surfaces with features of defined dimensions demonstrated that the size of the surface feature and the area of contact between the cell and the surface influenced the amount of bacteria attachment to a surface. This effect was suggested to be due to the lowered resistance of the cell attachment to the surface due to lower cell-surface binding energies.

Interestingly, with the exception of the silicon surfaces, the lowest amount of attachment was demonstrated on the surfaces that had been spin coated, and this may have been due to mobility on the co-polymer surfaces. Berglin at al., (2008) demonstrated that by changing the side chains in poly(alkylmethacrylate), they were able to systematically vary the mobility of the polymer chains. It may be that the movement of the polymer chains in this system resulted in decreased available surface area binding, reducing the interactions between the conidia or spheres and the surfaces.

The results using the glass spheres demonstrated that the attachment measurements were of a higher force and range on all the surfaces and there was no relationship between surface roughness or surface wettability and the force of probe attachment. It may be that the chemical heterogeneity of the glass surface was an additional factor that may account for the higher force of attachment observed when using the glass spheres or that the shape and hydrophobicity of the spheres influenced the findings with the larger, more hydrophilic glass spheres producing a more diverse range of results. In agreement with our work, it has been suggested that one reason why surfaces do not always perform as expected is due to the chemical properties of the substrata and an influencing factor might be a relatively small number of chemically variable areas. These act as highly adhesive sites and may influence the overall response of the system to initial microbial deposition (Ma et al., 2008). Work by Webb et al. (1999) further confirmed that chemical variation in the surface polymer affected the initial adhesion of fungi. When the adhesion of *A. pullulans* was tested against plasticized polyvinyl chloride (pPVC) and unplasticized PVC (uPVC), it was demonstrated that fungal

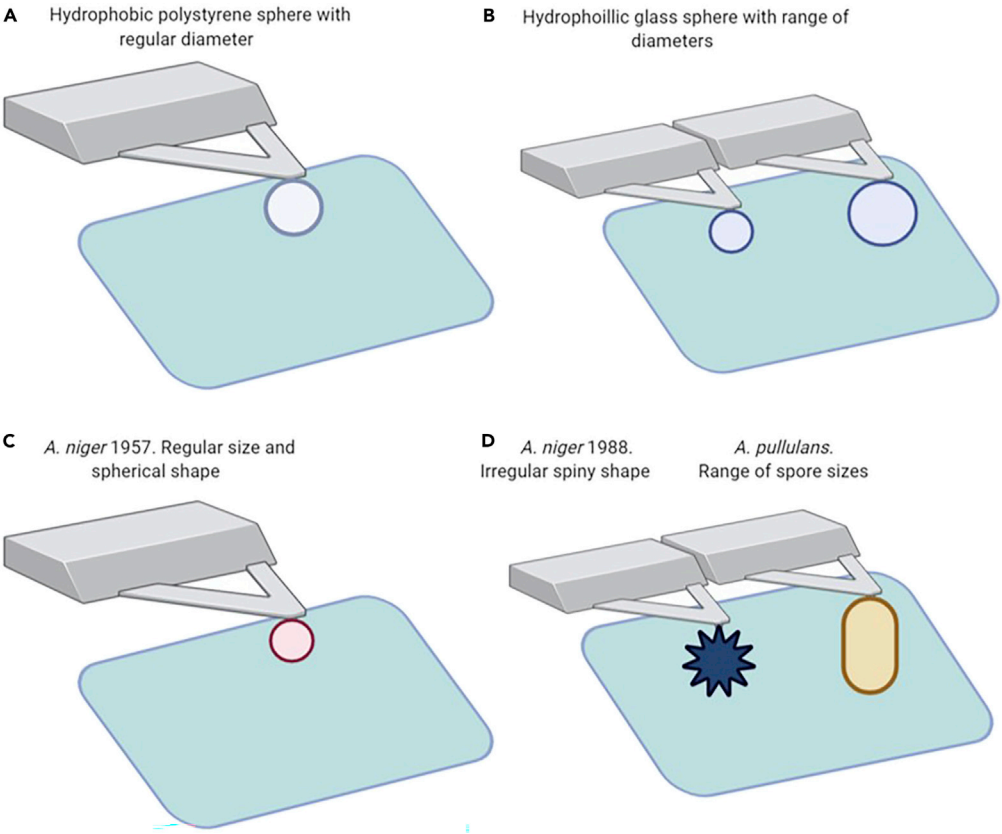

Figure 5. Representation of the sphere : surface and spore : surface interactions

Tipless cantilevers fixed with (A) hydrophobic polystyrene sphere of regular size and shape, (B) hydrophilic glass sphere which were a range of sizes, (C) *A. niger* 1957 conidia of regular size and with a spherical shape, and (D) *A. niger* 1988 which was of irregular spiny morphology and *A. pullulans* conidia which were of a range of spore sizes.

spore adhesion to pPVC was greater than that to uPVC by a maximum of 280% after a 4 hr incubation period (Webb et al., 1999).

Comparisons were made with the AFM measurements of fungal spores from a previous system (Whitehead et al., 2011) to demonstrate if a non-biological system using polystyrene and glass modified spheres could provide results reproducible of biological systems. When the strength of attachment measurements for the unmodified tipped cantilever and the surfaces were plotted against the spore attachment measurements, a similar trend was shown, in that *A. niger* 1957 had a more narrow distribution of results similar to the results for the polystyrene spheres than either the *A. niger* 1988 or the *A. pullulans*. It might be speculated that this result is due to the regular round shape and size of the spore (*A. niger* 1957) and the polystyrene spheres. In contrast, *A. niger* 1988 has a variable surface: spore contact area due to its spikey structure, and although *A. pullulans* is a bigger spore, it is irregularly shaped which could result in its range of differences in contact with the surface. Thus, it was suggested that the biological aspect of the spore did influence the force of attachment. This was confirmed by the measurements of the glass spheres being similar to those seen in the *A. niger* 1988 and *A. pullulans*.

It has been suggested that the attachment of a particle to a surface may depend on the rigidity or deformability of the spheres since for a deformable polystyrene sphere will undergo slow conformational changes during the process of attachment (Sharma et al., 2008). Observations by others have suggested that the response of a polystyrene spheres is not purely elastic (Thormann et al., 2008). The results from the current work suggest that the attachment forces were most influenced by the size of the spheres and spores and in this scenario and this may have occurred due to a matter of scale whereby the application to the biological conditions negated the observation of the events at the atomic scale. In addition, when the adhesion

between polystyrene spheres and a flat silica substrate in a dry atmosphere (Reitsma et al., 2000) and the adhesion between two polystyrene spheres in water (Hodges et al., 2004) were investigated, in both cases it was found that the Johnson-Kendall-Roberts theory[=], which describes how two bodies adhere together and what deformation they undergo when in contact with each other (Johnson et al., 1971) failed to provide a reliable prediction of the adhesion force (Thormann et al., 2008). Hence, such contradictions to theoretical expectations may occur. The interesting finding of the *A. niger* 1988 resembling the force measurements of the glass sphere rather than the polystyrene sphere might be due the spiny surface of the spore since it is known that small interfacial gaps, due to surface asperities or dust spheres, will strongly influence the adhesion because attractive surface forces decrease rapidly with increasing separation (Thormann et al., 2008).

This work suggests that the spheres and spore sizes strongly influenced the results observed. When using the polystyrene spheres of a defined size, the surface roughness was found to influence the attachment force to the surface. The glass spheres demonstrated more widely distributed attachment measurements consistent with conidia morphologies of an irregular shape and size.

CONCLUSION

The polystyrene sphere with the defined size demonstrated a narrower range and lower force of attachment than the glass spheres to the surfaces which coincided with the force measurements demonstrated by *A. niger* 1957 conidia. The polystyrene sphere force of attachment was least on the spin coated surfaces which could possibly have been due to the mobility of the co-polymer surfaces. The glass sphere demonstrated results that revealed a wider range of higher force measurements, similar to those seen with *A. niger* 1988 and *A. pullulans* conidia. Thus, it is suggested that the observed results could be due to the size of the spheres and conidia utilized on the cantilevers and their binding energies with the surfaces. Overall, the PMMAsc surface demonstrated the lowest force of attachment measurements for both the polystyrene and glass spheres.

Limitations of the study

The main limitations of this study included that although this non-biological system demonstrates force measurements that could be related to fungal spore force of attachment measurements, there are many caveats since fungal spores are incredibly complex and the amount of influence of the spore biochemistry on the measurement of interactions will be dependent on whether they are determined at the macroscale, microscale, or nanoscale. Thus, this system may not be applicable to all spore types and would have to be correlated accordingly.

Resource availability

Lead contact

Professor Kathryn Whitehead.

Material 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.2020.101962.

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AUTHOR CONTRIBUTIONS

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

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

Use of spherical particles to understand conidial attachment to surfaces using atomic force microscopy

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Supplemental Information.

Figure Legend

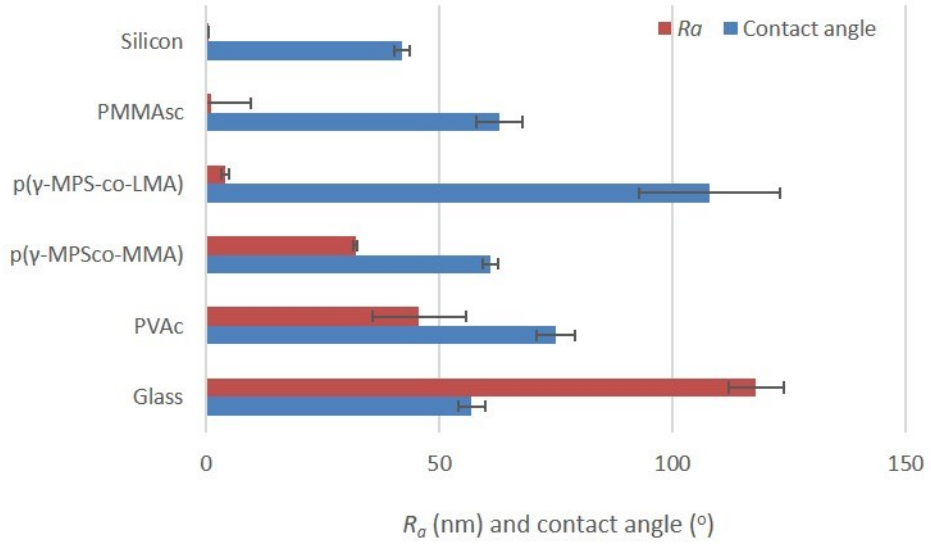

Figure S1. R_a and contact angle values of surfaces described as in previous work, related to Figure 1 (Whitehead et al., 2011; Liauw et al., 2020; Whitehead et al., 2020).

Transparent Methods

Surface production and analysis

Nine different surfaces presenting different properties were utilised in this study. Silicon wafers were purchased from Montco Technologies (PA, USA). Glass cover slips were purchased from Scientific Laboratory Supplies (20 mm x 20 mm) (UK). The poly(vinyl acetate) (PVAc) surfaces were produced using compression moulding. A hydraulic heated press (Press type 202B-50 ton Bradley and Turton Ltd., UK), was heated to 50 ˚C for PVAc. A steel frame mould (outer dimensions: 16.3 cm x 19.5 cm, inner dimensions: 8.8 cm x 15.4 cm) was placed in the centre of a 0.5 mm FEP film (FEP Shelman, UK) and two stainless steel sheets (30.2 cm x 23 cm) were placed on either side of the release sheets. The assembly was heated for five min and the sheets were removed from the press and separated. Granules of PVAc (13.5 g) (BDH, UK) were spread into the centre of the mould and the sheets were placed together and placed in the electric press for 10 min. These were removed and immediately transferred into a water cooled press (Francis Shaw and Company, UK) for 5 min. The sample was removed from the cold press and released from the mould. The spin coated surfaces were fabricated by dropping the dissolved polymer onto silicon wafer disks ensuring the entire disk surface was covered. The samples were spun at 2000 rpm for 10 - 15 s. The PLMA surface used lauryl methacrylate copolymerised with γ- MPS (p(γ-MPS-co-LMA). The PMMAsc surfaces used spin coating of 3-methacryloxypropyltrimethoxysilane (γ- MPS) copolymerised with methyl methylacrylate (MMA). *p*(γ-MPS-co-MMA) was produced via the copolymerisation of MMA with γ-MPS (in a ratio of 90 to 10) (Whitehead et al., 2011; Liauw et al., 2020; Whitehead et al., 2020).

Strength of attachment measurements

Strength of attachment measurements using regular cantilevers

All the strength of attachments were carried out using an Explorer AFM (Veeco Instruments, UK). To measure the adhesion force between the particle probes and the substratum, the probe was brought into momentary contact with the surface. AFM strength of attachment measurements were obtained from force-distance curves. To convert the cantilever deflection to a force, the spring constant of the cantilever and the zero of the force were defined and the cantilever deflection (d) was converted into a force (F) through the use of Hooke's law;

$$F = kd \quad (1)$$

where k is the cantilever spring constant, which was determined for each cantilever (Whitehead et al., 2011). The cantilever was deflected by a distance (d). The corrected curve was determined by plotting F as a function of ($z - d$), where z is the vertical displacement of the piezoelectric scanner (Whitehead et al., 2011). To calculate the force, the spring constant was multiplied by the displacement (Hookes Law), and the zero of the force was subtracted from the image setpoint. The resultant value was converted to nN from nA by multiplying the applied force by the reciprocal of the slope and the cantilever spring constant (Whitehead et al., 2011).

Modification of tipless cantilevers

Tipless cantilevers (Veeco, UK) were glued onto cantilever stubs (Veeco, UK) using a two-phase silver mounting adhesive. Ten microlitres of glass colloid particles in sterile distilled water or 10 μL of suspended polystyrene colloid particles were pipetted into a new, sterile, clean Petri dish, and the diluent was evaporated off in a class II flow hood. After the diluent had been evaporated, the samples were thoroughly dried in a phosphorous pentoxide desiccator for three days. Using double sided tape, a 20 cm x 20 cm glass cover slip was attached to an AFM mounting disc (Veeco, UK). A small number of dried conidia were removed from the Petri dish placed onto a coverslip. Next, a small amount of cyanoacrylate gel (Bostik, UK) was added to the coverslip, attached to the mounting disk and placed into the AFM (Whitehead et al., 2011). Using the AFM camera and XY automated translation stage,

the tipless cantilever was moved to the edge of the cyanoacrylate gel and was lowered in the z plane until the cantilever was in momentary contact with the gel, then quickly moved in the z plane. The cantilever was then moved across the coverslip until a suitable colloid particle was found and was again lowered in the z plane until in contact with a colloid particle. The cantilever was left in contact with the colloid particles for 10 s before being lifted vertically. Next, the cantilever was removed from the AFM and left for 24 h to allow the adhesive to fully cure. All cantilevers with attached colloid particles were examined using light microscopy prior to use. Before each experiment the spring constant of the cantilever was determined by measuring the mechanical response of the cantilever to thermal noise as a function of time using the AFM software and the exact spring constant recorded was incorporated into the perpendicular force equations.

Light microscopy of spores

The spores were prepared as in previous work (Whitehead et al., 2020). Light microscopy of the fungal spores was carried out using either a x 400 or x 1000 working distance objective using a Nikon Eclipse E600 (Nikon, UK). The microscope was mounted with a Hitachi HV-D37P colour camera and used a Lucia Image Analysis package (Nikon, UK).