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Molybdenum Disulphide Surfaces to Reduce *Staphylococcus aureus* and *Pseudomonas aeruginosa* Biofilm Formation

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ABSTRACT: The reduction of bacteria and biofilm formation is important when designing surfaces for use in industry. Molybdenum disulphide surfaces ($\text{MoS}_{2\text{SUR}}$) were produced using MoS_2 particle ($\text{MoS}_{2\text{PAR}}$) sizes of 90 nm, 2 μm and 6 μm containing $\text{MoS}_{2\text{PAR}}$ concentrations of 5%, 10%, 15% and 20%. These were tested to determine the efficacy of the $\text{MoS}_{2\text{SUR}}$ to impede bacterial retention and biofilm formation of two different types of bacteria, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The $\text{MoS}_{2\text{SUR}}$ were characterised using Fourier Transform InfraRed Spectroscopy, Ion Coupled Plasma Atomic Emission Spectroscopy, Scanning Electron Microscopy, Optical Profilometry and Water Contact Angles. The $\text{MoS}_{2\text{SUR}}$ made with the smaller 90 nm $\text{MoS}_{2\text{PAR}}$ sizes demonstrated smaller topographical shaped features. As the size of the incorporated $\text{MoS}_{2\text{PAR}}$ increased, the $\text{MoS}_{2\text{SUR}}$ demonstrated wider surface features, and they were less wettable. The increase in $\text{MoS}_{2\text{PAR}}$ concentration within the $\text{MoS}_{2\text{SUR}}$ groups did not affect the surface topography but did increase wettability. However, the increase in $\text{MoS}_{2\text{PAR}}$ size increased both the surface topography and wettability. The $\text{MoS}_{2\text{SUR}}$ with the smaller topographical shaped features, influenced the retention of the *S. aureus* bacteria. Increased $\text{MoS}_{2\text{SUR}}$ topography and wettability resulted in the greatest reduction in bacterial retention and the bacteria became more heterogeneously dispersed and less clustered across the surfaces. The surfaces that exhibited decreased bacterial retention (largest particle sizes, largest features, greatest roughness, most wettable) resulted in decreased biofilm formation. Cytotoxicity testing of the surface using cell viability demonstrated that the $\text{MoS}_{2\text{SUR}}$ were not toxic against HK-2 cells at $\text{MoS}_{2\text{PAR}}$ sizes of 90 nm and 2 μm . This work demonstrated that individual surfaces variables ($\text{MoS}_{2\text{SUR}}$ topographic shape and roughness, $\text{MoS}_{2\text{PAR}}$ size and concentration) decreased bacterial loading on the surfaces, which then decreased biofilm formation. By optimising $\text{MoS}_{2\text{SUR}}$ properties, it was possible to impede bacterial retention and subsequent biofilm formation.

KEYWORDS: *Molybdenum disulphide surfaces, Bacteria, Biofilms, Retention, Antifouling, Cytotoxicity*

INTRODUCTION

Biofouling is an issue that is of great concern to many industries including the healthcare sector, water and food industries. In the food industry, microbial contamination of surfaces can result in product spoilage, and ultimately lead to health issues of the consumer.¹ Thus, the preference of bacteria to become retained onto a surface is highly undesirable due to the ever present possibility of biotransfer.²

The retention of bacterial cells to materials is an initial step in which contamination of surfaces, food products or medical equipment can occur.³ Bacterial cells typically attach to a surface following a two-step process. Initial, reversible attachment, is thought to be influenced more by physicochemical forces while irreversible retention may be more influenced by surface roughness.⁴ Planktonic bacteria prefer to be attached to a surface, and once they have attached they form a biofilm.⁵ The aggregation of biofilms on abiotic or biotic surfaces are a ubiquitous behaviour of bacteria. The biofilms which bacteria produce are covered by a hydrophilic, exopolymeric substance, which protects the bacteria making them more resilient to antimicrobials.⁶ Thus, bacteria in such formations are much more difficult to treat and kill than planktonic bacterial species. This makes biofilm formation a much more difficult problem to treat and/or eradicate. The formation of biofilms in the food processing industries is a leading cause of foodborne disease spread.⁷ In clinical settings, the continual presence of biofilms increases the risk of healthcare associated infections (HCAI).⁸ Biofilm formation of medical instrumentation or via transmission from water systems may lead to potential pathogen transfer and subsequent increased morbidity and mortality, posing a significant impact on public health and wellbeing.⁹ Moreover, the cleaning of surfaces is a substantial financial burden on both the industrial and medical professions.¹⁰

Key strategies for disrupting bacterial retention may be targeted towards the development of novel surfaces. Although some 2D-materials containing metals have been suggested (reduced graphene oxide/silver in nanocomposite and zinc-graphite composite coatings) there is also a growing requirement for surfaces to be more ecological friendly, and potentially less cytotoxic by using materials that do not leach their individual components.^{11,12} Two dimensional (2D) materials and particles have been suggested to demonstrate antifouling activity with low toxicity.^{5,13,14} Although much work has been carried out on the antifouling properties of graphite and graphene/graphene oxide amongst nanomaterials, the transition metal dichalcogenides have received little attention in the determination of their ability to impede bacterial retention and subsequent biofilm formation, with minimal cytotoxic properties.^{11,15-18} One such material with the potential to be used as a low fouling surface is

MoS₂_{SUR}. Molybdenum disulphide is mainly obtained as a secondary product from the mining of copper.¹⁸ Since both molybdenum and sulphur are earth abundant elements, this combined with there being several commercial scale methodologies for producing MoS₂, allow it to be a relatively cheaply sourced material. MoS₂_{SUR} potentially has inherent antifouling properties, better than those of other materials such as graphene oxide due to its surface properties. MoS₂_{SUR} and MoS₂_{PAR} are composed of a monolayer of molybdenum atoms that are between parallel sulphur atom layers which are held together loosely by van der Waals forces.^{19,20} MoS₂_{SUR} has unique physicochemical and mechanical properties, with a lowered amount of functional groups.^{21,22} MoS₂_{SUR} also have extremely low friction coefficients which means that fouling it likely to be lowered on such substrates.²³ Thus, the use of such surfaces as antifouling materials may result due to their surface and anti-frictional properties. Investigation into the antifouling nature of such modified surfaces is required, since factors such as surface topography, chemistry and physicochemistry have been demonstrated to influence microbial retention, with microbial retention and subsequent biofilm formation.

Often the relationship between the surface properties and the retention and distribution of bacteria across surfaces is determined using factors such as percentage coverage.²⁴ However, in order to understand such interactions in more detail, mathematical analysis, such as using multifractal analysis can be used to give further insight into the surface interface:bacterial relationships.²⁴⁻²⁶ The relationship between initial bacterial load and the rate of biofilm formation is also unclear. Studies investigating the effects antifouling effects of 2D materials including MoS₂_{SUR} are weighted heavily towards testing against planktonic bacteria. However, it is also important to determine the effects that such surfaces have towards bacterial biofilms, since biofilm formations are more prevalent on surfaces in industry and the environment and they are extremely difficult to impede.

The aim of this work was to determine the effect of MoS₂_{SUR} on the retention and subsequent biofilm formation of *Staphylococcus aureus* and *Pseudomonas aeruginosa* and determine the cytotoxic effect of the MoS₂_{SUR}.

MATERIALS AND METHODS

MoS₂_{SUR} Surface Screen Printing. The MoS₂_{PAR} were analytical grade and were used as received from Sigma-Aldrich (UK) without further purification. The average lateral width, purity of 98-99%, and a molybdenum content of 58.4% - 61.4% of the MoS₂_{PAR} in powder was determined according to the manufacturer specifications (Sigma-Aldrich, UK).²⁷ To produce MoS₂_{PAR} containing surfaces, MoS₂_{PAR} sizes of 90 nm, 2 µm and 6 µm (Sigma-Aldrich, UK)

were incorporated into commercially available graphite ink (Gwent Electronic Materials, UK) using the weight percent of MoS₂PAR within the mass of the ink formulation used in the printing process;

$$\% = (M_P / M_I) \times 100 \quad [1]$$

where M_P is the mass of the particulate added and M_I is the total combined weight of the ink formulation. The MoS₂PAR containing ink was screen-printed onto an underlying layer of a cured carbon graphite ink formulation, which was printed onto a polyester flexible film (Autostat, UK 250 μ m thickness) using the in-house fabricated screen print stencils (microDEK1760RS DEK, Weymouth). The percentage mass of MoS₂PAR was 5%, 10%, 15% and 20% for each of the three different MoS₂PAR sizes. The MoS₂SUR screen printed surfaces were cured for 30 min at 60 °C, to remove trace solvents.

MoS₂SUR Surface Preparation. The MoS₂SUR printed surfaces were cut using scissors to form a 1 cm x 1 cm square. The surface was sterilized using 70% ethanol (Sigma-Aldrich, UK) for 10 min, and rinsed using a pipette and 2.5 mL of sterile distilled water at a 45° angle, then placed in a class II airflow cabinet to dry for 1 h.

Fourier Transform Infra-Red Spectroscopy (FTIR). A Thermo-Winslet Continuum FTIR microscope was used for analysis of the MoS₂SUR. The attachment used was a type A MCT detector. The aperture was used at 200 mm x 200 mm and the spectra of the MoS₂SUR was acquired using Omnic 5.2 software.

Inductively coupled plasma atomic emission spectroscopy (ICP-AES). The MoS₂SUR were placed into 15 mL Falcon tubes (Fisher Scientific, UK). Eight millilitres of sterile distilled water was added, and the samples were incubated at 37 °C for 24 h with agitation (150 *rpm*). The solution was removed using a syringe and filtered using leur lock syringes (ThermoFisher, UK) with 0.2 μ m sterile syringe filters (Starstead, UK). The solutions were analysed using an ICP–AES (Thermo scientific, iCAP6300 DUO, UK). The analysis parameters used were pump settings: flush pump rate – 50 *rpm*, analysis pump rate – 50 repetitions per min (*rpm*) and pump tubing – Tygon Orange / White. The source setting were as follows; RF power – 1250W, auxiliary gas flow – 0.5 L / min and nebuliser gas flow – 0.55 L / min with a sample flush time of 46 s ($n = 3$).

Scanning Electron Microscopy (SEM). The MoS₂SUR were mounted on aluminium SEM mounts (Agar Scientific, UK) with double-sided conducting carbon tabs (Agar Scientific, UK). The MoS₂SUR were characterised using a Zeiss Supra 40VP field emission gun scanning electron microscope (Zeiss, UK) and the following parameters; acceleration voltage -2.00kV, working distance 4.1 mm - 4.5 mm, SE2 detector, magnification at 10,000x.

Surface Roughness. Optical profilometry (Zemetrics, Germany), was used to determine the surface topography of the MoS₂_{SUR}. Analysis of the surface roughness was carried out qualitatively via images, and quantitatively via *S* values; *S_a*, *S_q* and *S_{pv}* (arithmetical mean height, mean square roughness and mean maximum height respectively). The average peak and valley values from the line profiles were also taken (*n* = 10).

Water Contact Angle Measurements. Contact angle measurements were determined of the MoS₂_{SUR} at room temperature using the sessile drop technique.¹⁹ HPLC grade water (BDH, UK) at a drop size of 5 µL was deposited onto the horizontal substrata and the measurements were determined using a goniometer (KRUS GMBH, Germany) (*n* = 3).

Stock Cultures. Stock cultures of *S. aureus* (NCTC 12981) and *P. aeruginosa* (PA01) were used for all microbiological assays. Stock solutions were stored in the freezer at -80°C. Cultures were thawed and inoculated onto tryptone soya agar (TSA) (Oxoid, UK) and incubated for 24 h at 37 °C. Stock cultures were re-frozen immediately after use. For maintenance of bacterial physiology, inoculated plates were stored in the fridge at 4 °C and replaced every month.

Preparation of Bacterial Suspensions. A single colony of bacteria was added to 10 mL of tryptone soya broth (TSB) and vortexed for 10 s. The bacterial inoculum was incubated at 37 °C for 24 h in an orbital shaking incubator at 200 *rpm*. Bacterial cultures were centrifuged (Sigma-ALdrich 3-16L, UK) at 1721 *g* for 10 min, and the bacterial pellet was washed in 10 mL of 0.85% saline solution (Oxoid, UK) and vortexed for 10 s. The washing procedure was carried out twice. The bacteria were re-suspended to an optical density (OD) of 1.0 ± 0.1 at 540 nm using a spectrophotometer (Jenway 6305, UK). Colony forming units (CFU/ mL) were determined using serial dilutions and were 8.40 x 10⁸ CFU/ mL for *S. aureus* and 2.88 x 10⁸ CFU/ mL for *P. aeruginosa*.

Scanning Electron Microscopy. For visualisation of the single cell species, 100 µL of washed, single species bacterial suspensions were added to 10 mm x 10 mm silicon wafer surfaces (Montco Silicon Technologies Inc., USA) and dried for 30 min at room temperature in a class 2 microbiology cabinet. The single cell species, or bacterial biofilms were placed in 4% v/v glutaraldehyde (Agar Scientific, UK) overnight, then dehydrated in an absolute ethanol series of 30%, 50%, 70%, 90% and 100% v/v ethanol for 10 min. The samples were stored in a desiccator until they were sputter coated with a gold-palladium mix and imaged using SEM.

Retention Assay and Epifluorescence Microscopy. Three replicate MoS₂_{SUR} were placed horizontally in a Petri dish and fixed using double sided tape. Thirty milliliters of standardized bacterial inoculation were added and incubated for 1 h at 37 °C without agitation.

Following incubation, the bacterial inoculum was removed and discarded using a sterile pipette. Non-adhered cells were gently removed from the MoS₂SUR with sterile distilled water (dH₂O) (2.5 mL), rinsing at a 45° angle for 5 s. The MoS₂SUR were dried in a class two airflow cabinet for 1 h before being stained with 0.03% acridine orange (Sigma-Aldrich, UK) dissolved in 2% glacial acetic acid (Sigma-Aldrich, UK). The stain was left on for 2 min before removing the MoS₂SUR and rinsing at a 45° angle for 5 s with 2.5 mL of dH₂O and air drying in the dark for 1 h. The substrata plus adhered microorganisms were visualized using epifluorescence microscopy and a F-View II black and white digital camera (Nikon Eclipse E600, Japan) using a 100 x oil immersion lens and a Nikon B-2A fluorescence filter. The MoS₂SUR were analysed using Cell-F software (Olympus, UK) ($n = 3$).

Multifractal Analysis to Determine Bacterial Coverage (Retention), Distribution, Density, Dispersion and Clustering. The epifluorescence images of the retention of the bacteria on the MoS₂SUR were analysed using multifractal analysis using the MATLAB®, Image Processing Toolbox® whereby the datasheets were converted to greyscale images. The properties of the typical theoretical multifractal datasets were computed for certain motifs using MATLAB.²⁴ Multifractal matrix (datasheets) of size 512 x 512 were computed by overlaying the given motifs one on top of another. In this case, a 2 x 2 matrix (motif) was overlaid (using iteration) to generate a 4 x 4 matrix image followed by an 8 x 8 matrix image until completion. The parameters α_{\max} and α_{\min} were used to measure the asymmetry of the curve ($\Delta\alpha_{AS}$). Symmetry of the $f(\alpha)$ curve indicates homogeneity and a lack of clustering. Asymmetry in the $f(\alpha)$ curve indicates clustering of gaps, if the curve is left skewed, and clustering of bright pixels, if the curve is right skewed. The height of the $f(\alpha)$ curve gives a measure of density of the pixels spread across the images, and the width gives a measure of dispersion. For the grayscale images used in this paper, D_0 , the maximum value of the $f(\alpha)$ curve (when $q = 0$), gives a numerical value for the density of the cells on the surface, whilst $\Delta\alpha = \alpha_{\max} - \alpha_{\min}$, describes the heterogeneity of the cell spread on the surface. A skewed curve to the right indicated a clustering of the bacteria, whilst a left skewed curve gives an indication of clustering of gaps. The width of the $f(\alpha)$ curves gave a measure of dispersion of the bacteria. Aanalysis of the images was undertaken to determine the total retention of cells as a percentage ($n = 3$).

Crystal Violet Biofilm Assay. A bacterial inoculum was prepared for both bacterial species, as in the preparation of bacterial suspensions, but with the second broth re-suspension using tryptone soya broth (Oxoid, UK) instead of saline. A 12 well plate (Fisher Scientific, UK) was used for each bacterium and 1 mL of inoculated broth was added to each well. Sterile MoS₂SUR were added to the wells and negative controls were carried out. The plates were

wrapped in Parafilm (Fisher Scientific, UK) and incubated at 37 °C for 24 h. After incubation, the broth containing the bacteria was removed from the wells using a pipette, leaving the MoS₂SUR with attached biofilm. The MoS₂SUR were gently rinsed with sterile water at a 45° angle for 5 s to avoid removing the attached biofilm. One millilitre of 0.03% crystal violet solution (Prolab, UK) was added to each well and left to stand for 30 min before removing the crystal violet from the well using a pipette. The biofilm attached to the MoS₂SUR was rinsed with sterile water. Washing of the wells was repeated a further two times to ensure removal of all excess stain and loosely adhered cells. One millilitre of 33% glacial acetic acid (Fisher Scientific, UK) was added to each well and left to stand for 30 min. The optical density of each sample was measured at 540 nm against a blank of 33% glacial acetic acid ($n = 3$).²⁸

Eukaryotic Cell Culture. Immortalised renal human proximal tubular (HK-2) cells (ATCC CRL-2190) were maintained in growth media medium (1:1 of Dulbecco's modified Eagle medium (DMEM): Ham's F-12 medium supplemented with 10% foetal bovine serum and penicillin/streptomycin (Life Technologies, UK). The cells were incubated at 37 °C in 5% CO₂ and the medium was refreshed every 48 h. At 80% confluence, the cells were rinsed with sterile phosphate buffered saline (PBS) and detached using a trypsin solution (Life Technologies, UK) to be counted by haemocytometer and seeded at required density in subsequent experiments.

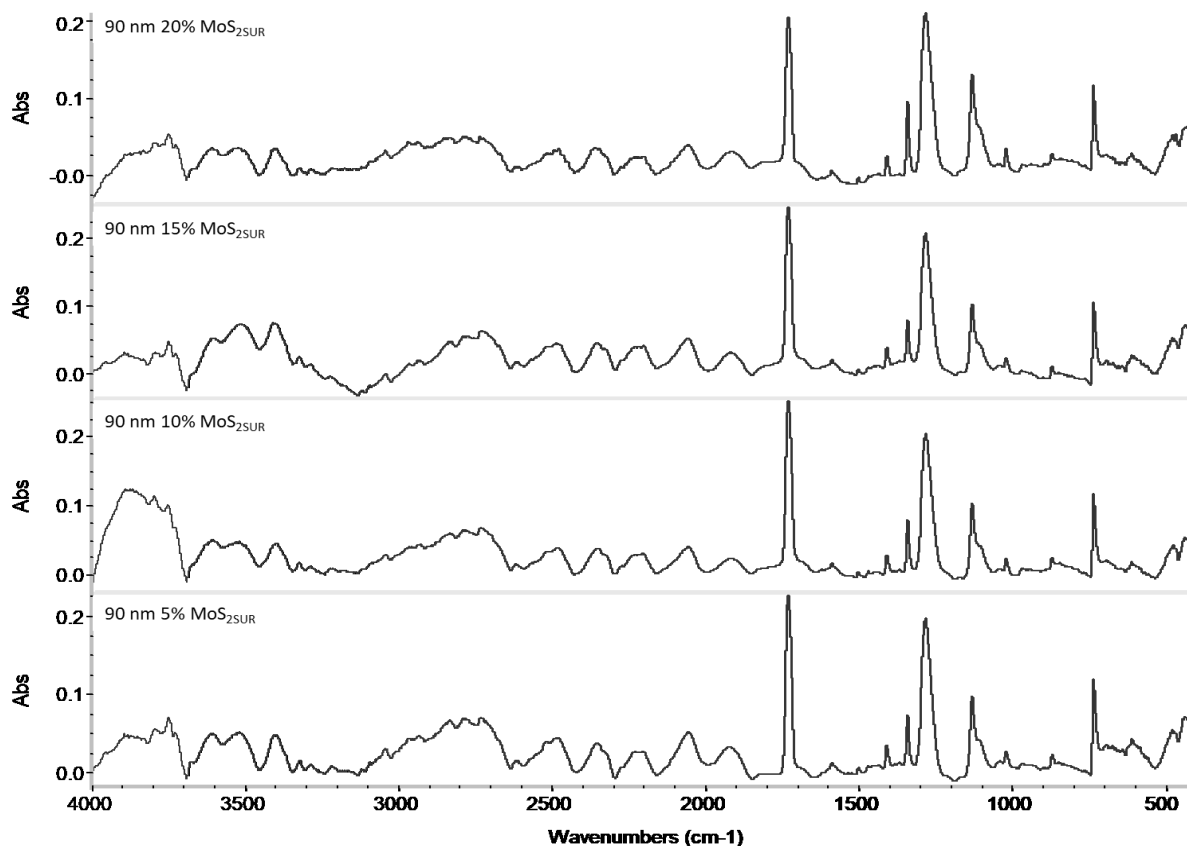
HK-2 Cell Viability Assays. To evaluate the effect of leaching of MoS₂ on the viability of HK-2 cells, sterile MoS₂SUR were inserted into wells of 96 well plates. Then 200 µL of serum-free medium (SFM) (DMEM: Ham's F-12 medium supplemented with penicillin/streptomycin) were added to each well and the plates were incubated for 48 h at 37 °C in 5% CO₂. Control medium used as a negative control were generated by incubation of polyester flexible film substrates with 200 µL of SFM.

HK-2 cells were seeded in separate 96 well plates (Thermo Scientific, UK) at a density of 5,000 cells per well. The cells were incubated for 48 h in growth medium whereby reaching 80% confluency, and then they were growth arrested for 24 h in 200 µL of SFM. The medium was removed from each well and the cells were then exposed to 170 µL of SFM for 48 h at 37 °C in 5% CO₂. Following incubation, 9 µL of viability reagent WST-8 (tetrazolium-based cell counting kit-8, Tebu-Bio, UK) was added to each well and incubated for 1 h at 37 °C as per manufacturers recommendations. The absorbance of each well was measured at 450 nm and 650 nm using a plate reader (Thermo Scientific, UK) ($n = 6$).

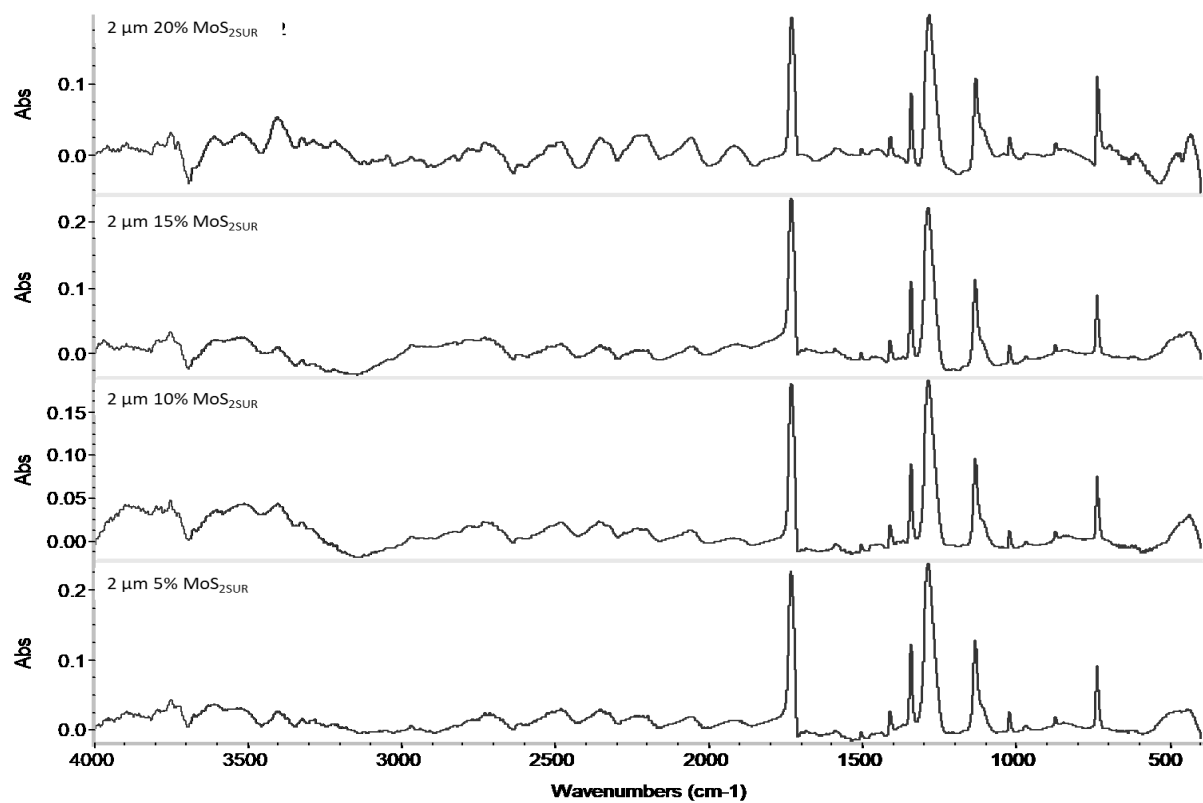
Statistical Analysis. *p* values were calculated at the 95% confidence limits using student *t*-tests. Graphs were drawn in Microsoft Excel 2016. The results were reported as \pm standard error. In all cases, $p < 0.05$ was considered statistically significant.

RESULTS

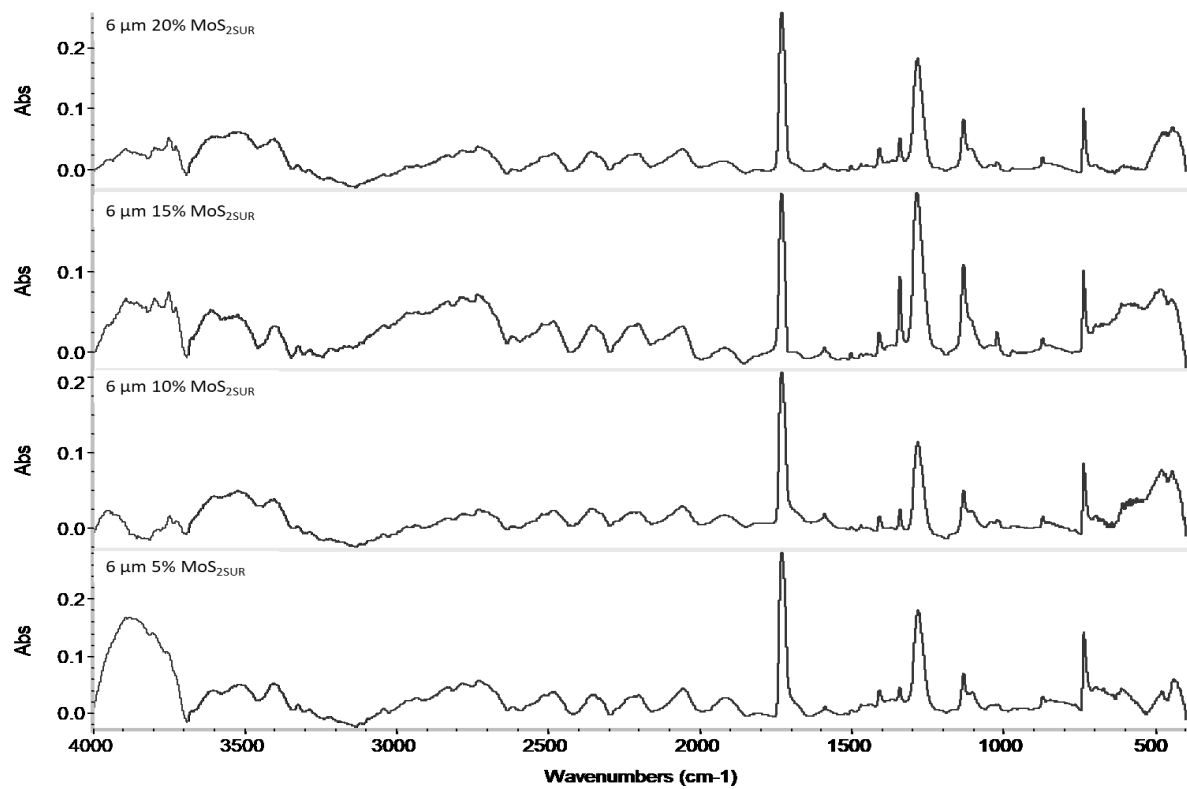
Fourier Transform Infrared Spectroscopy (FTIR) of the MoS₂SUR. The FTIR analysis of the MoS₂SUR demonstrated similar chemical moieties for all the MoS₂SUR (Figure 1). Analysis of the FTIR spectra extended from 4000 cm⁻¹ to 400 cm⁻¹. Spectra between 3969 cm⁻¹ – 3512 cm⁻¹ were attributed to O-H stretching. N-H groups were determined in the 3405 cm⁻¹ region. Other C dominated groups were demonstrated including C-H stretching, (2790 cm⁻¹, 2732 cm⁻¹, 736 cm⁻¹), O=C=O and C-O (2356 cm⁻¹, 1283 cm⁻¹ respectively), C-N and C=N (2235 cm⁻¹, 1410 cm⁻¹, 1343 cm⁻¹ and 2056 cm⁻¹ respectively) were determined which may be indicative of the presence of the vinyl fillers. A graphite peak was observed at 2483 cm⁻¹ for all the surfaces tested. Sulphur containing species were demonstrated at 1410 cm⁻¹ and 1134 cm⁻¹. The molybdenum species were determined at 613 cm⁻¹ and the Mo-S characteristic band was observed at 479 cm⁻¹. The bands in the lower frequency of the 400 cm⁻¹ spectra may be characteristic of residual solvents used to make the graphitic inks.



a)



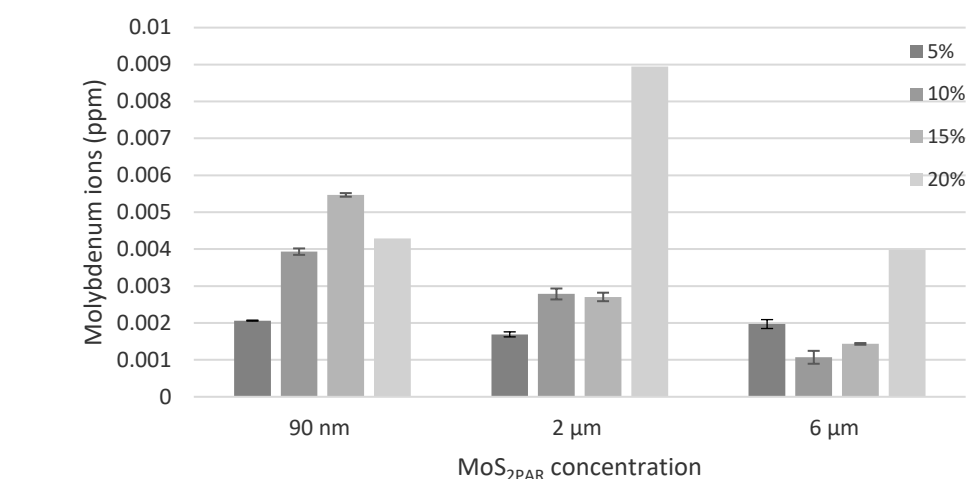
b)



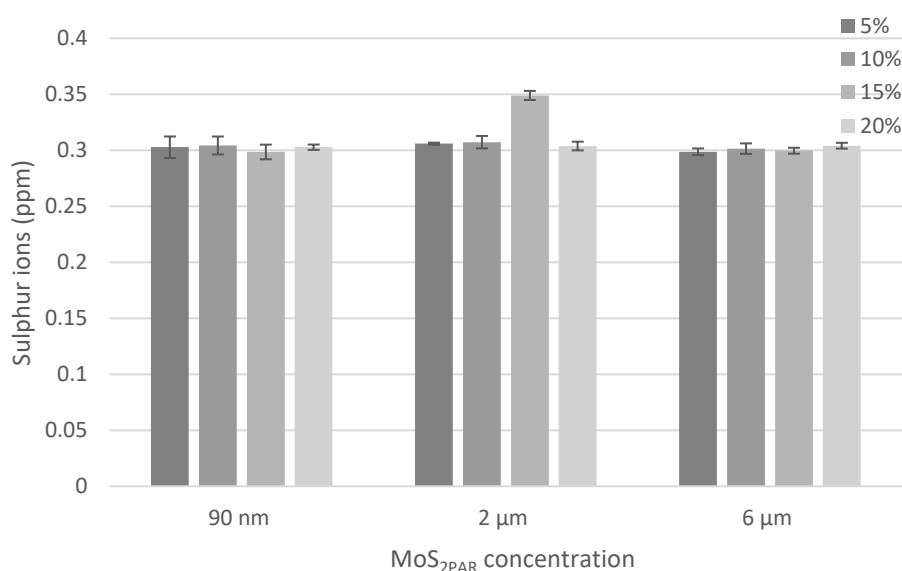
c)

Figure 1. FTIR spectra of the MoS₂_{SUR} incorporated with MoS₂_{PAR} of 90 nm b) 2 µm and c) 6 µm at MoS₂_{PAR} loadings of 5%, 10%, 15% and 20%.

Inductively Coupled Plasma Atomic Emission Spectroscopy. ICP-AES was carried out using the surfaces to determine the leaching of the ions. It was demonstrated that there was no significant difference in the leaching of either the molybdenum ions (Figure 2a), or the sulphur ions (Figure 2b) from the surfaces. For both molybdenum and sulphur ions, the amount detected leaching from the surfaces was considered to be negligible.



a)



b)

Figure 2. ICP-AES of the MoS₂_{SUR} demonstrating the leaching of molybdenum and sulphur ions (ppm).

Scanning Electron Microscopy of MoS₂. SEM was used to quantitatively analyse the MoS₂_{SUR} at MoS₂_{PAR} sizes of 90 nm, 2 μ m and 6 μ m, and at each concentration (5%, 10%, 15% and 20%). The shape of the MoS₂_{PAR} were irregular in shape and size, and they were distributed throughout the matrix of the graphitic ink used to make the MoS₂_{SUR}. The size of the MoS₂_{PAR} varied and it was estimated that there was a 10% margin of error from the average MoS₂_{PAR} size. The coverage of the MoS₂_{PAR} was regular throughout the MoS₂_{SUR}, but complete coverage of the MoS₂_{SUR} was not evident. (Figure 3a - c). On the scanning electron micrographs a powder like coating was observed, which was the binder used to prevent the MoS₂_{SUR} from flaking.

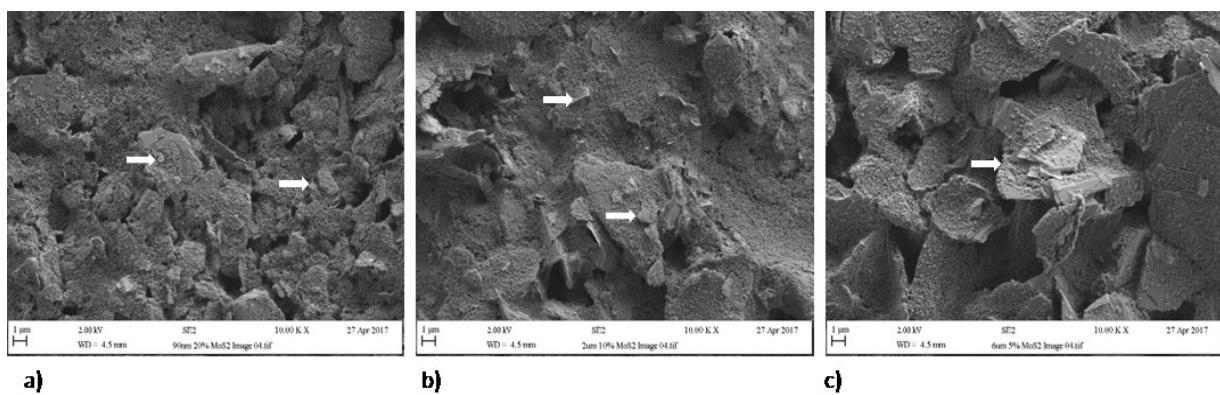


Figure 3. SEM images of the MoS₂_{SUR} made with the a) 90 nm, b) 2 μ m and c) 6 μ m MoS₂_{PAR}. The white arrows demonstrate the MoS₂_{PAR} within the graphite ink matrix.

Surface Roughness Characterisation. Optical profilometry was carried out to determine the roughness (Figure 4 a-c) and shape of the topography of the MoS₂_{SUR} (Figure 4d-f). It was demonstrated that there was complete coverage across the surfaces of the graphitic ink and in agreement with the SEM images, there were peaks on the topographical profiles corresponding to the protruding MoS₂_{PAR}. It was evident from the linear profiles that the shape of the topographical features were sharper and more pointed for the MoS₂_{SUR} made with the 90 nm MoS₂_{PAR} (Figure 4b), than with the 2 μ m MoS₂_{PAR} (Figure 4d). The MoS₂_{SUR} which incorporated the 6 μ m MoS₂_{PAR}, clearly demonstrated the largest and most square shaped surface topographical features (Figure 4f).

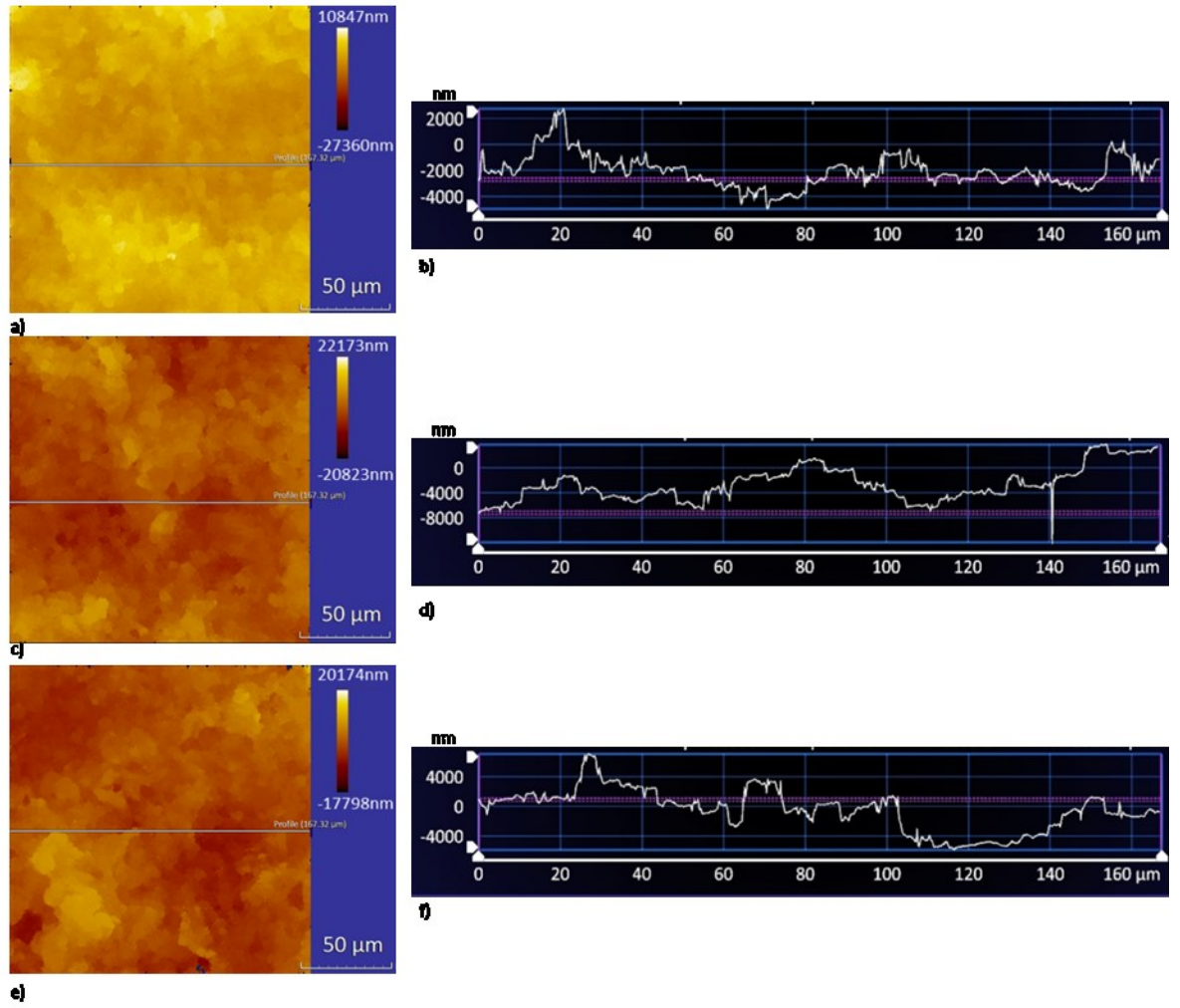


Figure 4. Optical profilometry maps and linear profiles demonstrating the surface coverage and shape of the topographic features on the MoS₂_{SUR} a/b) 90 nm, c/d) 2 μ m and e/f) 6 μ m MoS₂_{PAR} incorporated surfaces.

Line Profiles. The line profiles of the surface roughness were analysed to determine the size of the MoS₂_{SUR} peaks and valleys (Table 1). The results showed that the MoS₂_{SUR} fabricated with the 6 μ m MoS₂_{PAR} at 5%, 10%, 15% and 20% MoS₂_{PAR} concentration possessed the largest average peak height (774.23 nm, 18.97 nm, 1097.14 nm and 1133.77 nm respectively) and the widest valleys (13.49 nm, 12.26 nm, 13.08 nm and 13.90 nm respectively). However, only the 6 μ m peak height was significantly different to the other surfaces, except for the 6 μ m MoS₂_{SUR} with a 10% loading of MoS₂_{PAR}, where a significant reduction in average peak height was demonstrated (18.97 nm). The lowest average peak height was observed on the 2 μ m MoS₂_{SUR} (27.46 nm, 17.74 nm, 16.53 nm and 16.7 nm respectively), whilst the 90 nm MoS₂_{SUR} demonstrated the smallest average valley widths (10.22 nm, 12.26 nm, 10.22 nm and 11.04 nm respectively).

Table 1. Peak and valley widths and heights of the MoS_{2SUR} incorporated with 90 nm, 2 μ m and 6 μ m MoS_{2PAR} at concentrations of 5%, 10%, 15% and 20%.

Surface MoS _{2PAR} size and concentration	Largest peak height (nm)	Smallest peak height (nm)	Largest valley width (nm)	Smallest valley width (nm)
5% 90 nm	22.81 \pm 2.9	0.01 \pm 0.002	83.39 \pm 6.2	10.22 \pm 0.7
10% 90 nm	17.17 \pm 2.1	0.02 \pm 0.003	129.58 \pm 10.1	12.26 \pm 0.0
15% 90 nm	23.41 \pm 3.5	0.01 \pm 0.002	105.87 \pm 7.3	10.22 \pm 0.7
20% 90 nm	34.05 \pm 4.3	0.01 \pm 0.005	88.71 \pm 4.7	11.04 \pm 0.6
5% 2 μ m	27.46 \pm 5.4	0.01 \pm 0.003	89.52 \pm 10.6	10.22 \pm 0.7
10% 2 μ m	17.74 \pm 2.3	0.01 \pm 0.002	121.41 \pm 9.4	12.26 \pm 0.0
15% 2 μ m	16.53 \pm 0.8	0.02 \pm 0.001	114.92 \pm 5.7	10.34 \pm 0.5
20% 2 μ m	16.70 \pm 3.0	0.02 \pm 0.002	109.14 \pm 4.9	12.26 \pm 0.0
5% 6 μ m	774.23 \pm 293	0.64 \pm 0.2	134.08 \pm 8.8	13.49 \pm 0.6
10% 6 μ m	18.97 \pm 2.7	0.01 \pm 0.002	104.65 \pm 6.0	12.26 \pm 0.0
15% 6 μ m	1097.14 \pm 410	0.36 \pm 0.1	123.04 \pm 10.3	13.08 \pm 0.5
20% 6 μ m	1133.77 \pm 146	0.21 \pm 0.1	142.26 \pm 8.8	13.90 \pm 0.7

MoS_{2SUR} Roughness Parameters. Optical profilometry was used to obtain the roughness values of the MoS_{2SUR} (Figure 5). It was determined that for the MoS_{2SUR} of each MoS_{2PAR} size group (90 nm, 2 μ m and 6 μ m), when the concentrations of each MoS_{2PAR} size was compared (5%, 10%, 15% and 20%) no significant differences ($p > 0.05$) were demonstrated in the S_a (arithmetical mean height) (Figure 5a), S_q (mean square roughness) (Figure 5b) and S_{pv} (mean maximum height) (Figure 5c) values. However, when the MoS_{2SUR} in order of MoS_{2PAR} size were compared with one another, it was demonstrated that as MoS_{2PAR} size increased, so did the MoS_{2SUR} roughness. The 90 nm, 2 μ m and 6 μ m MoS_{2SUR} demonstrated the same trends in S_a , S_q and S_{pv} values, with the exception of the 6 μ m MoS_{2PAR} sized MoS_{2SUR}, which demonstrated a different S_{pv} trend from those previously seen. The 2 μ m MoS_{2SUR} at the 15% concentration demonstrated decreased S values (S_a of 1703.0 nm; S_q of 2119.2 nm and a S_{pv} of 31668.7 nm respectively), but these were not significantly different in comparison to the other 2 μ m MoS_{2SUR} concentrations.

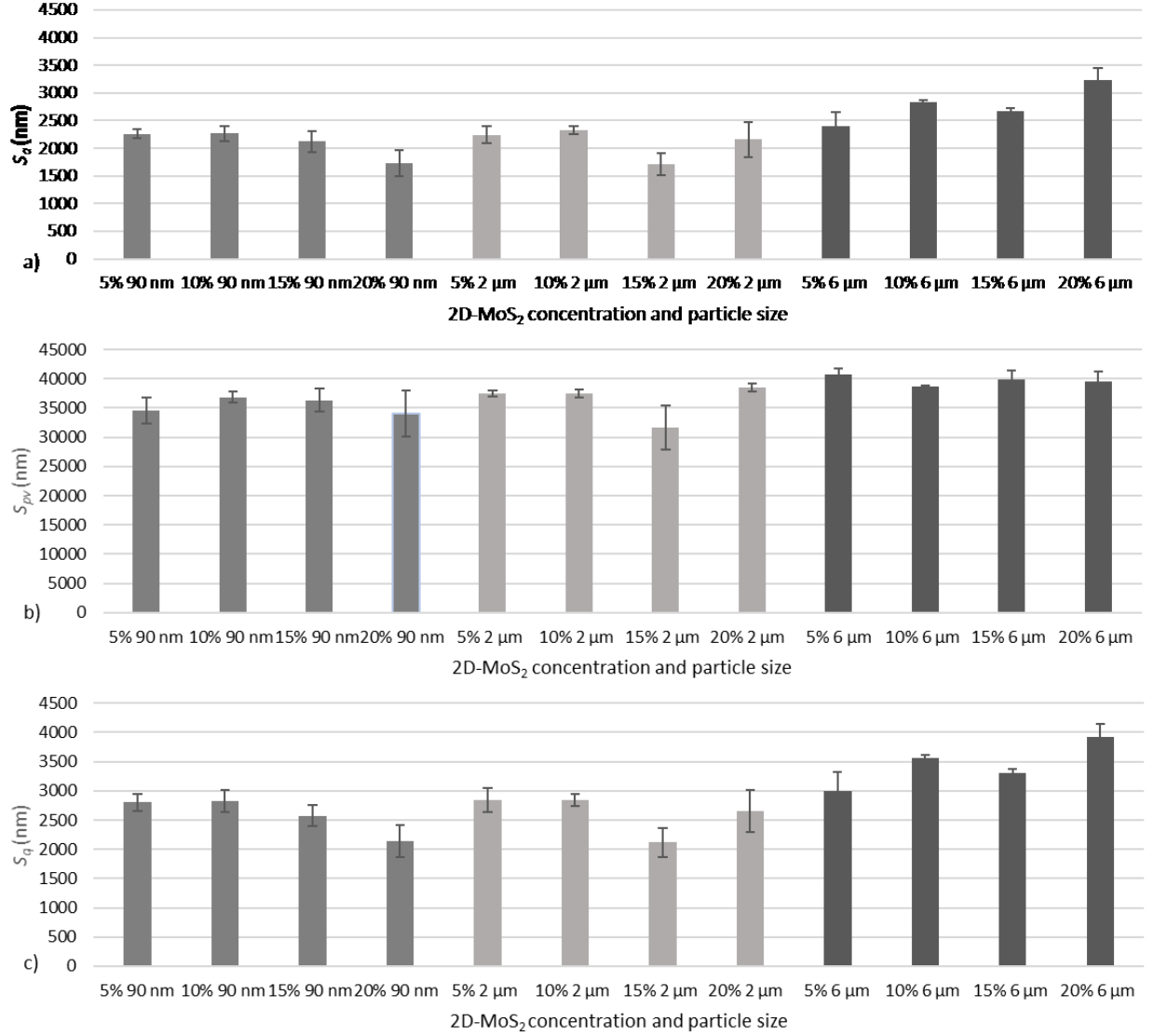


Figure 5. MoS₂_{SUR} roughness parameters a) average MoS₂_{SUR} roughness (S_a) values, b) MoS₂_{SUR} mean square roughness values (S_q) and c) average maximum height of MoS₂_{SUR} (S_{pv}) at increasing MoS₂_{PAR} concentrations of 5%, 10%, 15% and 20%.

MoS₂_{SUR} Water Contact Angles. Water contact angles were determined using the sessile drop technique (Figure 6). The MoS₂_{SUR} demonstrated properties of a non-wettable nature, at the lowest concentrations of the MoS₂_{PAR} in the MoS₂_{SUR}. As MoS₂_{PAR} concentration increased, the MoS₂_{SUR} contact angle decreased and became more wettable. The 2 μm (100.0° – 106.7°) and 6 μm (96.3° – 106.7°) MoS₂_{SUR} demonstrated significant differences when compared to the 90 nm (90.2° – 95.1°) MoS₂_{SUR} ($p = 0.003$ and 0.005 respectively), whilst the wettability of the MoS₂_{SUR} between the MoS₂_{PAR} concentrations of the same MoS₂_{PAR} sizes were not significantly different.

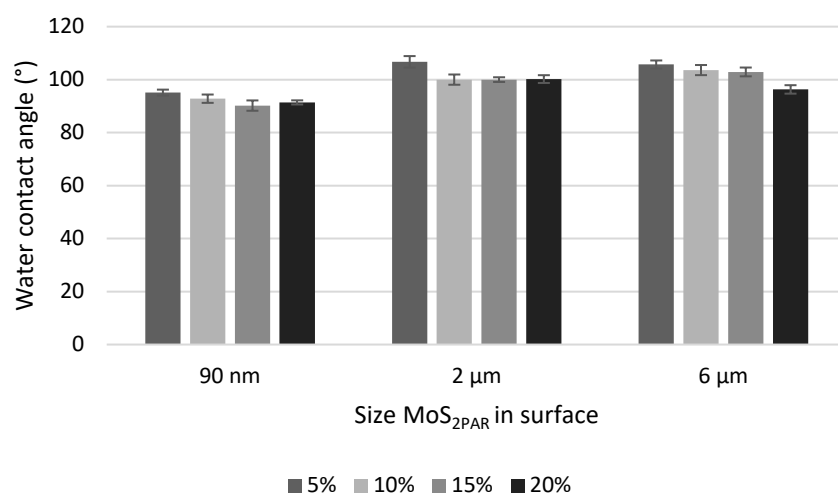


Figure 6. MoS₂SUR water contact angles incorporated with 90 nm, 2 μm and 6 μm MoS₂PAR at concentrations of 5%, 10%, 15% and 20%.

Bacteria. The bacteria used in this study were of different shapes and sizes (Figure 7). *P. aeruginosa* are rod shaped, 0.5 μm to 1 μm in width and 1 μm to 3 μm in length (Figure 7a). *S. aureus* is cocci in shape and around 0.5 μm to 1 μm in diameter (Figure 7b). Once the bacteria form biofilms, the cells cluster and are bound by exopolymeric substance as can be observed with *P. aeruginosa* (Figure 7c) and *S. aureus* (Figure 7d).

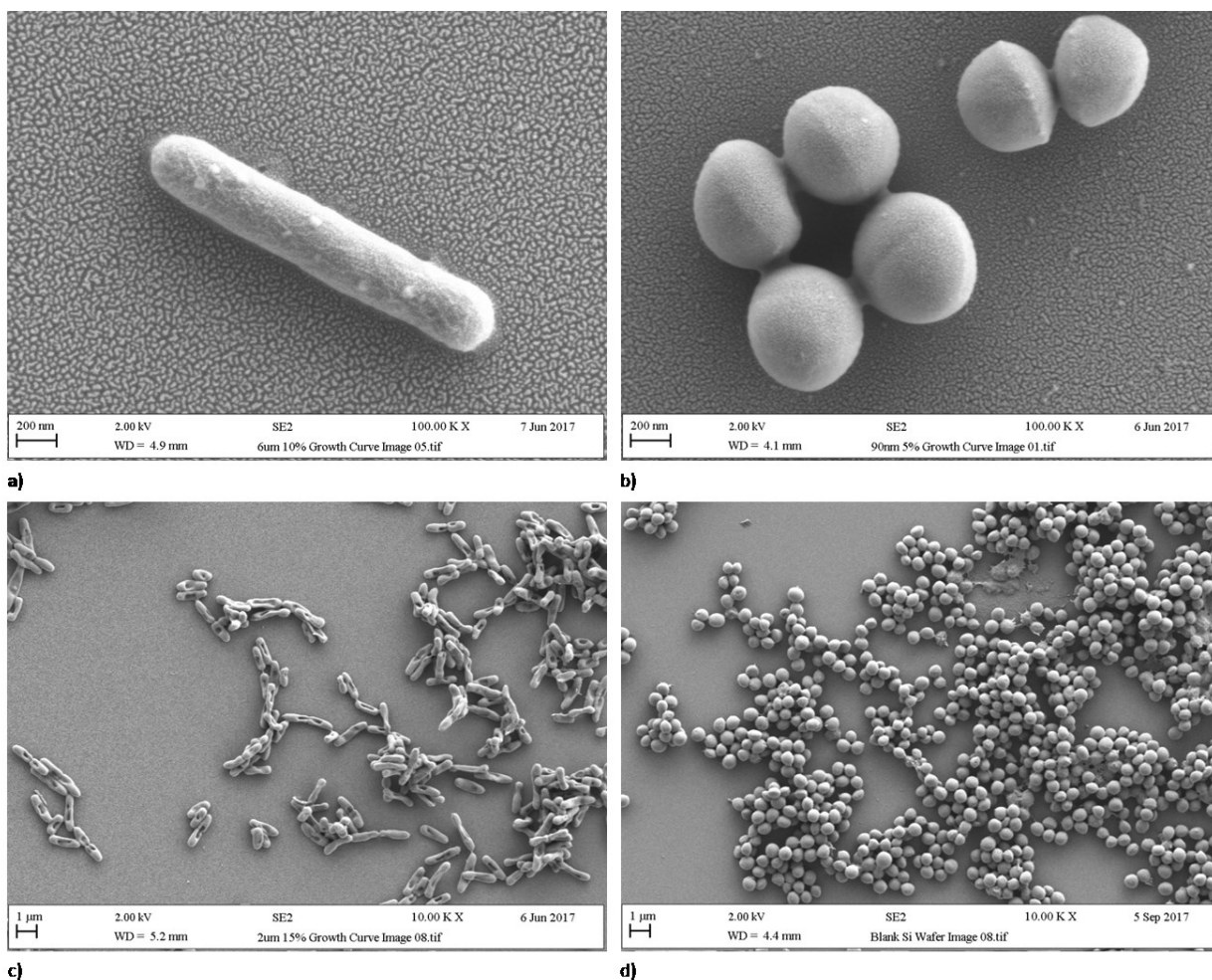


Figure 7. Morphology of different bacteria. Planktonic bacteria used in retention assays a) *P. aeruginosa* b) *S. aureus* and in biofilm formation c) *P. aeruginosa* and d) *S. aureus*.

Retention, Distribution, Density, Dispersion and Clustering of Bacteria Retained on MoS₂SUR. Epifluorescence microscopy and multifractal analysis was used to quantitatively determine the retention, density, dispersion and clustering of bacteria on the MoS₂SUR. The $f(\alpha)$ curves were used to determine the spreading parameters of the bacteria on the MoS₂SUR (ESI Figure 1). The curves for the *P. aeruginosa* retained on the 90 nm (ESI Figure 1a/d) and 6 μ m (ESI Figure 1c/f) MoS₂SUR were skewed to the left-hand side demonstrating that the image was densely packed with cells. The longer leg of the $f(\alpha)$ curve (ESI Figure 1c) and increased skewness for the *P. aeruginosa* retained on the 6 μ m MoS₂SUR evidenced that the number of cell clusters on the MoS₂SUR was greater, which was reflected in the microscopy image (ESI Figure 1f) when compared to the spread of the bacteria on the 90 nm MoS₂SUR (ESI Figure 1d). The *P. aeruginosa* retained on the 2 μ m MoS₂SUR was skewed to the right-hand side demonstrating that the cells were more sparsely distributed, and this was reflected in the

microscopy image (ESI Figure 1e). The $f(\alpha)$ curves for the *S. aureus* were more symmetrical than for *P. aeruginosa* (ESI Figure 2) demonstrating that generally the density, dispersion and clustering elements of the microbial retention patterns were less pronounced than for the *P. aeruginosa*.

Retention of the bacteria across the MoS₂SUR. Multifractal analysis was used to quantify the retention, density, dispersion and clustering of the bacteria across the MoS₂SUR (Figure 8). An increase in retention of the bacterial cells (28.46% at 5%; 28.60% at 10% and 32.25% at 15% respectively) was shown at the 90 nm MoS₂SUR at each MoS₂PAR concentration (5%, 10% and 15%) with the exception of 20% MoS₂PAR (20.17%), and this followed the same pattern as the trend demonstrated in the S_a , S_q and S_{pv} values. On the 2 μ m MoS₂SUR, the retention of *P. aeruginosa* the greatest numbers of bacteria retained were demonstrated on the 5% (55.30%) and 15% (37.84%) MoS₂SUR, whilst the least number of bacteria retained were demonstrated on the 10% (21.01%) and 20% (20.14%) MoS₂SUR. The retention patterns on these MoS₂SUR followed the opposite to the trend seen in the S_a , S_q and S_{pv} values of the MoS₂SUR. The MoS₂SUR at a MoS₂PAR of 6 μ m demonstrated a decreasing trend of *P. aeruginosa* retention (45.97% at 5%, 45.11 at 10%; 26.72% at 15% and 24.30% at 20% respectively) as the concentration of MoS₂PAR increased along with an increase in MoS₂SUR roughness and wettability.

The retention of *S. aureus* followed a trend whereby, the percentage coverage of bacteria retained on the MoS₂SUR decreased with MoS₂PAR size, with the exception of the 5% 90 nm MoS₂SUR. The 2 μ m MoS₂SUR demonstrated the lowest *S. aureus* retention on the 5%, 10% and 15% MoS₂PAR MoS₂SUR (23.44%, 20.80% and 14.69% respectively) when compared to the 90 nm and 6 μ m MoS₂PAR MoS₂SUR at the same concentration of MoS₂PAR loadings. The retention of *S. aureus* on the MoS₂SUR demonstrated the overall highest retention on the 5% and 10% 6 μ m MoS₂PAR MoS₂SUR (52.24% and 52.37% respectively). The lowest retention was demonstrated on the 6 μ m MoS₂PAR size surface with a 20% concentration (6.11%) (Figure 8a). Across all MoS₂ MoS₂PAR sizes tested, the 20% MoS₂ concentrations demonstrated the lowest *S. aureus* retention (11.98% at 90 nm; 13.84% at 2 μ m and 6.11% at 6 μ m respectively). The retention of the bacteria on the MoS₂SUR followed the same trend as the S_a , S_q and S_{pv} values for the MoS₂SUR made with the 90 nm MoS₂PAR, and for the 2 μ m and 6 μ m MoS₂PAR MoS₂SUR, the increase in MoS₂SUR wettability, also resulted in a decrease in bacterial retention.

Density of bacteria across the MoS₂SUR. The $f(\alpha)$ curves were used to calculate the density of the *P. aeruginosa* across the MoS₂SUR (Figure 8b). None of the MoS₂SUR made with

the different MoS₂PAR sizes (90 nm, 2 µm and 6 µm) demonstrated trends in the density of the *P. aeruginosa* that could be related to the MoS₂SUR properties. The 90 nm MoS₂SUR demonstrated the lowest average density across all MoS₂PAR concentrations (range 1.85 to 1.88), but only the density of the bacteria across the 15% MoS₂SUR was significantly different. For the 2 µm MoS₂SUR there was only a significant difference between the highest density on the 5% MoS₂SUR (1.89) and the lowest density on the 10% (1.87) MoS₂SUR. On the 6 µm MoS₂SUR, the highest level of *P. aeruginosa* density (1.91 at 15%) was also significantly different to the lowest level of density demonstrated on the 20% MoS₂SUR (1.87%).

When comparing the measurements on the MoS₂SUR made with the 90 nm MoS₂PAR size, the density of the *S. aureus* increased (1.89 at 5%; 1.90 at 10% and 1.90 at 15%) with the exception of the 20% MoS₂ (1.85) (Figure 8b). The 2 µm MoS₂SUR demonstrated a trend whereby *S. aureus* density decreased marginally (1.9 at 10%; 1.9 at 15% and 1.89 at 20%) with increasing MoS₂PAR concentration, with the exception of the 5% MoS₂SUR (1.87). At the largest MoS₂PAR size of 6 µm, the density of bacteria remained constant (1.88 at 5%; 1.87 at 15% and 1.87 at 20%) with the exception of 10% MoS₂PAR (1.91) concentration, although this was not statistically significant ($p < 0.05$). In summary, the greatest density of *S. aureus* was demonstrated on the 6 µm 10% MoS₂PAR concentration MoS₂SUR and lowest on the 90 nm MoS₂PAR size 20% concentration MoS₂SUR, but overall bacterial density did not demonstrate a trend with MoS₂SUR properties.

Distribution of the bacteria across the MoS₂SUR. On all the MoS₂SUR, *P. aeruginosa* were heterogeneously spread across the MoS₂SUR. The most heterogeneously spread bacteria were on the 90 nm MoS₂PAR, 15% MoS₂SUR (0.66), and on the 6 µm, 5% MoS₂PAR (0.67) and 10% MoS₂PAR (0.69) MoS₂SUR (Figure 8c). Only on the 6 µm MoS₂SUR did *P. aeruginosa* demonstrate a trend between the surface properties and bacterial dispersion, whereby as the MoS₂SUR increased their MoS₂PAR size and became more wettable, so the bacteria became more dispersed.

S. aureus demonstrated an increase in dispersion (0.67 at 5%; 0.70 at 10%; 0.78 at 15% and 0.95 at 20% respectively) (Figure 8c) on the 6 µm MoS₂SUR as MoS₂PAR concentrations increased. This was not observed with the *S. aureus* on the 90 nm or 2 µm MoS₂SUR, and the 90 nm and 2 µm MoS₂PAR sizes demonstrated no significant differences in the dispersion of bacteria. With regards to the MoS₂SUR properties, the MoS₂SUR made with the 90 nm and 2 µm MoS₂PAR did not demonstrate a trend with the S_a , S_q , S_{pv} or wettability values. However, the 6

μm MoS_{2SUR} demonstrated an increase in bacterial dispersion with increased MoS_{2SUR} roughness.

Clustering of bacteria across the MoS_{2SUR}. Clustering of the bacteria across the MoS_{2SUR} demonstrated that on the 90 nm MoS_{2SUR}, the clustering of *P. aeruginosa* decreased with the concentration of MoS_{2PAR} loading (0.03 to 0.48). A different trend was demonstrated on the 2 μm MoS_{2SUR}, whereby the clustering of the *P. aeruginosa* on the 5% MoS_{2SUR} (0.77) was significantly different to the other MoS_{2SUR} (0.11 at 5%, 0.23 at 15% and 0.12 at 20%). Only on the 6 μm MoS_{2SUR} was a clear trend observed, whereby *P. aeruginosa* clustering decreased with increased MoS_{2PAR} loadings (0.94 at 5%, 0.75 at 10%, 0.31 at 15% and 0.04 at 20%) (Figure 8d). Overall, *P. aeruginosa* demonstrated less trends in their distribution across the MoS_{2SUR} related to the MoS_{2SUR} properties than did *S. aureus*.

The underlying MoS_{2PAR} size and concentration produced different trends in the *S. aureus* clustering patterns on the MoS_{2SUR} (Figure 8d). On the 90 nm MoS_{2SUR}, clustering of the *S. aureus* was greatest on the 10% MoS_{2SUR} (0.83), and lowest on the 20% MoS_{2SUR} (-0.24), whilst on the 2 μm MoS_{2SUR}, clustering of the bacteria was greatest on the 10% (0.56), but lowest on the 15% MoS_{2SUR} (0.09). Only the 6 μm MoS_{2SUR} demonstrated decreased clustering of the *S. aureus* with increased MoS_{2PAR} size and concentration (1.07 at 5%, 0.94 at 10%, 0.36 at 15% and -0.68 at 20% respectively).

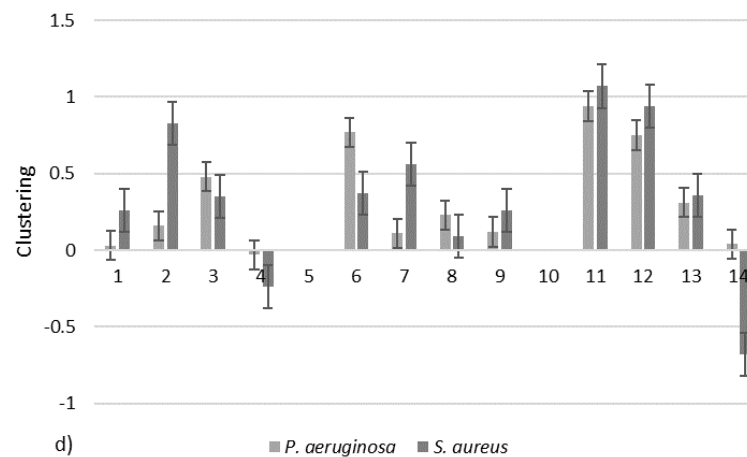
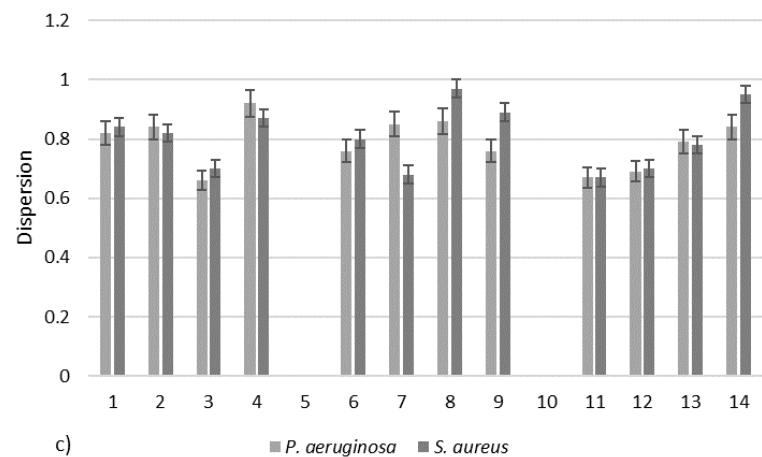
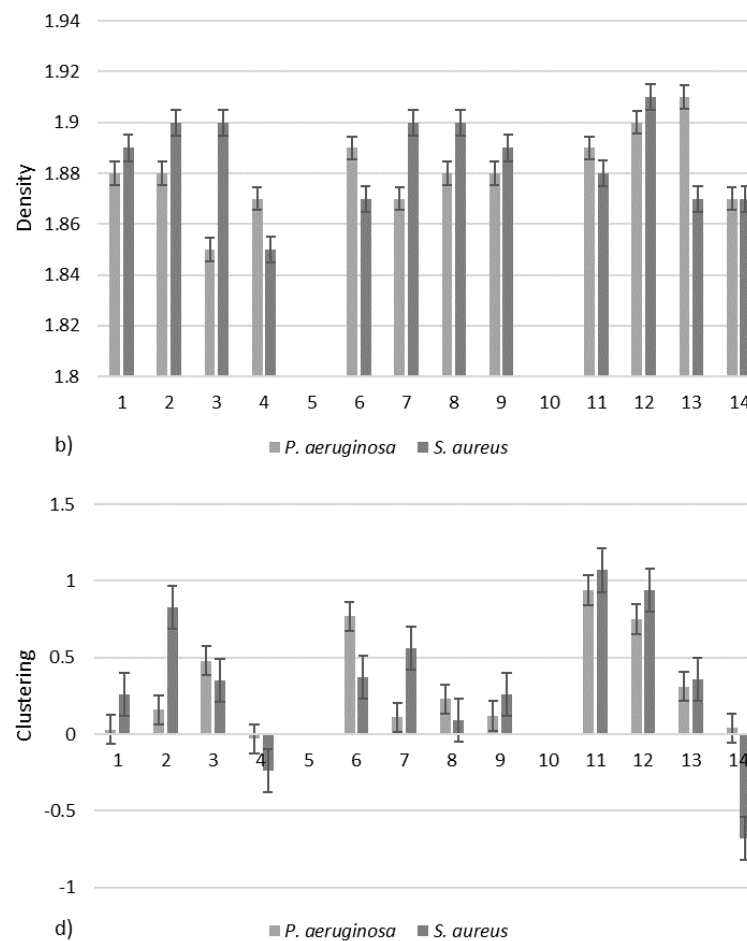
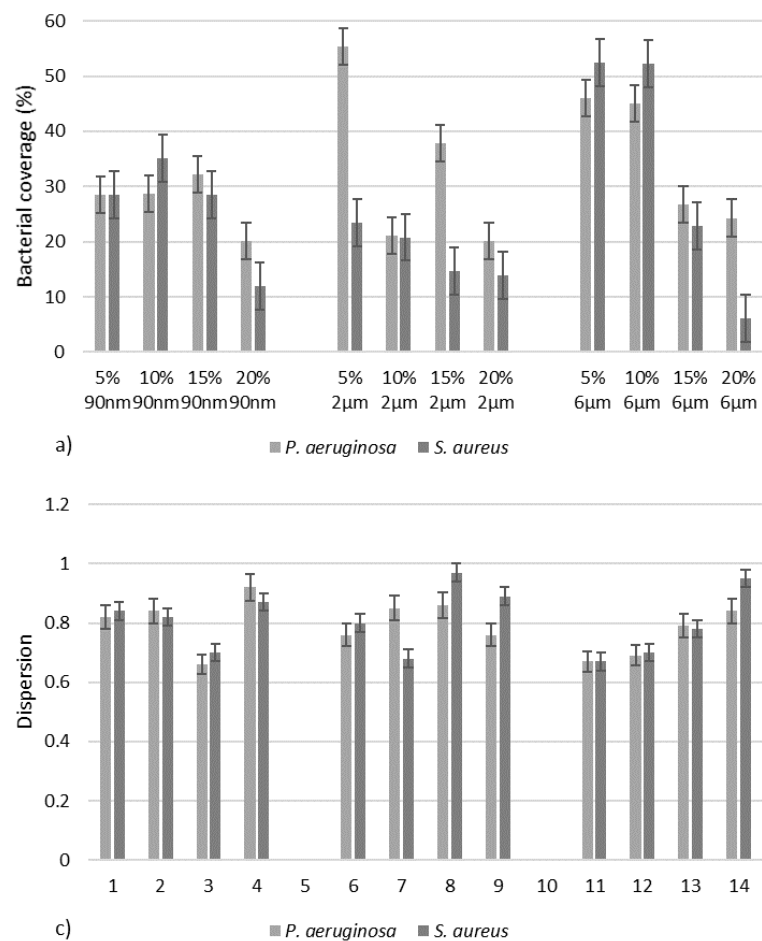


Figure 8. Pattern of *P. aeruginosa* and *S. aureus* a) retention (percentage coverage) b) density, c) dispersion and d) clustering across the MoS₂_{SUR} with MoS₂_{PAR} loadings of 5%, 10%, 15% and 20%.

Crystal Violet Biofilm Assay. The 2 μm MoS_2SUR were tested to determine if they had an effect on bacterial biofilm formation over 24 h (Figure 9). The MoS_2SUR demonstrated a reduction in biofilm formation for *S. aureus* (1.27, 1.61, 1.15 and 0.93) and *P. aeruginosa* (1.53, 1.53, 1.33 and 1.03) with increasing MoS_2PAR size incorporated into the MoS_2SUR . The total biofilm growth was reduced on MoS_2SUR made with increasing MoS_2PAR sizes and concentration of MoS_2 by 28.5% for *S. aureus* and 34.8% for *P. aeruginosa*. These MoS_2SUR also demonstrated a corresponding trend of increased wettability with decreased biofilm formation.

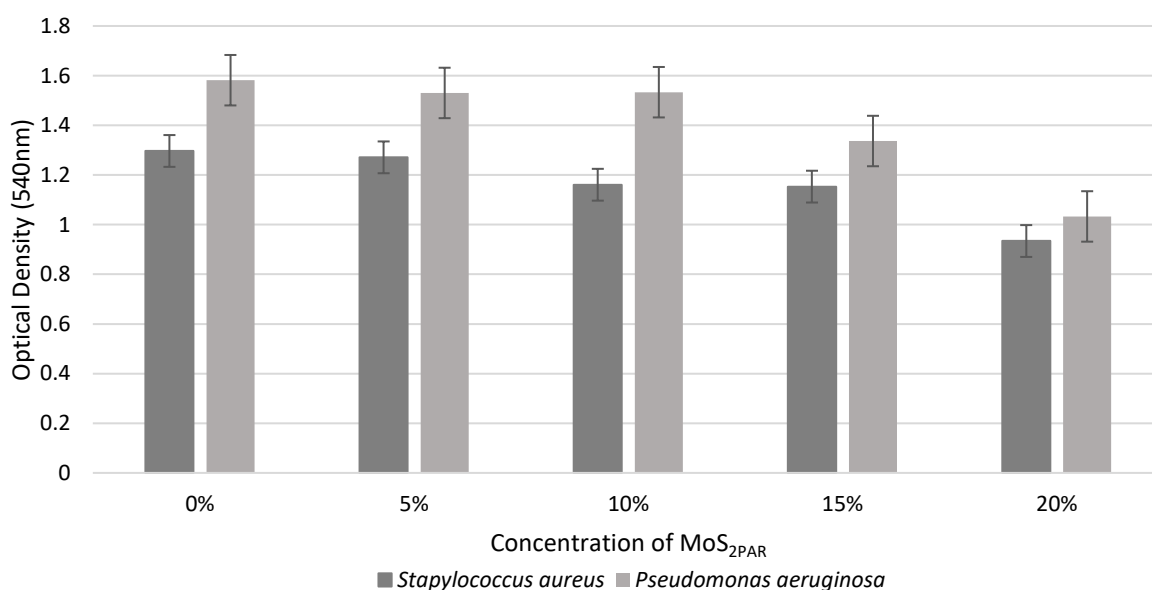


Figure 9. Biofilm of *S. aureus* and *P. aeruginosa* against MoS_2SUR at increasing concentrations of MoS_2PAR (5%, 10%, 15% and 20%) ($n = 3$).

HK-2 Cell Cytotoxicity. Immortalised renal human proximal tubular cells (HK-2) were used to determine the cytotoxicity of the MoS_2SUR . Cell viability was measured after 48 h of exposure of cells to SFM incubated with the control surfaces, or the 90 nm, 2 μm or the 6 μm MoS_2SUR at a concentration of 20% MoS_2PAR (Figure 10). The results demonstrated a decrease in HK-2 cell viability with increasing MoS_2PAR size in the MoS_2SUR (102.4%, 99.1% and 83.9% for 90 nm, 2 μm , and 6 μm surfaces respectively), in comparison to the control. The reduction in viability was significant only in response to media incubated with 6 μm MoS_2SUR , when compared the control, and the 90 nm MoS_2PAR sized MoS_2SUR .

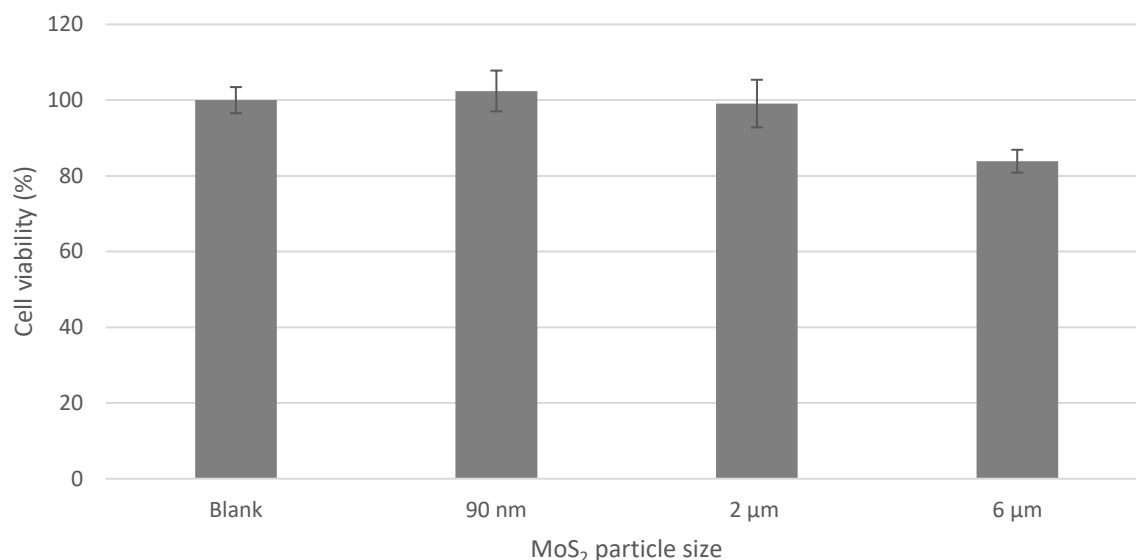


Figure 10. The effect of molybdenum and sulphur leached from MoS₂_{SUR} on HK-2 cell viability. Cells were incubated for 48 h with SFM exposed to a control or MoS₂_{SUR} at 20% MoS₂_{PAR} concentration, and MoS₂_{PAR} sizes of 90 nm, 2 μm or 6 μm. Cell viability was expressed as a percentage of the control cells. MoS₂_{SUR} of 6 μm sized MoS₂_{PAR} demonstrated significant reductions in HK-2 cell viability in comparison to the control ($p = 0.006$) and the 90 nm MoS₂_{PAR} ($p = 0.013$) ($n = 6$).

DISCUSSION

Due to the prevalent issue of bacterial retention, which subsequently leads to biofilm formation, the food, water and medical industries require novel ways to combat biofouling.^{25,26,29,30} A range of 2D materials have gained significant attention in recent years for their unique physical and chemical properties.¹⁸ The ICP-AES results demonstrated that there was negligible leaching of the molybdenum or sulphur from the MoS₂_{SUR}, thus consolidating the fact that, the mechanism of action of bacterial retention and biofilm formation was an effect determined by surface properties, rather than being due to a biocidal action. The FTIR spectra of the MoS₂_{SUR} demonstrated that the spectral peaks for the MoS₂_{PAR} and other components such as the graphite, binder and residual solvents in the graphitic ink were detected. The shifts in the peaks may be due to binding of other molecules in the MoS₂_{SUR} to the components of the graphitic ink. In agreement with the results in the FTIR, previous work had analysed the MoS₂_{SUR} using X-ray Diffraction Spectroscopy, X-ray Photoelectron Spectroscopy and Raman Spectroscopy analysis had demonstrated the chemistry of the MoS₂_{SUR} and the robustness of the MoS₂_{SUR} when used as electrodes³¹. The results were found

to demonstrate that the surfaces exhibited no degradation in current when used in over a 1000 repeat scans, thus supporting the stability of these MoS₂_{SUR} over time.³¹ The FTIR analysis and SEM images demonstrated that the MoS₂_{SUR} were chemically heterogeneous. The thickness of MoS₂_{SUR} was dependent on the printing parameters and ink viscosity, but previous work had demonstrated that the thickness of the MoS₂_{SUR} was 13.5 ± 1.5 microns.³¹ The MoS₂_{SUR} demonstrated different MoS₂_{PAR} size affected surface wettability and roughness, whereas changes of MoS₂_{PAR} concentration only affected surface wettability.

The properties of a surface can influence the retention of bacteria and hence subsequent biofilm formation.³² Different facets of the MoS₂_{SUR} properties, and also the bacterial shape were found to affect the bacterial retention to the MoS₂_{SUR} and these included the shape and size of the MoS₂_{SUR} features and microorganisms, the different MoS₂_{PAR} sizes incorporated into the MoS₂_{SUR} and the concentration of the MoS₂_{PAR} in the MoS₂_{SUR}. The MoS₂_{SUR} with the smaller topographical shaped features, and the concentration of the MoS₂_{PAR} incorporated into the MoS₂_{SUR} influenced the pattern of retention of the *S. aureus* bacteria, with some trends in retention seen for the *S. aureus* on the MoS₂_{SUR} made with the 90 nm and 2 μ m MoS₂_{PAR}. *P. aeruginosa* are rod shaped bacteria (1 μ m diameter by 1 μ m – 3 μ m length) and are larger than the cocci shaped *S. aureus*. in size (0.5 μ m – 1 μ m diameter). The smaller MoS₂_{SUR} features of the MoS₂_{SUR} made with the 90 nm or 2 μ m MoS₂_{PAR} may have enabled the smaller bacteria to be retained. This is one explanation as to why increasing the concentrations of the MoS₂_{PAR} in the MoS₂_{SUR} would result in the different behaviour of the bacteria. Although there is controversy regarding the influence of surface topography on bacterial retention, in agreement with our work, it has demonstrated that the size of the surface features can significantly affect bacterial retention.^{6,33-35}

The increase in the MoS₂_{PAR} used to make the MoS₂_{SUR} increased the MoS₂_{SUR} topography and wettability which resulted in the greatest bacterial reduction on the roughest, most wettable MoS₂_{SUR}. The water contact angle of MoS₂_{SUR} has previously been reported to be between 75.8° and 88.37°.³⁶⁻³⁸ However, graphite is known to be a less wettable material, with a water contact angle between 75°- 95°.³⁹ Thus, the increase of the MoS₂_{SUR} wettability with an increase in the concentration in the MoS₂_{PAR} in the MoS₂_{SUR} would be expected. As the MoS₂_{SUR} become more wettable with increasing MoS₂_{PAR} concentration, if the physicochemical forces between the bacteria and the MoS₂_{SUR} become similar, this may result in repulsive interactions and hence the MoS₂_{SUR} with the higher concentrations of MoS₂_{PAR} would repel the bacteria. This may explain why increasing the concentrations of the MoS₂_{PAR} in the MoS₂_{SUR} would result in the different behaviour of the bacteria. In agreement with our

work it has been suggested that a way to reduce fouling, particularly on membranes is increase the surface hydrophilicity.⁴⁰

As the MoS₂_{SUR} topography and wettability increased with increasing MoS₂_{PAR} size and concentration, the bacteria became more heterogeneously dispersed and less clustered across the MoS₂_{SUR}. *P. aeruginosa* demonstrated different patterns of retention, distribution, density, dispersion and clustering compared to the *S. aureus* suggesting that the interaction of the different MoS₂_{SUR} properties in conjunction with the morphology and physiology of the cells had a role to play.

Although much work has been carried out on the antibacterial properties of 2D materials, very little work has been carried out on the capabilities of such materials to impede biofouling. Alam et al., (2018) demonstrated that MoS₂_{SUR} performed as well as graphene oxide in reducing *Escherichia coli* adherence to MoS₂_{SUR}.²² which was suggested to be due to the lowered amount of functional groups on the MoS₂_{SUR}. Using QCM-D when natural organic matter was injected onto MoS₂_{SUR} and graphene oxide surfaces, a lesser frequency shift was observed on the MoS₂_{SUR}, indicating that the MoS₂_{SUR} would be less prone to fouling.²²

The advantage of using MoS₂_{SUR} is that they are cheap to produce and generally considered non-toxic and since they are antifouling rather than antimicrobial, they do not require the addition of other chemicals or metallic elements that may result in an increase in their toxicity profiles. MoS₂_{SUR} may also be more beneficial than other 2-D material surfaces since they been reported to contain less functional groups. Since the functional groups in graphene oxide forms hydrogen bonds with the lipopolysaccharides of Gram-negative bacteria, such as *P. aeruginosa*, the lack of functional groups on MoS₂_{SUR} may result in lowered bacterial:surface interactions.^{41,42} There is also a need to look for antifouling surfaces that do not contain metals. Although metals are a regular component of antifouling coatings, such as partially reduced graphene oxide/silver in nanocomposite and zinc-graphite composite coatings, there is concern over the toxic effects of these materials.^{11,12} Another factor is the uncontrolled use of silver as an antimicrobial in a wide range of applications and products and concerns are now being raised regarding the development of bacterial resistance to silver.⁴³ Thus the development of such surfaces that impede bacterial retention and biofilm formation with lowered toxicity profiles is highly advantageous.

Evaluation of the biofilm reducing properties of the MoS₂_{SUR} demonstrated the same trend for each bacterial type, whereby a decrease in biofilm formation for *P. aeruginosa* and *S. aureus* was positively correlated with an increase in the concentration of MoS₂_{PAR} in the MoS₂_{SUR}. The overall biofilm growth was reduced with increasing MoS₂_{PAR} sizes and

concentration of MoS₂_{SUR} by 34.8% for *P. aeruginosa* and 28.5% for *S. aureus*. This may have resulted since as the biofilm forms on the surface, exopolymeric substance (EPS) is produced by the cells which will coat both the surface of the MoS₂_{SUR} and the bacterial cell walls, resulting in similar chemistries on both the different types of cells. The exopolymeric substance have different patches or domains that can have a hydrophobic, hydrophilic, and positively or negatively charged nature, and the covering of the bacteria and MoS₂_{SUR} may have mitigated any bacterial:surface interaction effects.^{44,45} This is one mechanism which may explain why in the biofilm study, although different shaped and sized cells were used, they are seen to act in the same manner. The differences in the amount of biofilm produced on the MoS₂_{SUR} may be therefore due to the initial bacterial load on the MoS₂_{SUR}. This correlates with the retention values whereby the least amount of retained bacteria was observed on the surfaces loaded with the greatest concentration of MoS₂_{PAR}. In agreement with the results presented in this work, Yuwen et al., (2018), demonstrated that increasing the concentration of molybdenum within a hybrid surface, significantly reduced the presence of *S. aureus* biofilms.⁴⁶ The results from this study clearly demonstrate that the retention of bacteria on surfaces and biofilm formation are influenced by different phenomenon.

The use of cell viability assays for investigating cytotoxicity of surfaces *in vitro* is of importance, particularly when such surfaces have a potential to be used in the healthcare, water and food sectors. Despite the existing uses of 2D materials in biomedical applications, research into their toxicity in human cell lines is limited, with differences in testing methods, or without sufficient materials characterisation.⁴⁷ Studies on two dimensional materials, such as reduced graphene oxide and MoS₂, have previously demonstrated conflicting effects on cell cytotoxicity in mammalian cell lines.^{48,49}

An *in vitro* rat endothelial cell model testing the cytotoxic effects of media incubated with MoS₂ nanosheets demonstrated that increasing MoS₂ concentrations did not impair the cell viability.⁵⁰ Furthermore, some of the MoS₂ concentrations tested increased proliferation of the cells after 24 h of incubation. The cell viability in their study was measured using sulforhodamine B assay by quantifying the cellular protein content in living cells, pre and post MoS₂ interaction. Studies utilising the WST-8 viability assay have demonstrated that MoS₂ does not impair the cell viability of human embryonic kidney cells and lung epithelial cells.^{50,51} Similar results were observed in this study, where media incubated with 90 nm and 2 μ m MoS₂_{PAR} were non-toxic to human kidney cells (HK-2) over a 48 h period of incubation. Furthermore, the viability of HK-2 cells exposed to the 90 nm MoS₂_{PAR} was increased as compared to the control media, possibly due to increased

proliferation and cell growth. In contrast, media incubated with the 6 μm MoS₂PAR significantly reduced HK-2 cell viability. However, some evidence suggests that the size of the MoS₂PAR may have an effect on cell viability.⁵² Chng and Pumera, (2015), have demonstrated a correlation between the surface area of MoS₂PAR and their cytotoxic effects in A549 human lung epithelial cells.⁵³ Similar effects were observed by Zhang et al, (2017), where the flake size of similar 2D materials correlated with cell death in NIH 3T3, HCoEpiC and 293T cell lines.⁵⁴ The reduction in viability is attributed to an increase in reactive oxygen species generation, which correlated with an increase in the flake size of the particulates. Therefore, it is likely that the cytotoxic effects of the 6 μm MoS₂SUR observed in this study are due to an increased reactive oxygen species generation, due to increased surface MoS₂PAR size.

The results from this study demonstrated that MoS₂SUR have the potential to impede surface fouling. Such surfaces may be useful for use for specific applications within certain industries or healthcare where biofilm proliferation is an issue.

CONCLUSION

This work demonstrated that increasing the MoS₂PAR size, resulted in MoS₂SUR that had the least sharp surface topographies, with wider width valleys. The 6 μm MoS₂SUR, demonstrated the most dominant effects on both bacteria in terms of retention whereby, the increase in the concentration of the MoS₂PAR in the MoS₂SUR resulted in increased surface wettability and roughness but a decrease in bacteria retention and clustering and an increase in bacterial dispersion. Surfaces with smaller features and increased wettability had an effect on the retention of *S. aureus*, due to the smaller bacteria being able to fit into the smaller surface features. When the efficacy of the MoS₂SUR was tested against the biofilms, it was demonstrated that the amount of bacterial loading influenced the amount of biofilm formation. These results suggest that the surface properties of materials influenced microbial retention, and the amount of initial bacterial load influenced biofilm formation. Such bacterial retention and biofilm reducing MoS₂SUR may be further developed for use in industries whereby surface biofouling is an issue.

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AUTHOR CONTRIBUTIONS

KW developed the idea conceptualisation and drafted the final manuscript. MA, SR-N, LS, SL, JW-N and ME-M contributed to the material and manuscript preparation, data collection and analysis. CB and SR-N lead the surface design. All authors read and approved the final manuscript.

CONFLICT OF INTERST

There is no conflict of interest in this work.

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There are no funders to report.

SUPPLEMENTARY INFORMATION

- $f(\alpha)$ curves derived for the a) 90 nm, b) 2 μm and c) 6 μm MoS₂PAR incorporated into the MoS₂SUR derived from the corresponding images following the bacterial retention assays of *P. aeruginosa* on the d) 90 nm, b) 2 μm and c) 6 μm surfaces demonstrating the mathematical information used to determine the percentage coverage, density dispersipon and clustering of *P. aeruginosa*. Error bars are representative of 50 μm .
- $f(\alpha)$ curves derived for the a) 90 nm, b) 2 μm and c) 6 μm MoS₂PAR loaded MoS₂SUR derived from the corresponding images following the bacterial retention assays of *S. aureus* on the d) 90 nm, b) 2 μm and c) 6 μm surfaces demonstrating the mathematical information used to determine the percentage coverage, density dispersipon and clustering of *S. aureus*. Error bars are representative of 50 μm .

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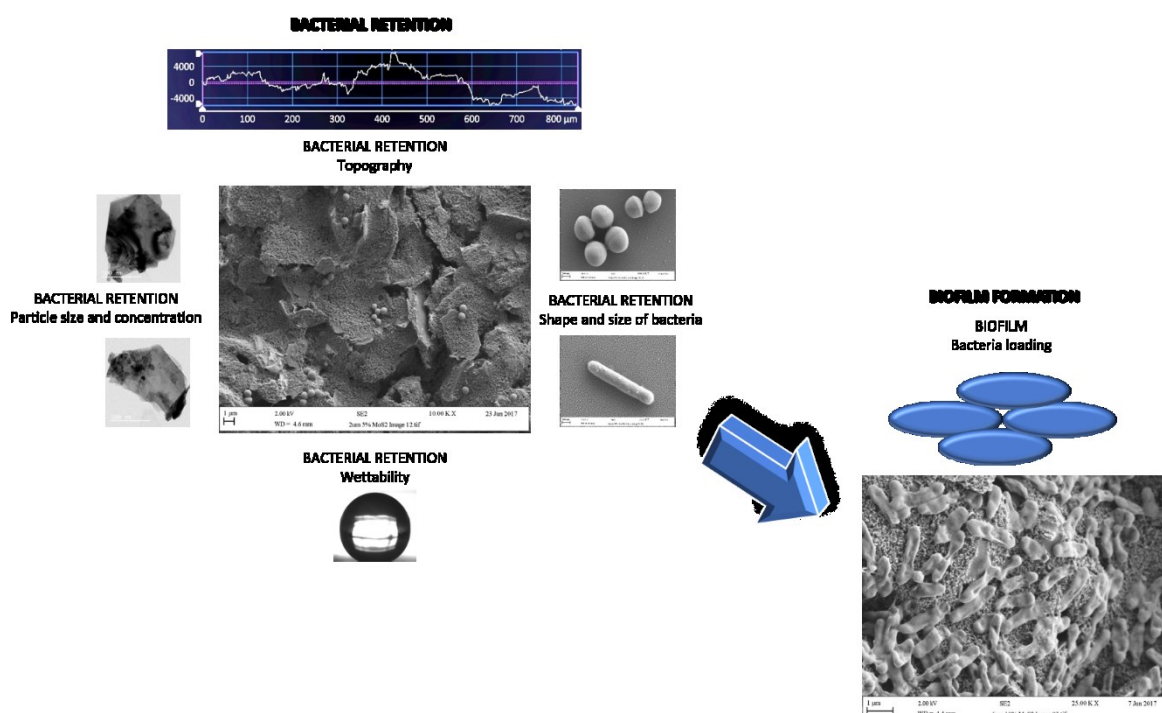
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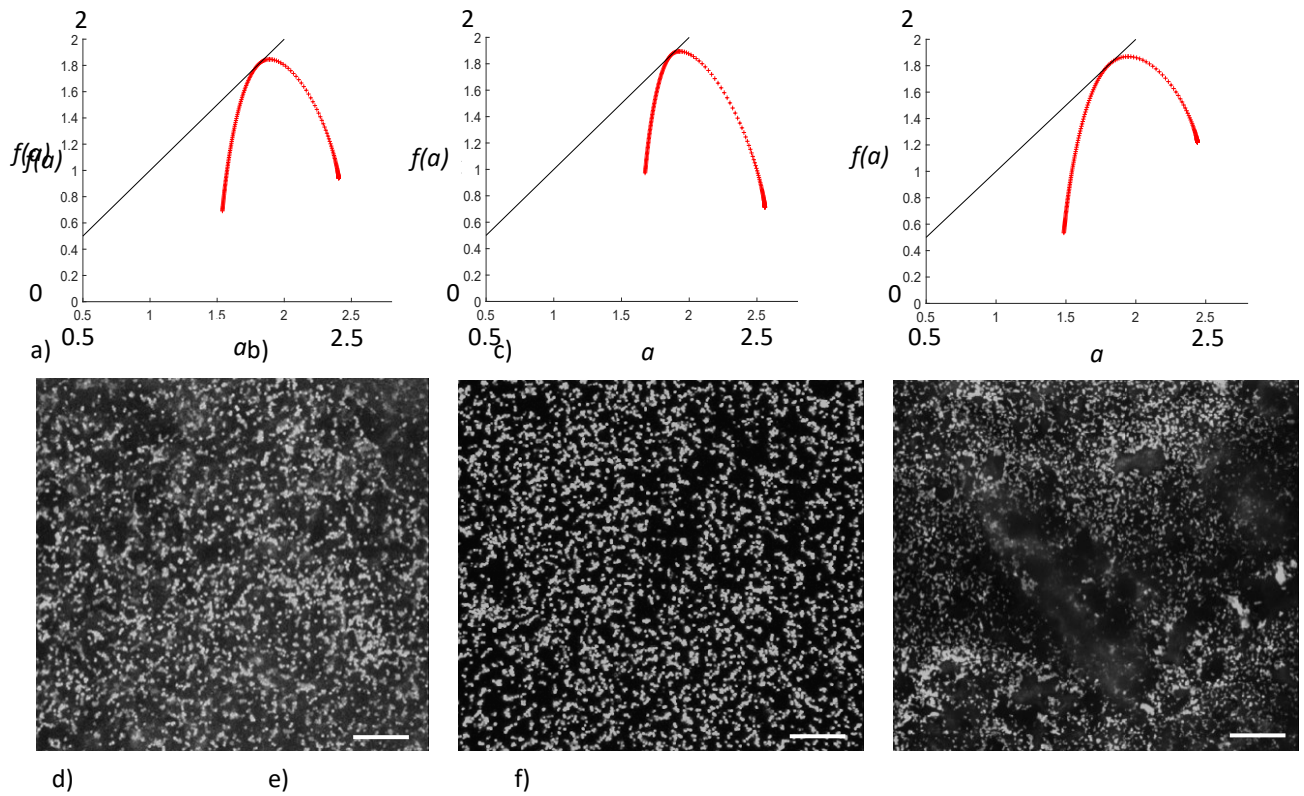


Figure 1. $f(\alpha)$ curves derived for the a) 90 nm, b) 2 μm and c) 6 μm MoS₂PAR incorporated into the MoS₂SUR derived from the corresponding images following the bacterial retention assays of *P. aeruginosa* on the d) 90 nm, b) 2 μm and c) 6 μm surfaces demonstrating the mathematical information used to determine the percentage coverage, density dispersipon and clustering of *P. aeruginosa*. Error bars are representative of 50 μm .

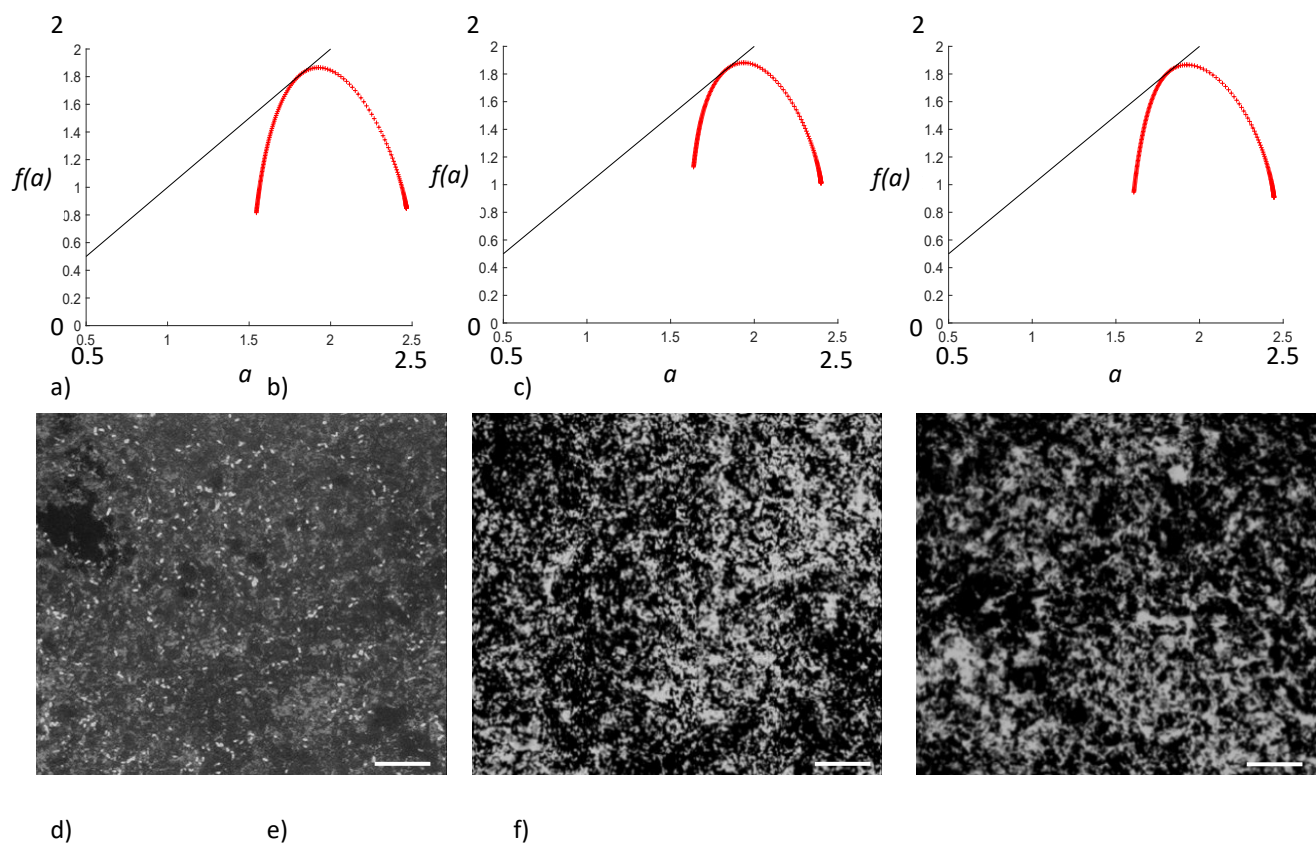


Figure 2. $f(\alpha)$ curves derived for the a) 90 nm, b) 2 μm and c) 6 μm MoS₂PAR loaded MoS₂SUR derived from the corresponding images following the bacterial retention assays of *S. aureus* on the d) 90 nm, b) 2 μm and c) 6 μm surfaces demonstrating the mathematical information used to determine the percentage coverage, density dispersion and clustering of *S. aureus*. Error bars are representative of 50 μm .