Effectiveness of titanium nitride silver coatings against Staphylococcus spp. in the presence of BSA and whole blood conditioning films

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ABSTRACT

Implanted medical devices are at risk of developing an infection at the surgical site. Once a medical implant is inserted, it initially becomes coated by a conditioning film, followed by bacterial retention. In the present study, medical grade stainless steel substrata were coated with titanium nitride (TiN) or titanium nitride/silver (TiN/14.94at.%Ag or TiN/19.04at.%Ag). Surface analysis determined that with increased silver concentration, silver nanoparticles were heterogeneously distributed throughout the coatings. The effect of bovine serum albumin or whole blood conditioning films on the antimicrobial activity and microbial retention were determined using Staphylococcus aureus or Staphylococcus epidermidis. The presence of the conditioning films reduced the antimicrobial effect of the surfaces against S. aureus. When the cells and conditioning films were applied together, a reduction in bacterial retention and
conditioning film was observed. These results suggest that the impact of conditioning films should be considered since conditioning films may reduce bacterial retention but may also decrease the antimicrobial properties of the surface coatings.

**Keywords:** Retention, antimicrobial, conditioning film, BSA, whole blood, titanium nitride silver

### 1. Introduction

External fixations are essential components of modern orthopaedic surgery. For example, orthopaedic devices such as fine-wire fixators and external fixators are commonly use for the treatment of longbone fractures and pelvic fractures for both adults and children (Ktistakis et al., 2015). However, external fixations are associated with a high incidence of pin tract infection rates (Ktistakis et al., 2015; Schalamon et al., 2007). Indeed, the surface of medical devices and implants provides an artificial interface on which bacteria can aggregate to form a biofilm (Gristina, 1987; Lindsay and von Holy, 2006). Some pathogenic strains of common skin microbiota species, such as *Staphylococcus aureus* and *Staphylococcus epidermidis*, can grow in these biofilms and also be involved in pin tract infections. For example, Schalamon et al., (2007) found that among 37 external fixations placed on 30 children, 19 (52%) led to at least one infection. *S. aureus* and *S. epidermidis* were found in 33 % and 22 % of paediatric pin tract infections respectively. Biofilm infections are associated with chronic infection, which are recalcitrant to traditional antimicrobial therapy (Costerton et al., 1999). In order to combat device related infections, the prevention of microbial attachment/retention on the surgical implants, or the use of an antimicrobial coating may provide a partial solution to this problem. Studies have shown that coating some metals, such as stainless steel with titanium can reduce bacterial attachment/retention, and/or have antimicrobial properties (Whitehead et al., 2015). Stainless steel and titanium have been considered in depth since they are the most common materials used to produce pins or wires used in bone fixing (Galanakos et al., 2009). It has been
suggested that infection rates seem to be higher for stainless steel alone, compared to titanium alloys (Veerachamy et al., 2014). Previous research has demonstrated that silver coated pins decreased bacterial colonisation and pin tract infection both in vitro and in vivo (Bosetti et al., 2002). However, some silver impregnated structures have also demonstrated stronger bacterial adhesion, whilst still presenting an increased incidence of dead cells (Whitehead et al., 2011). Thus, there is some debate as to which substrata provide the most beneficial surfaces.

Following insertion of the implant, a conditioning film forms rapidly on the surface, as proteins such as fibrinogen are adsorbed onto the substratum of the device (Hohmann et al., 2015). The exact format of the conditioning film is dependent on the surface properties of the implanted biomaterial, such as hydrophobicity and topography (Whitehead and Verran, 2015). Organic films may also modify the impact of the coatings on microbial attachment/retention and may alter their antimicrobial activities. To the authors knowledge, the effect of a conditioning film on bacterial retention and antimicrobial activity on TiNAg, coatings, i.e., nanocomposite coatings containing silver particles in a titanium nitride matrix, has not been previously described. For this study, two conditioning films were used; bovine serum albumin (BSA) and whole blood (WB). BSA was used since it is representative of plasma proteins. Whole blood proteins are involved in conditioning film formation on implant surfaces (Hohmann et al., 2015).

The aim of this work was to determine the effect of two conditioning films on the retention and antimicrobial activity of a range of surfaces (medical grade stainless steel, titanium nitride (TiN), TiN/14.94 at.% Ag and TiN/19.04 at.% Ag). This information may help to determine if such coatings have the potential to be used to reduce infections used in bone fixation devices.

2. Materials and Methods

2.1 Substrata
The surfaces were prepared according to Whitehead et al., 2010. In brief, using a guillotine, 10 mm x 10 mm coupons of stainless steel (SS) were cut. Coatings were deposited onto the stainless steel coupons, (titanium nitride (TiN), titanium nitride with 14.94% silver (TiN/14.94at.%Ag) and 19.04 % (TiN/19.04at.%Ag) using an adapted magnetron sputtering method (Whitehead et al., 2010).

2.2 Energy Dispersive X-ray Spectroscopy (EDX)

The chemical analysis of the coupons was penetrated up to a 1 µm depth (Link Pentafet detector), and the analysis used Inca software with a windowless system and resolution of 133 eV (Oxford Instruments, UK) (n = 15).

2.3 Atomic Force Microscopy

An atomic force microscope (AFM) (Explorer, Veeco Instruments, UK) was used in contact mode using pyramidal shaped, silicon nitride tips to obtain the images using a scan rate of 20.03 µm s⁻¹ with 300-pixel resolution. Cantilever spring constants 0.05 N m⁻¹ were defined by the manufacturer (n = 15).

2.4 Bacterial Preparation

*Staphylococcus epidermidis* NCTC 11047 and *Staphylococcus aureus* NCTC 3048 were incubation with shaking at 200 rpm at 37 °C for 24 h in 100 mL nutrient broth (NB) (Oxoid, UK) and inoculated onto nutrient agar plates and incubated at 37 °C for 24 h. *S. aureus* or *S. epidermidis* was inoculated into 15 mL of nutrient broth and incubated overnight at 37 °C, then centrifuged at 567 g for 10 min. The supernatant was removed and the cell pellet was washed in sterile distilled water (10 mL) and diluted until an optical density (OD) reading of 1.0 ± 0.1 was reached (540 nm). Colony forming units (CFU) corresponding to 9.72 ± 1.3 x 10⁷ cells for *S. epidermidis* and 1.2 ± 0.2 x 10⁸ cells for *S. aureus* were obtained.

2.5 Conditioning film preparation
Powdered bovine serum albumin (BSA) (Sigma, UK) was dissolved in sterile dH₂O to obtain a 10% solution and was mixed, then filter sterilised (PALL R Acrodisc R 32 mm syringe filter, 0.2 µm Supor membrane R). Sterile horse blood, donated as whole blood (WB) (TCS Biosciences, UK) was diluted to 10% solution using sterile dH₂O.

2.6 Retention assays

Twenty five millilitres of cell suspension alone or mix with conditioning film was gently poured over the coupons which had been placed into glass Petri dishes. To obtain the cell suspension with conditioning film, 12.5 mL of the OD of 1.0 bacterial suspension and 12.5 mL of either 10% BSA or 10% blood plasma solution was mixed together and incubated without agitation for 1 h at 37 °C. Following incubation, the coupons were rinsed once for 5 s using a drip lock bottle at a 45 ° angle with sterile dH₂O and air dried in a microbiological class 2 hood. The numbers of cells retained was adjusted to take into account the dilution effect of the conditioning film.

A 1:1 ratio of Rhodamine B (0.1 mg/L) and 4',6-diamidino-2-phenylindole (DAPI) (0.1 mg/L) was prepared (Whitehead et al., 2009a) and 10 µL of the mix was spread across the coupons. Following staining, the coupons were viewed using either DAPI 330–380 nm or rhodamine B 590–650 nm filters (Nikon Eclipse E600, UK with F view-II black and white digital camera, Soft Imaging System, UK). The percentage coverage of cells was calculated (Cell F software, UK) and recorded (n = 60). Calculations were used to take into account the dilution factor of the conditioning films.

2.7 Microbial adhesion to solvents (MATS) assays (based on an assay by Bellon-Fontaine et al., 1996).

In order to determine the relative hydrophobicity of the microbial cells, MATS assays were carried out. Bacteria were centrifuged at 3500 rpm for 10 min, then washed 3 times using pH 7.1 PUM buffer (22.2 g L⁻¹ K₂HPO₄·3H₂O, 7.26 g L⁻¹ KH₂PO₄, .8 g L⁻¹ urea and 0.2 g L⁻¹
$1\text{MgSO}_4\cdot 7\text{H}_2\text{O}$. Cells were re-suspended to an OD 1.0 at 400 nm. To a round bottomed test tube 15 mm in diameter, 1.5 mL volume of washed cells suspended in PUM buffer was added. Two hundred and fifty microliters of one of the test chemicals (Decane (BDH, UK); Hexadecane (Sigma, UK); Ethyl Acetate (Sigma, UK) or Chloroform (Sigma, UK)) was added to the suspension which was incubated at 37°C for 10 min. Following vortexing for 2 min the mixture was incubated again at 37°C for 30 min. The optical density of the lower aqueous phase was determined (400 nm). To determine the cell surface adhesion to the solvent:

$$Adhesion = \left(1 - \frac{A}{A_0}\right) \times 100$$ [1]

Where $A$ is the optical density measured at 400 nm of the extracted lower aqueous phase; $A_0$ is the optical density of the microbial suspension ($n = 3$).

2.8 MATH assay in the presence of a conditioning film

The microbial adhesion to hydrocarbons assay was followed with the following modifications. Prior to testing, 7.5 mL of standardised bacterial suspension (OD 1.0 at 540 nm) and 3.25 mL of 10 % bovine serum albumin (Sigma, UK) was vortexed for one minute. The mixture was centrifuged at 3000 rpm for 10 minutes and rinsed once with 10 mL PUM buffer, and was then re-centrifuged. The final pellet was diluted to an OD of 1.0 at 400 nm in PUM buffer before testing. ($n = 3$). This assay was not carried out using whole blood, since the presence of the blood cells interfered with the results.

2.9 Zone of inhibition assays (ZoI)

*S. aureus* or *S. epidermidis* (100 µL) was spread across the surface. For the ZoI with conditioning films, 10 µl of 10 % conditioning film (BSA or WB) was spread onto the coupon surfaces using a sterile pipette tip and dried in a microbiological class II flow hood for 1 h. The substrata with or without conditioning films was placed surface down on to the bacterial lawn.
The agar plates were incubated overnight at 37 °C. The presence of bacterial clearance around the coupons was measured with callipers correct to 0.01 mm (n = 6).

2.10 Statistical analysis

Statistics were carried out using two tailed T-tests using Excel. The results were reported as a mean +/- standard error. Variance seen within the data was considered significant if $p < 0.05$.

3. Results

3.1 Surface Characterisation

EDX analysis of the surfaces demonstrated the chemical composition of the coatings (% atomic weight) including the concentration of the silver which was determined to be 14.94%at.wt and 19.04%at.wt (Table 1). AFM images showed that with increased silver concentration, the silver nanoparticles were heterogeneously distributed throughout the coatings, thus demonstrating that the surface coatings were not chemically homogenous in composition (Figure 1). Addition of the TiNAg coating resulted in surfaces with Ag nanoparticles that were segregated from the TiN matrix (Figure 1c and d). In order to quantitively assess the topographical heterogeneity of the surfaces, line profiles were taken from the AFM images (Figure 1 and Figure 4) was observed that the addition of the TiN and TiNAg coatings changed the nanotopography of the surface of the stainless steel (Figure 1) and that the addition of the coating resulted in higher/wider peaks and deeper valleys than was found on the stainless steel (Figure 2). The stainless steel demonstrated the smallest width (0.8 nm – 8.86 nm) and depth of the valleys (0.52 nm – 7.3 nm) and the smallest peak widths (4.43 nm – 17.72 nm) and peak heights (2.4 nm – 8 nm) (Figure 2a). This was followed by the TiN19.04at.%Ag which demonstrated valley widths of 23 nm – 104.23 nm, valley depths of 18 nm – 26.53 nm, peak heights of 42 nm – 61.74 nm and peak widths of 60 nm – 271.91 nm (Figure 2d). The TiN14.94at.%Ag surface demonstrated the greatest valley widths of 86 nm – 418.25 nm (Figure 2c). The TiN demonstrated the largest surface features for all the parameters tested (valley depth 17.5 nm –
128.7 nm, peak width 4.33 nm – 316.09 nm and peak height 3.58 nm – 139.43 nm), with the exception of the valley width parameter (8.67 nm – 95.13 nm) (Figure 2b).

3.2 Retention Assays

Retention assays were carried out in the presence of the bacteria or conditioning films alone, or in bacterial – conditioning film combinations (Figure 3). In the absence of conditioning film, bacterial numbers were greater for *S. aureus* retained on the different coatings (range 4.29% - 6.11%), than for *S. epidermidis* (range 1.75% - 3.14%). The conditioning films were retained in greatest amounts on the stainless steel surfaces (BSA = 34.80 ± 6.54 %, WB = 17.28 ± 3.95 %), but in lower amounts on the titanium coatings with silver (BSA = 4.23 ± 0.11 %, WB = 6.24 ± 1.55 %, and BSA = 3.32 ± 0.53 %, WB = 11.38 ± 2.25 %, for TiN/14.94at.%Ag and TiN/19.04at.%Ag respectively). On the TiN coating without silver no conditioning film was detected. Interestingly, when the cells and conditioning films were tested together, the percentage coverage of both was low on the TiN and TiNAg surfaces for both conditioning films (*S. aureus* and *S. epidermidis* between 0.88 ± 0.04 % – 0.14 ± 0.01 % and 0.88 ± 0.04 – 0.16 ± 0.01 % respectively) and significantly lower than when the cells were tested alone, with the exception of *S. epidermidis* in the presence of BSA on the SS surface (4.22 ± 0.82 %).

Overall, most fouling, except for the TiN coatings, occurred when the conditioning films were used alone.

3.3 Microbial adhesion to solvent (MATS) assays

The MATS assays was used to determine the physicochemistry of the bacteria in the presence and absence of conditioning films (Figure 4). Both species demonstrated the greatest adhesion to the acidic polar solvent chloroform (94.82 ± 1.25 % and 92.05 ± 5.27 % for *S. aureus* and *S. epidermidis* respectively). Both species also demonstrated high adhesion to the apolar n-alkanes decane (94.27 ± 1.22 % and 85.78 ± 5.85 %) and hexadecane (89.37 ± 4.46 % and 90.21 ± 3.33 %) (*S. aureus* and *S. epidermidis* respectively). The affinity of both species to
adhere to the non-polar hydrocarbons decane and hexadecane was high (> 55 %),
demonstrating that both bacterial species were highly hydrophobic and were strong electron
donors (Figure 4a). However, the electron status of the organisms could be argued to be both
donating and accepting, since both organisms adhered to the acidic solvent chloroform in
greater numbers than to the basic solvent, ethyl acetate, demonstrating a higher likelihood of
donating electrons rather than accepting them; however, the microorganisms are likely to be
capable of both. The hydrophobicity of the organisms was also demonstrated by the higher
combined affinity to the non-polar hydrocarbons (decane and hexadecane) than to the polar
solvents (chloroform and ethyl acetate).

When both species were exposed to a sterile 10 % BSA solution prior to performing the MATS
assay (Figure 4b), they demonstrated significant reductions in the adherence to chloroform
(46.08 ± 11.96 % and 21.65 ± 3.95 % for *S. aureus* and *S. epidermidis* respectively), decane
(29.78 ± 8.71 % and 15.93 ± 2.33 %) and hexadecane (46.87 ± 13.00 % and 17.22 ± 3.33 %).
However, the adherence to ethyl acetate decreased only in the case of the *S. epidermidis* strain.
These results demonstrate both a reduction of the hydrophobicity and a reduction in the ability
to donate electrons, for both strains. Further, the combined adhesion to the polar solvents
(chloroform and ethyl acetate) exceeded that to the non-polar hydrocarbons (decane and
hexadecane), confirming an increase in the hydrophilicity for both strains.

3.4 Antimicrobial Activity

Zones of inhibition were carried out to determine the antimicrobial activity of the surfaces in
the presence and absence of conditioning films (Figure 5). Stainless steel and titanium nitride
coupons did not demonstrate antibacterial properties against the bacteria in the presence or
absence of conditioning film (Figure 5). In the absence of conditioning films, the
TiN/19.04at.%Ag (0.31 ± 0.02 mm) coating demonstrated a significantly more pronounced
effect than the TiN/14.94at.%Ag coating (0.06 ± 0.003 mm) against *S. epidermidis*, but not in
the case of S. aureus. In the presence of WB or BSA, there was no ZoI effect demonstrated for either the TiN/14.94at.%Ag or TiN/19.04at.%Ag coatings against S. aureus (0.1 ± 0.005 mm on TiN/14.94at.%Ag; 0.07 ±0.004 mm on TiN/19.04at.%Ag). However, in contrast, in the presence of conditioning films, the BSA conditioning film did have an enhanced antimicrobial effect against S. epidermidis. On the TiN/14.94at.%Ag coating, in the presence of BSA (0.1 ± 0.005 mm), the results demonstrated similar ZoI to when BSA was not present (0.06 ± 0.003 mm). On the TiN/19.04at.%Ag coating, when BSA was present, a significantly greater ZoI was demonstrated (0.85 ± 0.04 mm). In the presence of WB, similar ZoI were demonstrated on both the TiN/14.94at.%Ag (0.06 ± 0.003) and TiN/19.04at.%Ag (0.31 ±0.016) coatings to those without WB present for S. epidermidis. Thus, overall the addition of the conditioning films did not affect the antimicrobial activity against S. epidermidis, but decreased it against S. aureus.

4. Discussion

The use of external fixators are common for the treatment of some fractures, such as long bone fractures and pelvic fractures, whereas infections related to the use of these biomedical devices have been recorded (Ktistakis et al., 2015; Schalamon et al., 2007). Preventing bacterial colonisation of biomedical devices is a key concept to reduce infection incidence after orthopedic surgery operations. However, it is important to determine the effect of conditioning films that may be retained on coatings or on surfaces that could be used to produce biomedical devices, since they may alter the antimicrobial properties of the surfaces, and increase/decrease bacterial retention. In this study, the retention and antimicrobial capabilities of stainless steel, TiN or TiN coated with different amounts of silver, in the presence and absence of conditioning films and in the presence/absence of microorganisms were determined.

4.1 The effect of surface properties on biofouling

Overall, it was demonstrated that the low surface roughness of the TiN surface may have reduced conditioning film attachment. More importantly, the addition of the conditioning film
and bacteria together to the surfaces reduced the number of bacteria and the amount of conditioning film retained. This could not be attributed to the surface topography but may be in part attributed to the changes in the physicochemistry demonstrated when the bacteria where subjected to the conditioning film. Further, the addition of the conditioning film reduced the antimicrobial activity of the silver containing surfaces against *S. aureus* but not against *S. epidermidis* suggesting that a component of the conditioning films may have protected the *S. aureus* against the antimicrobial action of the surfaces. However, further work is necessary to determine the action of these biochemical processes. The reduction in the antimicrobial activity of the surfaces against *S. aureus* and the decrease in the numbers of cells retained on the surfaces demonstrated that novel coatings should be tested in the presence of a conditioning film to determine their effect on the retention of the bacteria and to ensure that the antimicrobial efficacy of the surface is maintained.

Although it is well known that the topography of a surface can affect the retention of microorganisms in our work, in our work, in the presence of a conditioning film there was no significant impact on the retention of the two strains tested. This is coherent with a previous study that showed that both the nano-topography and the physicochemistry of metallic surfaces had no significant impact on bacterial retention (Whitehead et al., 2015).

### 4.2 The effect of physicochemistry on surface biofouling

In previous studies it has been shown that the presence or absence of a conditioning film could increase, decrease, or even have no impact on bacterial retention (Linnes et al., 2012). One explanation might be that conditioning film and cell retention are influenced, at least in this study by the effects that the conditioning film has on the physicochemistry of the surface and the cells. In the presence of a conditioning film, the surfaces could become more wettable (Whitehead et al., 2009b). This may in part explain why the presence of conditioning films reduced the bacterial adhesion to the surfaces. Therefore, rather than encourage microbial
adhesion, the presence of conditioning film proteins marginally reduced cellular adhesion to a surface, and this interesting factor should be taken into consideration when selecting materials for use.

Bacterial retention might also rely on hydrophobic properties of wall cell proteins, which were modified by the conditioning film components. Our results showed that before the addition of the conditioning film, the species were highly hydrophobic. When both species were exposed to the BSA conditioning film, they became more hydrophilic and electron accepting. Since less cells were retained on the surfaces in presence of BSA, this may be due to the confirmation of the proteins on the metallic surfaces and on the cells; if they exhibit similar properties when exposed to BSA they may repel one other.

4.3 Competition of binding sites

Another explanation for these results is that the conditioning film components and *Staphylococcus* cell wall proteins might compete with each other for binding sites on the surfaces. Indeed, cells and conditioning films interact with surfaces with both specific (ligand-receptor) and non-specific interactions (van der Waals, electrostatic, receptor-ligand and hydrophobic interactions) (Senaratne et al., 2005). Albumin has been shown to suppress initial bacterial adhesion to surfaces, which has been suggested to be due to the lack of specific interactions between the albumin and the bacteria (Linnes et al., 2012). Kinnari et al., (2005) demonstrated that binding of *S. aureus* on human serum albumin-coated surfaces was significantly inhibited (from 82 to 95% depending on concentration). Xu et al., (2008) reported that BSA adsorption to either fibronectin-coated substrata or *S. aureus* cell surfaces reduced *S. aureus* bacterial adhesion on fibronectin, and suggested that BSA blocked both nonspecific and specific adhesion/adsorption sites. Grzeškowiak et al., (2011) also suggested that mechanisms other than hydrophobic interactions were involved in the binding process between bacteria and
BSA, which led to the inhibition of bacterial adhesion to this protein. Similar interactions might occur between WB and Staphylococcus spp.

4.4 Conditioning film effects on the antimicrobial properties of the coatings

In addition to the retention capability, the antimicrobial properties of surfaces play a key role to avoid surface contamination (Cyphert and von Recum, 2017). Following the ZoI assays it was demonstrated that stainless steel and TiN did not display antimicrobial activity against the bacteria. However, an antimicrobial effect was observed when the TiN was incorporated with silver, and the higher silver concentration (TiN/19.04at.%Ag) displayed an higher antimicrobial activity when compared with a lower silver concentration (TiN/14.94at.%Ag). Previous studies have demonstrated that concentration of silver higher than 4.6 % in TiN/Ag coatings significantly reduced the amount of viable Pseudomonas aeruginosa and Staphylococcus aureus cells compared with TiN coatings without silver (Kelly et al., 2009). In this study, the presence of BSA increased the antimicrobial activity of the TiN/19.04at.%Ag 3-fold compared with the absence of a conditioning film or presence of whole blood against S. aureus. This suggests that the BSA may have resulted in an adjuvant effect on the action of silver against S. epidermidis. In the presence of conditioning films, no antimicrobial effect was demonstrated on S. aureus. This may suggest a specific protective effect from the conditioning film on the S. aureus bacteria, suggesting that each strain may act differently in the presence of conditioning films and thus they need independent consideration.

5. Conclusion

The presence of the conditioning films resulted in differences in the antimicrobial effect of the surfaces, and even though the bacteria used in this work were both Staphylococcus sp.. The conditioning films also interacted with the bacteria in different ways, resulting in differences in retention. This is important since the addition of the conditioning film on the surfaces clearly affects the cell surface properties which in turn affects the amount of bacterial retention, in this
case deterring it. These results suggest that the impact of conditioning films should be considered when designing new surfaces since conditioning films may either enhance or impair bacterial initial adhesion and the antimicrobial properties of surface coatings.

**Declaration of interest**

None.

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**References**


Lindsay, D., von Holy, A. 2006. Bacterial biofilms within the clinical setting: what healthcare professionals should know. Journal of Hospital Infection 64, 313–325.


Table 1. EDX analysis of the metal coupons in percentage atomic weight ± standard error.

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Figure 1. Atomic force microscopy and line profiles of a) stainless steel, b) titanium nitride, c) TiN/14.94at.%Ag and d) TiN/19.04at.%Ag demonstrating surface microtopographies and shape of the surface features.
Figure 2. Dimensions of surface peak widths and heights and valley depth and widths.
Figure 3. Percentage coverage of cells and/or conditioning film retained on the surfaces. *S. aureus* (Sa) or *S. epidermidis* (Se) without conditioning film (CF), in the presence of BSA, or in the presence of whole blood (WB), or BSA or WB conditioning film alone. 3
Figure 4. MATS assays for a) *S. aureus* and b) *S. epidermidis* in the presence of BSA.
Figure 5. Zone of inhibition assays of the surfaces against *S. aureus* (Sa) or *S. epidermidis* (Se) without conditioning film (CF), in the presence of BSA, or in the presence of whole blood (WB).