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

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14

15 **ABSTRACT**

16 Implanted medical devices are at risk of developing an infection at the surgical site. Once a
17 medical implant is inserted, it initially becomes coated by a conditioning film, followed by
18 bacterial retention. In the present study, medical grade stainless steel substrata were coated
19 with titanium nitride (TiN) or titanium nitride/silver (TiN/14.94at.%Ag or TiN/19.04at.%Ag).
20 Surface analysis determined that with increased silver concentration, silver nanoparticles were
21 heterogeneously distributed throughout the coatings. The effect of bovine serum albumin or
22 whole blood conditioning films on the antimicrobial activity and microbial retention were
23 determined using *Staphylococcus aureus* or *Staphylococcus epidermidis*. The presence of the
24 conditioning films reduced the antimicrobial effect of the surfaces against *S. aureus*. When the
25 cells and conditioning films were applied together, a reduction in bacterial retention and

26 conditioning film was observed. These results suggest that the impact of conditioning films
27 should be considered since conditioning films may reduce bacterial retention but may also
28 decrease the antimicrobial properties of the surface coatings.

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

31 **1. Introduction**

32 External fixations are essential components of modern orthopaedic surgery. For example,
33 orthopaedic devices such as fine-wire fixators and external fixators are commonly use for the
34 treatment of longbone fractures and pelvic fractures for both adults and children (Ktistakis et
35 al., 2015). However, external fixations are associated with a high incidence of pin tract
36 infection rates (Ktistakis et al., 2015; Schalamon et al., 2007). Indeed, the surface of medical
37 devices and implants provides an artificial interface on which bacteria can aggregate to form a
38 biofilm (Gristina, 1987; Lindsay and von Holy, 2006). Some pathogenic strains of common
39 skin microbiota species, such as *Staphylococcus aureus* and *Staphylococcus epidermidis*, can
40 grow in these biofilms and also be involved in pin tract infections. For example, Schalamon et
41 al., (2007) found that among 37 external fixations placed on 30 children, 19 (52%) led to at
42 least one infection. *S. aureus* and *S. epidermidis* were found in 33 % and 22 % of paediatric
43 pin tract infections respectively. Biofilm infections are associated with chronic infection, which
44 are recalcitrant to traditional antimicrobial therapy (Costerton et al., 1999). In order to combat
45 device related infections, the prevention of microbial attachment/retention on the surgical
46 implants, or the use of an antimicrobial coating may provide a partial solution to this problem.
47 Studies have shown that coating some metals, such as stainless steel with titanium can reduce
48 bacterial attachment/retention, and/or have antimicrobial properties (Whitehead et al., 2015).
49 Stainless steel and titanium have been considered in depth since they are the most common
50 materials used to produce pins or wires used in bone fixing (Galanakos et al., 2009). It has been

51 suggested that infection rates seem to be higher for stainless steel alone, compared to titanium
52 alloys (Veerachamy et al., 2014). Previous research has demonstrated that silver coated pins
53 decreased bacterial colonisation and pin tract infection both *in vitro* and *in vivo* (Bosetti et al.,
54 2002). However, some silver impregnated structures have also demonstrated stronger bacterial
55 adhesion, whilst still presenting an increased incidence of dead cells (Whitehead et al., 2011).
56 Thus, there is some debate as to which substrata provide the most beneficial surfaces.
57 Following insertion of the implant, a conditioning film forms rapidly on the surface, as
58 proteins such as fibrinogen are adsorbed onto the substratum of the device (Hohmann et al.,
59 2015). The exact format of the conditioning film is dependent on the surface properties of the
60 implanted biomaterial, such as hydrophobicity and topography (Whitehead and Verran,
61 2015). Organic films may also modify the impact of the coatings on microbial
62 attachment/retention and may alter their antimicrobial activities. To the authors knowledge,
63 the effect of a conditioning film on bacterial retention and antimicrobial activity on TiN/Ag,
64 coatings, i.e., nanocomposite coatings containing silver particles in a titanium nitride matrix,
65 has not been previously described. For this study, two conditioning films were used; bovine
66 serum albumin (BSA) and whole blood (WB). BSA was used since it is representative of
67 plasma proteins. Whole blood proteins are involved in conditioning film formation on
68 implant surfaces (Hohmann et al., 2015).

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

73 **2. Materials and Methods**

74 *2.1 Substrata*

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

80 *2.2 Energy Dispersive X-ray Spectroscopy (EDX)*

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

84 *2.3 Atomic Force Microscopy*

85 An atomic force microscope (AFM) (Explorer, Veeco Instruments, UK) was used in contact
86 mode using pyramidal shaped, silicon nitride tips to obtain the images using a scan rate of
87 $20.03 \mu\text{m s}^{-1}$ with 300-pixel resolution. Cantilever spring constants 0.05 N m^{-1} were defined
88 by the manufacturer (n = 15).

89 *2.4 Bacterial Preparation*

90 *Staphylococcus epidermidis* NCTC 11047 and *Staphylococcus aureus* NCTC 3048 were
91 incubation with shaking at 200 rpm at 37 °C for 24 h in 100 mL nutrient broth (NB) (Oxoid,
92 UK) and inoculated onto nutrient agar plates and incubated at 37 °C for 24 h. *S. aureus* or *S.*
93 *epidermidis* was inoculated into 15 mL of nutrient broth and incubated overnight at 37 °C, then
94 centrifuged at 567 g for 10 min. The supernatant was removed and the cell pellet was washed
95 in sterile distilled water (10 mL) and diluted until an optical density (OD) reading of 1.0 ± 0.1
96 was reached (540 nm). Colony forming units (CFU) corresponding to $9.72 \pm 1.3 \times 10^7$ cells for
97 *S. epidermidis* and $1.2 \pm 0.2 \times 10^8$ cells for *S. aureus* were obtained.

98 *2.5 Conditioning film preparation*

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

103 *2.6 Retention assays*

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

112 A 1:1 ratio of Rhodamine B (0.1 mg/L) and 4',6-diamidino-2-phenylindole (DAPI) (0.1 mg/L)
113 was prepared (Whitehead et al., 2009a) and 10 µL of the mix was spread across the coupons.
114 Following staining, the coupons were viewed using either DAPI 330–380 nm or rhodamine B
115 590–650 nm filters (Nikon Eclipse E600, UK with F view-II black and white digital camera,
116 Soft Imaging System, UK). The percentage coverage of cells was calculated (Cell F software,
117 UK) and recorded (n = 60). Calculations were used to take into account the dilution factor of
118 the conditioning films.

119 *2.7 Microbial adhesion to solvents (MATS) assays (based on an assay by Bellon-Fontaine et* 120 *al., 1996).*

121 In order to determine the relative hydrophobicity of the microbial cells, MATS assays were
122 carried out. Bacteria were centrifuged at 3500 rpm for 10 min, then washed 3 times using pH
123 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

124 ¹MgSO₄·7H₂O). Cells were re-suspended to an OD 1.0 at 400 nm. To a round bottomed test
125 tube 15 mm in diameter, 1.5 mL volume of washed cells suspended in PUM buffer was added.
126 Two hundred and fifty microliters of one of the test chemicals (Decane (BDH, UK);
127 Hexadecane (Sigma, UK); Ethyl Acetate (Sigma, UK) or Chloroform (Sigma, UK)) was added
128 to the suspension which was incubated at 37 °C for 10 min. Following vortexing for 2 min the
129 mixture was incubated again at 37 °C for 30 min. The optical density of the lower aqueous
130 phase was determined (400 nm). To determine the cell surface adhesion to the solvent:

$$131 \quad Adhesion = \left(1 - \frac{A}{A\emptyset}\right) \times 100 \quad [1]$$

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

134 *2.8 MATH assay in the presence of a conditioning film*

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

142 *2.9 Zone of inhibition assays (ZoI)*

143 *S. aureus* or *S. epidermidis* (100 µL) was spread across the surface. For the ZoI with
144 conditioning films, 10 µl of 10 % conditioning film (BSA or WB) was spread onto the coupon
145 surfaces using a sterile pipette tip and dried in a microbiological class II flow hood for 1 h. The
146 substrata with or without conditioning films was placed surface down on to the bacterial lawn.

147 The agar plates were incubated overnight at 37 °C. The presence of bacterial clearance around
148 the coupons was measured with callipers correct to 0.01 mm (n = 6).

149 *2.10 Statistical analysis*

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

152 **3. Results**

153 *3.1 Surface Characterisation*

154 EDX analysis of the surfaces demonstrated the chemical composition of the coatings (% atomic
155 weight) including the concentration of the silver which was determined to be 14.94%at.wt and
156 19.04%at.wt (Table 1). AFM images showed that with increased silver concentration, the silver
157 nanoparticles were heterogeneously distributed throughout the coatings, thus demonstrating
158 that the surface coatings were not chemically homogenous in composition (Figure 1). Addition
159 of the TiNAg coating resulted in surfaces with Ag nanoparticles that were segregated from the
160 TiN matrix (Figure 1c and d). In order to quantitatively assess the topographical heterogeneity
161 of the surfaces, line profiles were taken from the AFM images (Figure 1 and Figure 4) and
162 observed that the addition of the TiN and TiNAg coatings changed the nanotopography of the
163 surface of the stainless steel (Figure 1) and that the addition of the coating resulted in
164 higher/wider peaks and deeper valleys than was found on the stainless steel (Figure 2). The
165 stainless steel demonstrated the smallest width (0.8 nm – 8.86 nm) and depth of the valleys
166 (0.52 nm – 7.3 nm) and the smallest peak widths (4.43 nm – 17.72 nm) and peak heights (2.4
167 nm – 8 nm) (Figure 2a). This was followed by the TiN19.04at.%Ag which demonstrated valley
168 widths of 23 nm – 104.23 nm, valley depths of 18 nm – 26.53 nm, peak heights of 42 nm –
169 61.74 nm and peak widths of 60 nm – 271.91 nm (Figure 2d). The TiN14.94at.%Ag surface
170 demonstrated the greatest valley widths of 86 nm – 418.25 nm (Figure 2c). The TiN
171 demonstrated the largest surface features for all the parameters tested (valley depth 17.5 nm –

172 128.7 nm, peak width 4.33 nm – 316.09 nm and peak height 3.58 nm – 139.43 nm), with the
173 exception of the valley width parameter (8.67 nm – 95.13 nm) (Figure 2b).

174 3.2 Retention Assays

175 Retention assays were carried out in the presence of the bacteria or conditioning films alone,
176 or in bacterial – conditioning film combinations (Figure 3). In the absence of conditioning film,
177 bacterial numbers were greater for *S. aureus* retained on the different coatings (range 4.29 % -
178 6.11 %), than for *S. epidermidis* (range 1.75 % - 3.14 %). The conditioning films were retained
179 in greatest amounts on the stainless steel surfaces (BSA = 34.80 ± 6.54 %, WB = 17.28 ± 3.95
180 %), but in lower amounts on the titanium coatings with silver (BSA = 4.23 ± 0.11 %, WB =
181 6.24 ± 1.55 %, and BSA = 3.32 ± 0.53 %, WB = 11.38 ± 2.25 %, for TiN/14.94at.%Ag and
182 TiN/19.04at.%Ag respectively). On the TiN coating without silver no conditioning film was
183 detected. Interestingly, when the cells and conditioning films were tested together, the
184 percentage coverage of both was low on the TiN and TiNAg surfaces for both conditioning
185 films (*S. aureus* and *S. epidermidis* between 0.88 ± 0.04 % – 0.14 ± 0.01 % and 0.88 ± 0.04 –
186 0.16 ± 0.01 % respectively) and significantly lower than when the cells were tested alone, with
187 the exception of *S. epidermidis* in the presence of BSA on the SS surface (4.22 ± 0.82 %).
188 Overall, most fouling, except for the TiN coatings, occurred when the conditioning films were
189 used alone.

190 3.3 Microbial adhesion to solvent (MATS) assays

191 The MATS assays was used to determine the physicochemistry of the bacteria in the presence
192 and absence of conditioning films (Figure 4). Both species demonstrated the greatest adhesion
193 to the acidic polar solvent chloroform (94.82 ± 1.25 % and 92.05 ± 5.27 % for *S. aureus* and *S.*
194 *epidermidis* respectively). Both species also demonstrated high adhesion to the apolar n-
195 alkanes decane (94.27 ± 1.22 % and 85.78 ± 5.85 %) and hexadecane (89.37 ± 4.46 % and
196 90.21 ± 3.33 %) (*S. aureus* and *S. epidermidis* respectively). The affinity of both species to

197 adhere to the non-polar hydrocarbons decane and hexadecane was high ($> 55 \%$),
198 demonstrating that both bacterial species were highly hydrophobic and were strong electron
199 donors (Figure 4a). However, the electron status of the organisms could be argued to be both
200 donating and accepting, since both organisms adhered to the acidic solvent chloroform in
201 greater numbers than to the basic solvent, ethyl acetate, demonstrating a higher likelihood of
202 donating electrons rather than accepting them; however, the microorganisms are likely to be
203 capable of both. The hydrophobicity of the organisms was also demonstrated by the higher
204 combined affinity to the non-polar hydrocarbons (decane and hexadecane) than to the polar
205 solvents (chloroform and ethyl acetate).

206 When both species were exposed to a sterile 10 % BSA solution prior to performing the MATS
207 assay (Figure 4b), they demonstrated significant reductions in the adherence to chloroform
208 ($46.08 \pm 11.96 \%$ and $21.65 \pm 3.95 \%$ for *S. aureus* and *S. epidermidis* respectively), decane
209 ($29.78 \pm 8.71 \%$ and $15.93 \pm 2.33 \%$) and hexadecane ($46.87 \pm 13.00 \%$ and $17.22 \pm 3.33 \%$).
210 However, the adherence to ethyl acetate decreased only in the case of the *S. epidermidis* strain.
211 These results demonstrate both a reduction of the hydrophobicity and a reduction in the ability
212 to donate electrons, for both strains. Further, the combined adhesion to the polar solvents
213 (chloroform and ethyl acetate) exceeded that to the non-polar hydrocarbons (decane and
214 hexadecane), confirming an increase in the hydrophilicity for both strains.

215 3.4 Antimicrobial Activity

216 Zones of inhibition were carried out to determine the antimicrobial activity of the surfaces in
217 the presence and absence of conditioning films (Figure 5). Stainless steel and titanium nitride
218 coupons did not demonstrate antibacterial properties against the bacteria in the presence or
219 absence of conditioning film (Figure 5). In the absence of conditioning films, the
220 TiN/19.04at.%Ag (0.31 ± 0.02 mm) coating demonstrated a significantly more pronounced
221 effect than the TiN/14.94at.%Ag coating (0.06 ± 0.003 mm) against *S. epidermidis*, but not in

222 the case of *S. aureus*. In the presence of WB or BSA, there was no ZoI effect demonstrated for
223 either the TiN/14.94at.%Ag or TiN/19.04at.%Ag coatings against *S. aureus* (0.1 ± 0.005 mm
224 on TiN/14.94at.%Ag; 0.07 ± 0.004 mm on TiN/19.04at.%Ag). However, in contrast, in the
225 presence of conditioning films, the BSA conditioning film did have an enhanced antimicrobial
226 effect against *S. epidermidis*. On the TiN/14.94at.%Ag coating, in the presence of BSA ($0.1 \pm$
227 0.005 mm), the results demonstrated similar ZoI to when BSA was not present (0.06 ± 0.003
228 mm). On the TiN/19.04at.%Ag coating, when BSA was present, a significantly greater ZoI was
229 demonstrated (0.85 ± 0.04 mm). In the presence of WB, similar ZoI were demonstrated on both
230 the TiN/14.94at.%Ag (0.06 ± 0.003) and TiN/19.04at.%Ag (0.31 ± 0.016) coatings to those
231 without WB present for *S. epidermidis*. Thus, overall the addition of the conditioning films did
232 not affect the antimicrobial activity against *S. epidermidis*, but decreased it against *S. aureus*.

233 **4. Discussion**

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

244 *4.1 The effect of surface properties on biofouling*

245 Overall, it was demonstrated that the low surface roughness of the TiN surface may have
246 reduced conditioning film attachment. More importantly, the addition of the conditioning film

247 and bacteria together to the surfaces reduced the number of bacteria and the amount of
248 conditioning film retained. This could not be attributed to the surface topography but may be
249 in part attributed to the changes in the physicochemistry demonstrated when the bacteria were
250 subjected to the conditioning film. Further, the addition of the conditioning film reduced the
251 antimicrobial activity of the silver containing surfaces against *S. aureus* but not against *S.*
252 *epidermidis* suggesting that a component of the conditioning films may have protected the *S.*
253 *aureus* against the antimicrobial action of the surfaces. However, further work is necessary to
254 determine the action of these biochemical processes. The reduction in the antimicrobial activity
255 of the surfaces against *S. aureus* and the decrease in the numbers of cells retained on the
256 surfaces demonstrated that novel coatings should be tested in the presence of a conditioning
257 film to determine their effect on the retention of the bacteria and to ensure that the antimicrobial
258 efficacy of the surface is maintained.

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

264 *4.2 The effect of physicochemistry on surface biofouling*

265 In previous studies it has been shown that the presence or absence of a conditioning film could
266 increase, decrease, or even have no impact on bacterial retention (Linnes et al., 2012). One
267 explanation might be that conditioning film and cell retention are influenced, at least in this
268 study by the effects that the conditioning film has on the physicochemistry of the surface and
269 the cells. In the presence of a conditioning film, the surfaces could become more wettable
270 (Whitehead et al., 2009b). This may in part explain why the presence of conditioning films
271 reduced the bacterial adhesion to the surfaces. Therefore, rather than encourage microbial

272 adhesion, the presence of conditioning film proteins marginally reduced cellular adhesion to a
273 surface, and this interesting factor should be taken into consideration when selecting materials
274 for use.

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

282 *4.3 Competition of binding sites*

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

296 BSA, which led to the inhibition of bacterial adhesion to this protein. Similar interactions might
297 occur between WB and *Staphylococcus spp.*

298 4.4 Conditioning film effects on the antimicrobial properties of the coatings

299 In addition to the retention capability, the antimicrobial properties of surfaces play a key role
300 to avoid surface contamination (Cyphert and von Recum, 2017). Following the ZoI assays it
301 was demonstrated that stainless steel and TiN did not display antimicrobial activity against the
302 bacteria. However, an antimicrobial effect was observed when the TiN was incorporated with
303 silver, and the higher silver concentration (TiN/19.04at.%Ag) displayed an higher
304 antimicrobial activity when compared with a lower silver concentration (TiN/14.94at.%Ag).
305 Previous studies have demonstrated that concentration of silver higher than 4.6 % in TiN/Ag
306 coatings significantly reduced the amount of viable *Pseudomonas aeruginosa* and
307 *Staphylococcus aureus* cells compared with TiN coatings without silver (Kelly et al., 2009). In
308 this study, the presence of BSA increased the antimicrobial activity of the TiN/19.04at.%Ag
309 3-fold compared with the absence of a conditioning film or presence of whole blood against *S.*
310 *aureus*. This suggests that the BSA may have resulted in an adjuvant effect on the action of
311 silver against *S. epidermidis*. In the presence of conditioning films, no antimicrobial effect was
312 demonstrated on *S. aureus*. This may suggest a specific protective effect from the conditioning
313 film on the *S. aureus* bacteria, suggesting that each strain may act differently in the presence
314 of conditioning films and thus they need independent consideration.

315 5. Conclusion

316 The presence of the conditioning films resulted in differences in the antimicrobial effect of the
317 surfaces, and even though the bacteria used in this work were both *Staphylococcus sp.*. The
318 conditioning films also interacted with the bacteria in different ways, resulting in differences
319 in retention. This is important since the addition of the conditioning film on the surfaces clearly
320 affects the cell surface properties which in turn affects the amount of bacterial retention, in this

321 case deterring it. These results suggest that the impact of conditioning films should be
322 considered when designing new surfaces since conditioning films may either enhance or impair
323 bacterial initial adhesion and the antimicrobial properties of surface coatings.

324 **Declaration of interest**

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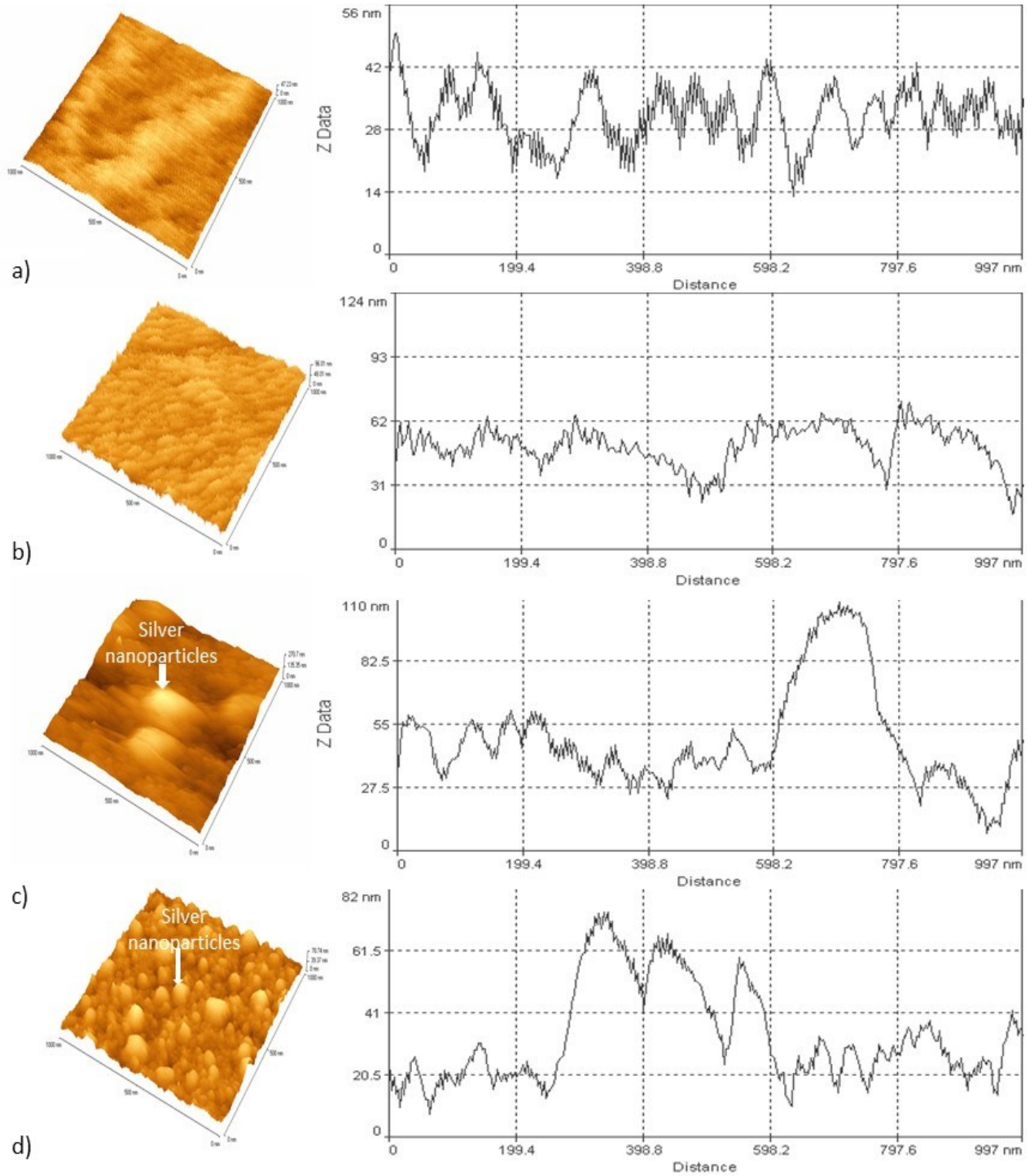
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397

398 Table 1. EDX analysis of the metal coupons in percentage atomic weight \pm standard error.

	C	O	Si	Cr	Fe	Ni	Ti	N	Ag
SS	5.91 ± 0.66	2.86 \pm 0.18	0.61 \pm 0.08	16.60 \pm 0.63	63.51 \pm 0.92	10.51 \pm 0.04			
TiN							62.94 \pm 0.91	37.06 \pm 0.91	
TiN/ 14.94 at.%Ag							58.34 \pm 0.17	26.72 \pm 0.23	14.94 \pm 0.14
TiN/ 19.04 at.%Ag							42.97 \pm 1.76	37.99 \pm 1.52	19.04 \pm 0.24

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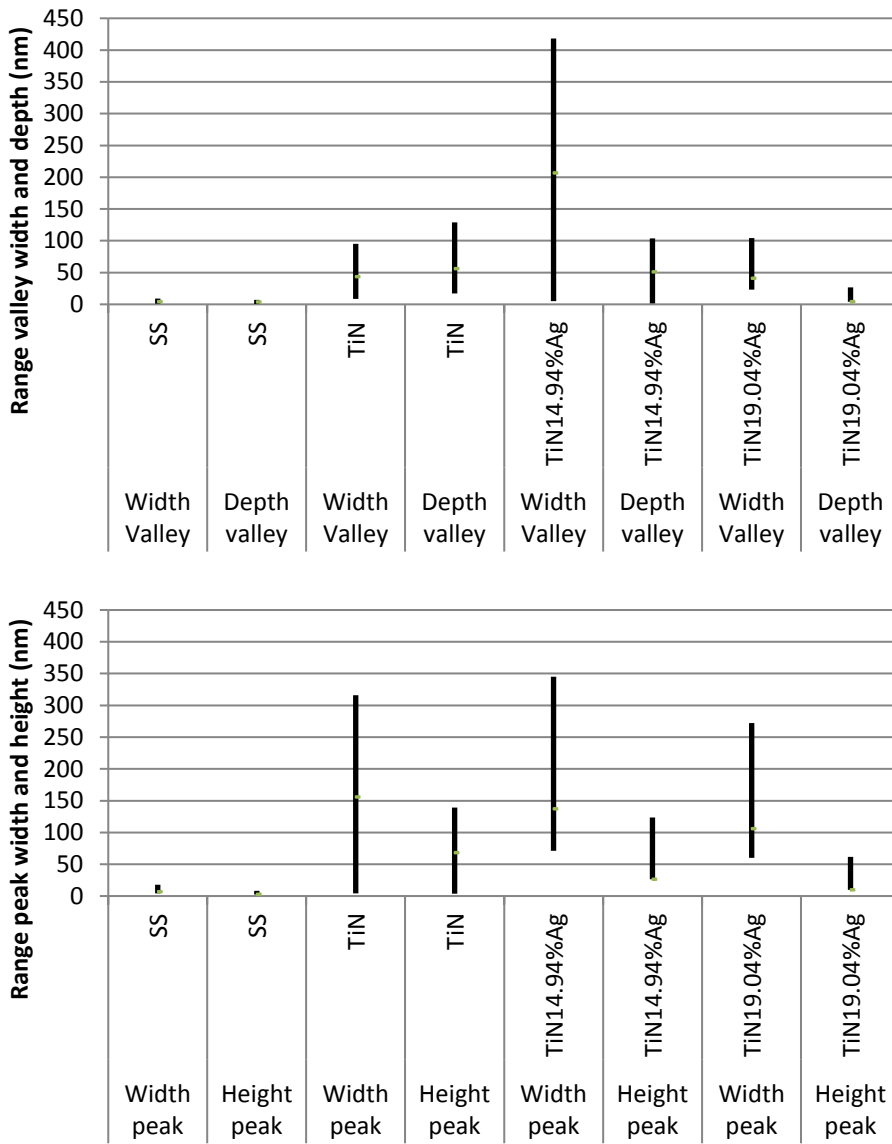
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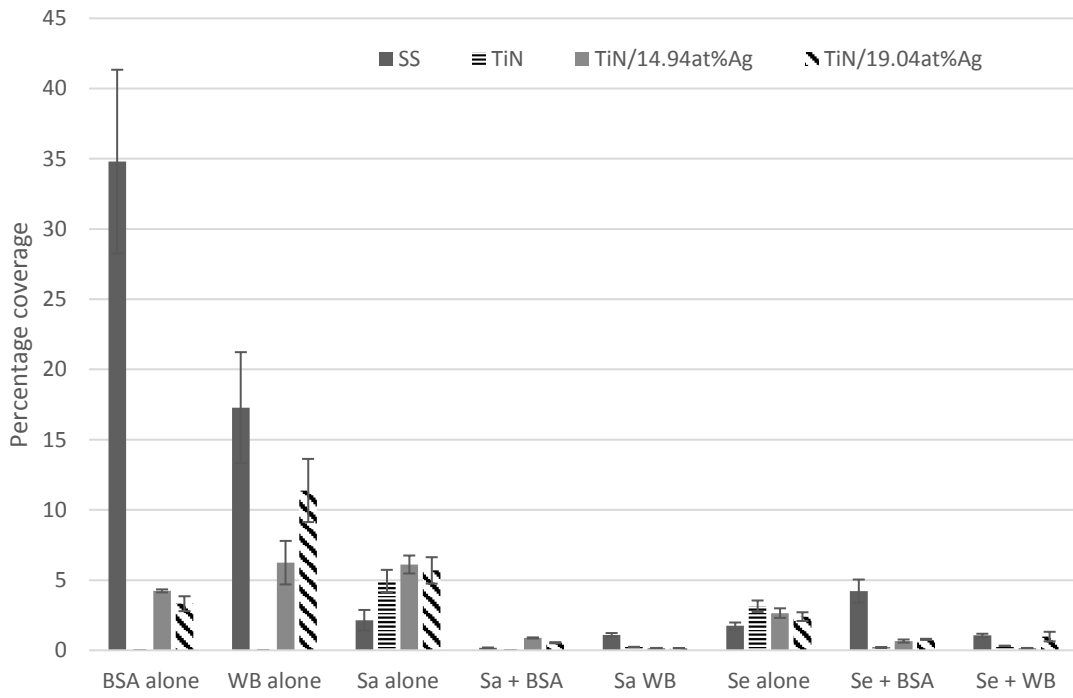
402 Figure 1. Atomic force microscopy and line profiles of a) stainless steel, b) titanium nitride,

403 c) TiN/14.94at.%Ag and d) TiN/19.04at.%Ag demonstrating surface microtopographies. and

404 shape of the surface features.



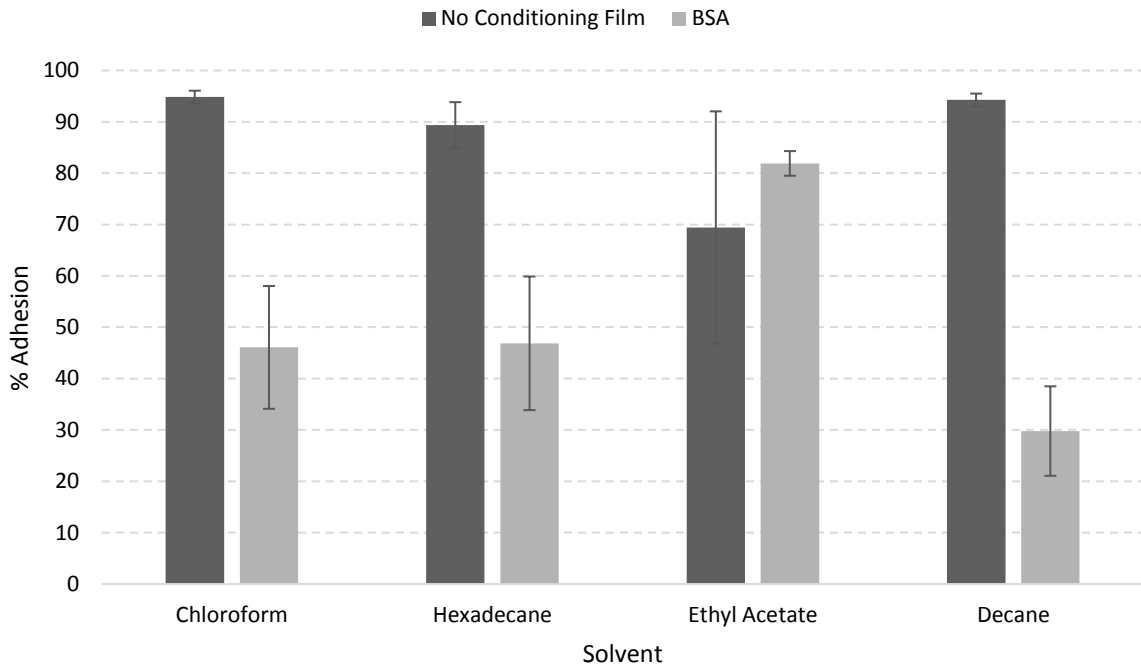
407 Figure 2. Dimensions of surface peak widths and heights and valley depth and widths. 2



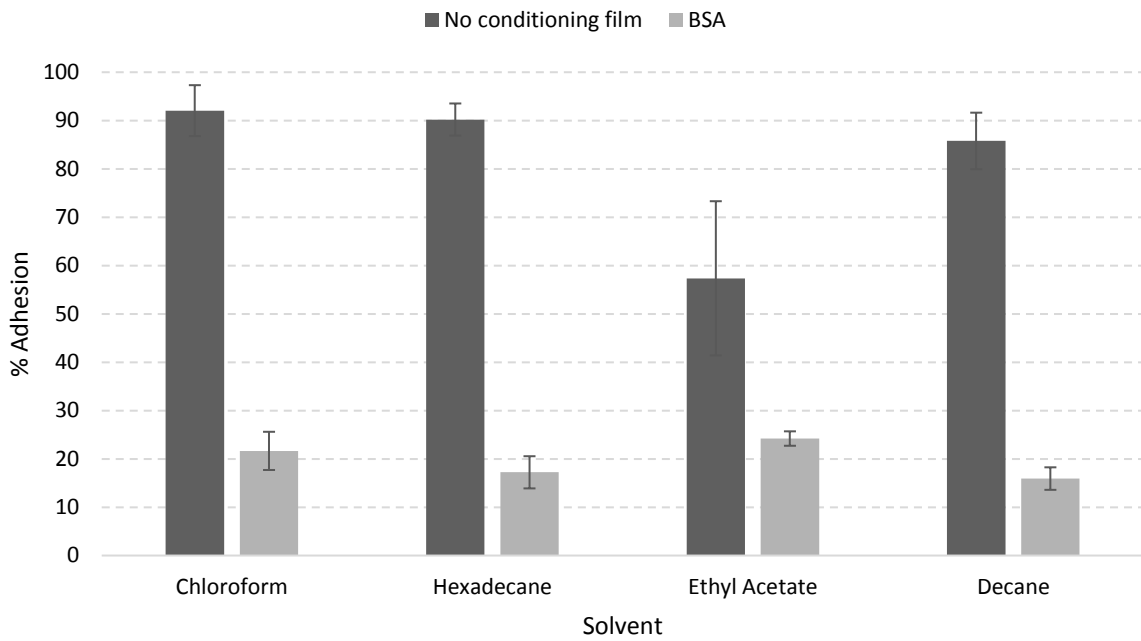
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412 Figure 3. Percentage coverage of cells and/or conditioning film retained on the surfaces. *S.*
 413 *aureus* (Sa) or *S. epidermidis* (Se) without conditioning film (CF), in the presence of BSA, or
 414 in the presence of whole blood (WB), or BSA or WB conditioning film alone. 3

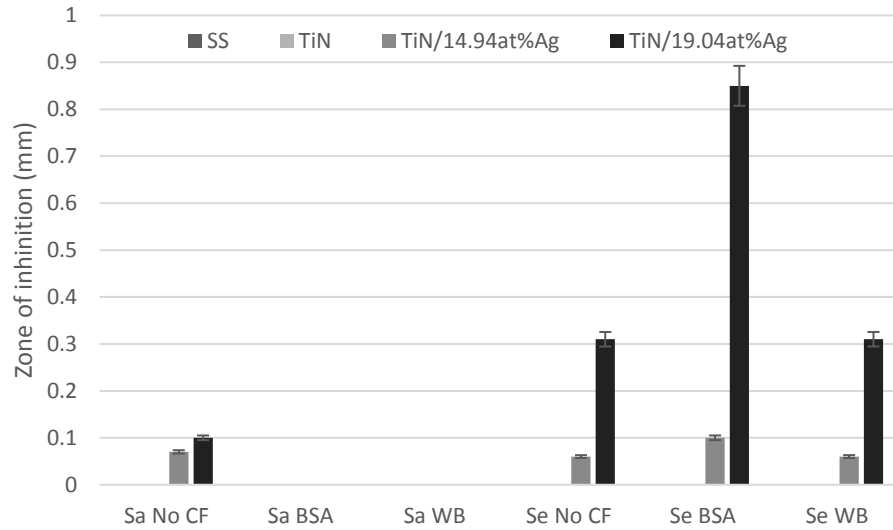


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417 Figure 4. MATS assays for a) *S. aureus* and b) *S. epidermidis* in in the presence of BSA. 4



418

419 Figure 5. Zone of inhibition assays of the surfaces against *S. aureus* (Sa) or *S. epidermidis*

420 (Se) without conditioning film (CF), in the presence of BSA, or in the presence of whole

421 blood (WB). 5