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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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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

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

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 μm 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 $^{\circ}$C for 24 h in 100 mL nutrient broth (NB) (Oxoid,

92 UK) and inoculated onto nutrient agar plates and incubated at 37 $^{\circ}$C for 24 h. *S. aureus* or *S.*

93 *epidermidis* was inoculated into 15 mL of nutrient broth and incubated overnight at 37 $^{\circ}$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 dH$_2$O 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 dH$_2$O.

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 dH$_2$O 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} K$_2$HPO$_4$.3H$_2$O, 7.26 g L^{-1} KH$_2$PO$_4$, .8 g L^{-1} urea and 0.2 g L$^-$

124 [1]MgSO$_4$.7H$_2$O). 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
$$Adhesion \ = \ \left(1 - \frac{A}{A\text{ø}}\right) x \ 100 \qquad\qquad [1]$$

132 Where *A* is the optical density measured at 400 nm of the extracted lower aqueous phase; *Aø*

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.

6

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 be14.94%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 segregatedfrom the

160   TiN matrix (Figure 1c and d). In order to quantitively assess the topographical heterogeneity

161   of the surfaces, line profiles were taken from the AFM images (Figure 1 and Figure 4) was

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 stainess 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 ± 11.96 % and 21.65 ± 3.95 % for *S. aureus* and *S. epidermidis* respectively), decane

209 (29.78 ± 8.71 % and 15.93 ± 2.33 %) and hexadecane (46.87 ± 13.00 % and 17.22 ± 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 prounounced

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 ±

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 where

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

11

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

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

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

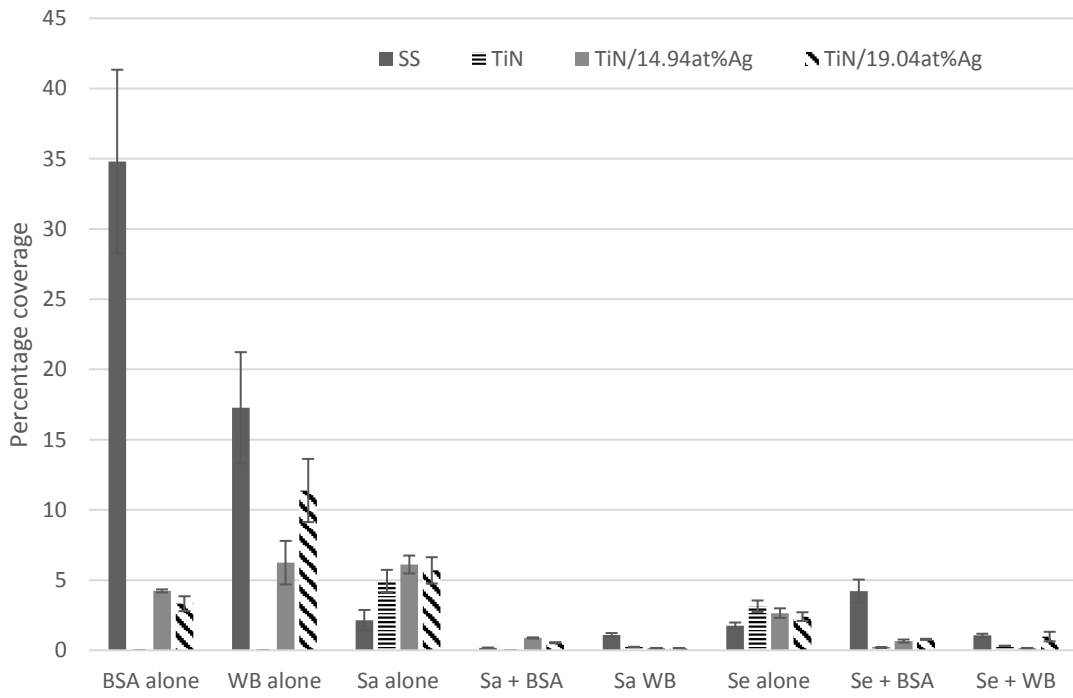c) TiN/14.94at.%Ag and d) TiN/19.04at.%Ag demonstrating surface microtopographies. and

shape of the surface features.

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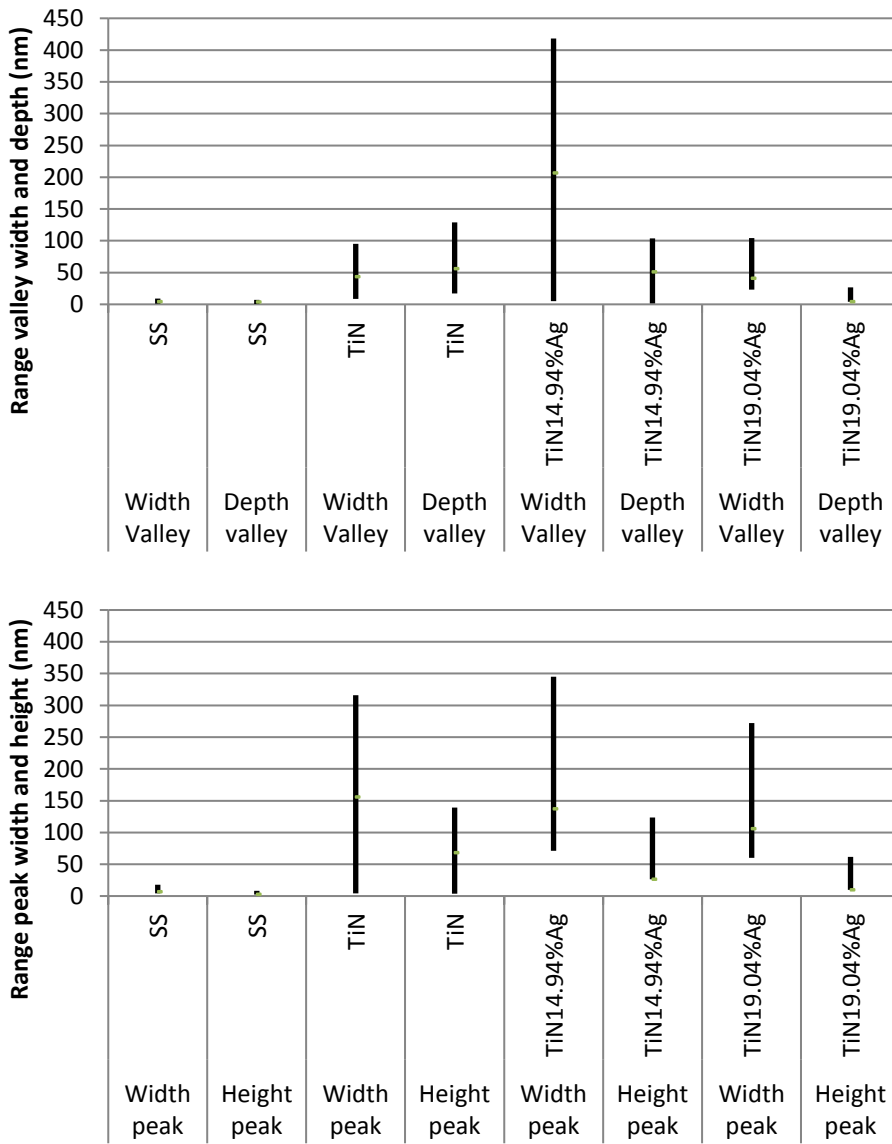
406

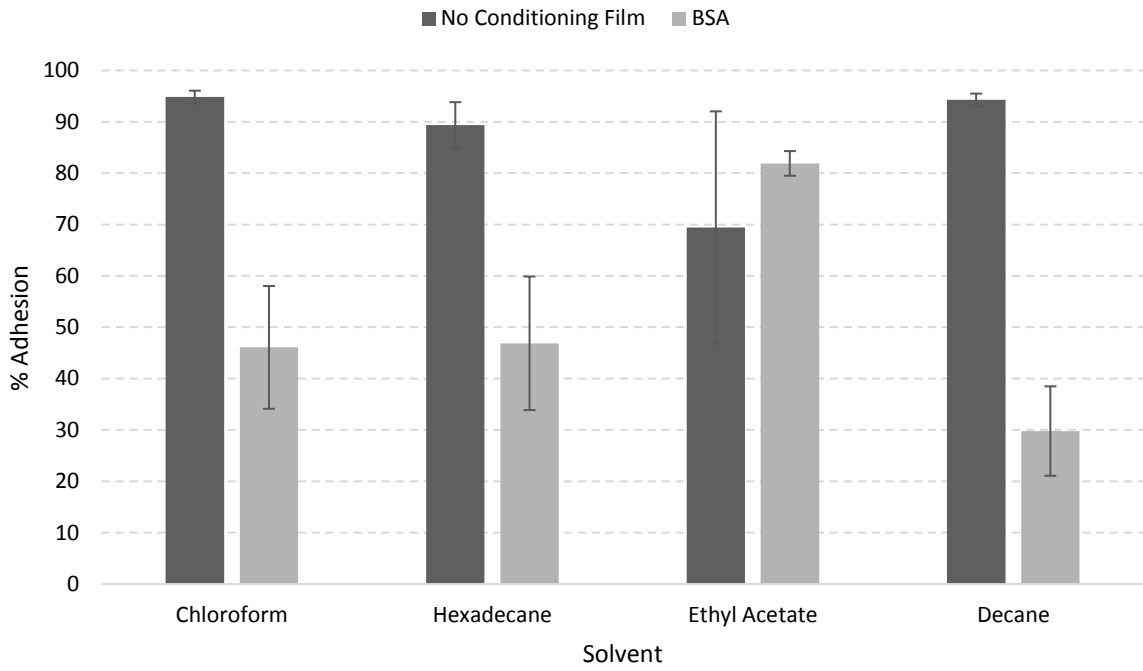407   Figure 2. Dimensions of surface peak widths and heights and valley depth and widths. 2
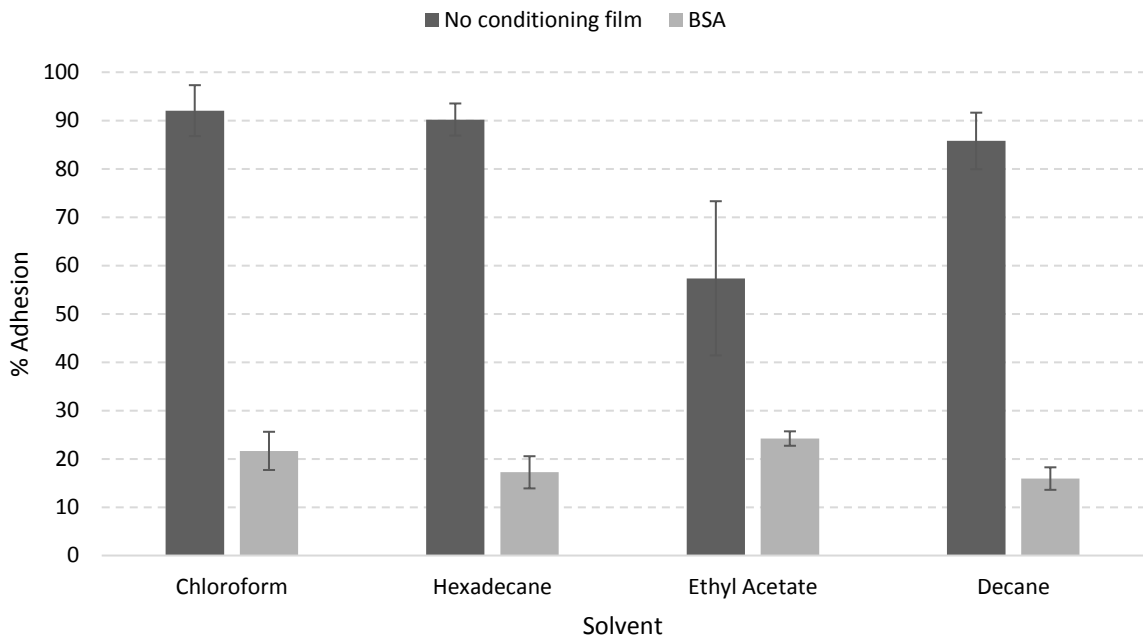
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Figure 3. Percentage coverage of cells and/or conditioning film retained on the surfaces. *S. aureus* (Sa) or *S. epidermidis* (Se) without conditioning film (CF), in the presence of BSA, or in the presence of whole blood (WB), or BSA or WB conditioning film alone. 3

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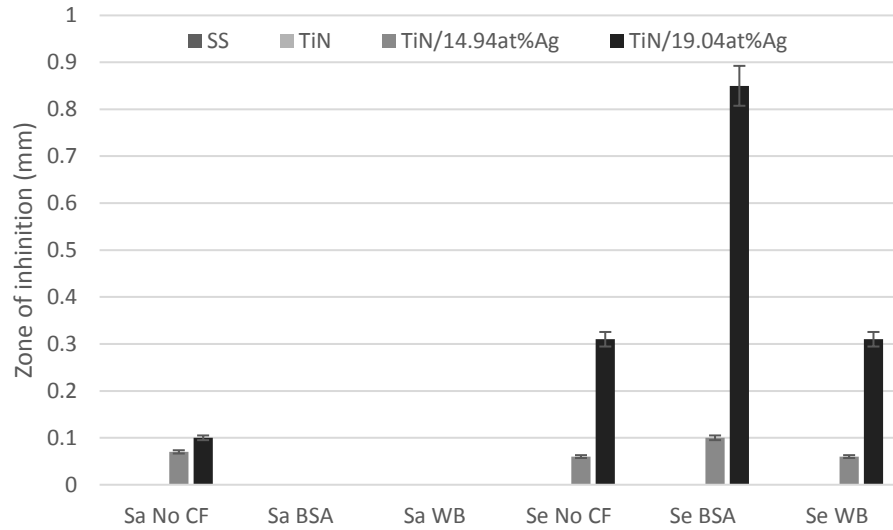

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Figure 4. MATS assays for a) *S. aureus* and b) *S. epidermidis* in in the presence of BSA. 4

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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