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The effects of blood conditioning films on the antimicrobial and retention properties of zirconium-nitride silver surfaces

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ABSTRACT

External bone fixation devices provide support and rehabilitation for severely damaged/broken bones, however, this invasive procedure is prone to infection. Zirconium nitride/silver (Ti-ZrN/Ag) coatings were characterised for surface topography, chemical composition, physicochemistry and antimicrobial efficacy (against *Staphylococcus aureus* and *Staphylococcus epidermidis*), in the presence of a blood conditioning film. The conditioning film altered the width of the microtopography of the surfaces however, the depth of the features remained relatively constant. The conditioning film also altered the coatings from hydrophobic to hydrophilic/partially hydrophilic surfaces. Following the MATH assay, the presence of a conditioning film reduced affinity towards the hydrocarbons for both microorganisms. The addition of a blood conditioning film reduced the antimicrobial efficacy of the Ti-ZrN/Ag coatings but also reduced the number of retained bacteria. This study suggests that the presence of a pre-defined blood conditioning film may result in surfaces with anti-adhesive properties, potentially leading to a reduction in bacterial retention. This, combined with the antimicrobial efficacy of the coatings, could reduce the risk of infection on biomaterial surfaces.

1. Introduction

External bone fixation is widely established throughout the field of orthopaedics utilised in a range of both, correctional and rehabilitation procedures [1,2]. External fixators involve the construction of an exterior frame that is attached to the bone *via* pins, which penetrate through the skin and soft tissue to apply skeletal traction or to hold bone fragments together in a suitable position to assist with fracture healing [3,4]. This procedure creates an internal-external interface, between the body and the surrounding environment, leading to a compromise of the innate immune system and an elevated risk of infection [5].

Pin tract infections commonly occur at the site of pin entry, often caused by opportunistic pathogens which are associated with the hosts own commensal flora. Left untreated, a pin tract infection may spread down the fixation pin, through the tissue to the bone, resulting in infection of the tissue and bone and mechanical pin loosening and serious complications such as septic arthritis, bacteraemia and osteomyelitis can arise [6,7].

One proposed approach to reduce the rate of infections associated with external bone fixation pins is to alter the surface characteristics of the devices *via* both physical and chemical techniques, in order to improve the surface properties so that they either resist colonisation or exhibit effective antimicrobial activity [8]. The most common bacterial strains isolated as the causative agent of pin-tract infections are *Staphylococcus aureus* and *Staphylococcus epidermidis* [9]. *S. aureus* infections are the most common causative agent of metal-based biomaterial associated infections, whilst *S. epidermidis* are more frequently associated with polymer-based biomaterial infections [10].

Zirconium nitride has many advantageous attributes making it suitable for biomedical implant materials, including excellent corrosion resistance, high tensile strength and good biocompatibility [11–13]. The antimicrobial efficacy of silver is well established and large varieties of silver-containing products are commercially available [14]. Co-deposition of zirconium nitride (ZrN) and silver creates a nanocomposite thin film, which combines the strength and corrosion resistance of the zirconium nitride, with the antimicrobial attributes of silver [12,15,16]. Previous studies have demonstrated the enhanced antimicrobial activity and reduction in bacterial retention on Ti-ZrN surface coatings [12,15,16].

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Surface properties, including topography, chemistry and physicochemistry play an important role in bacterial retention. Bacterial cells do not bind directly to the surface, instead they bind to a conditioning film which is produced following exposure to the surrounding environment whereby the surface initially adsorbs various organic and inorganic matter [17–20]. The structure of a conditioning film is ultimately dependent upon the composition of surrounding liquids, products and the surface properties of the implanted biomedical device [21]. Human blood constituents enhance microbial retention and biofilm formation in both, Gram-positive and Gram-negative bacterial species [22]. Physicochemical alteration of surfaces *via* the production of a conditioning film affects both the rate and the extent of bacterial retention [23,24]. Therefore, evaluation of surfaces in the presence of a relevant fouling material (*e.g.* blood) gives more insight towards the behaviour of bacterial cells and the antimicrobial efficacy of external fixation pin devices.

To the authors knowledge, the effect of a blood conditioning film on the antimicrobial activity and bacterial retention on Ti-ZrNAg, coatings has not been previously described. The aim of this study was to determine the antimicrobial efficacy of Ti-ZrN/Ag surface coatings against *S. aureus* and *S. epidermidis* in the presence of blood conditioning films.

2. Materials and methods

2.1. Surface coatings

The Ti-ZrN/Ag coatings were fabricated as per Wickens et al. [15]. The titanium in the title indicates that a titanium-interlayer was used to improve the adhesion of the coating to stainless steel. The application of a titanium-interlayer has previously demonstrated no significant impact on the action of the active surface [16]. Further, following surface and chemical analysis the titanium was not evident as part of the surface coating. Previous research carried out by this research group has characterised the coatings utilised in this study *via* Energy Dispersive X-Ray Spectroscopy (EDX) [12].

2.2. Conditioning films

For analysis of the surfaces in the presence of conditioning film, defibrinated sheep blood (Oxoid, UK) was used in all assays except the MATH assay, wherein sterile human blood plasma NHS Blood and Transplant (NHSBT) was used. The sheep blood was collected by the manufacturers using sterile techniques blood *via* venous puncture into specially designed collection bags. The blood was then defibrinated by gentle mechanical agitation; no chemicals were added. The blood was transferred from the fibrin clot and the packed cell volume adjusted to specification prior to dispensing into final containers. Sterile defibrinated sheep blood was diluted 1:1 with sterile distilled water and mixed using a vortex for 20 s. Twenty microliters were pipetted onto the surfaces and spread using the side of the pipette tip then placed in a class II microbiological flow cabinet for one hour to dry. The sterile human blood plasma was prepared from citrated whole blood collected by standard NHSBT methods. The whole blood was pooled and then centrifuged by NHSBT. The resulting plasma was 0.45 µm filtered and lyophilized and kept at 4 °C before use. The sterile human blood plasma was only used in place of the sheep blood in the Microbial Adhesion to Hydrocarbons (MATH) assay because the cells in the sheep blood interfered with the hydrocarbon assay.

2.3. Bacterial preparation

Throughout this study *S. aureus* (NCTC 8532) and *S. epidermidis* (NCTC 11047) were used. Cultures were inoculated onto nutrient agar (Oxoid, UK) and incubated at 37 °C for 24 h. Aliquots of 10 mL sterile brain heart infusion (BHI) broth (Oxoid, UK) were inoculated with either *S. aureus* or *S. epidermidis* and incubated overnight in an orbital shaker (200 rpm) at 37 °C for 24 h. Following incubation, cells were washed in 10 mL sterile distilled water (MilliporeElix, USA) by centrifugation (1721 *g* for 8 min). The supernatant was discarded and the cells were re-suspended in sterile distilled water. The suspension was then adjusted to an optical density (OD) of 1.0 ± 0.1 at 540 nm, using a spectrophotometer (Jenway 6305, UK). This spectrophotometer model is a UV/visible spectrophotometer covering a wavelength range from 198 nm to 1000 nm with a resolution of 1 nm. Cell numbers were determined *via* colony forming units (CFU/mL). For experiments containing the conditioning agents, a mixture of bacteria and conditioning film was made to ensure that the number of cells was equal on both the non-conditioned and the conditioned surfaces.

2.4. Atomic force microscopy

AFM scans of the coatings, were undertaken to display the surface topography of the coating. 3D images were obtained using an explorer AFM (Veeco, CA, USA) operated in contact mode using a force constant of 0.12 Nm^{-1} and a silicon nitride tip. Scan sizes were 20 μm^2 and three replicate scans were performed on three separate coupons.

2.5. White light interferometry

Medical grade 316 L stainless steel coupons (Aalco, UK) were coated with Ti-ZrN/Ag surfaces as per Wickens et al. [15]. Surface roughness values were obtained using a White Light Interferometry (Omniscan, UK). Five images of three replicates ($n = 15$) of each surface topography, were taken using a MicroXAM (phase shift) surface mapping microscope on the highest magnification setting (x101.61 magnification) with an ADE phase shift (XYZ 4400 mL system) and an AD phase shift controller (Omniscan, Wrexham, UK). The image analysis software used was Mapview AE 2Æ17 (*Z* range 210.5 nm – 1.169 µm) (Omniscan, UK). Analysis was carried out using EX mode. The '*Z* range' is the term used as a height descriptor on a surface topography map. The value determines the maximum height difference throughout the scanned area. In order to test the surfaces in the presence of the conditioning film, the surfaces were prepared as above.

2.6. Hydrophobicity, surface free energies and components

The hydrophobicity was calculated as per van Oss (2005), by obtaining the surface contact angles of two polar liquids, HPLC grade water (Fisher Scientific, UK) and Formamide (Sigma Aldrich, UK) and one non-polar solvent, 1-bromonaphthelene (Sigma Aldrich, UK) [25]. The contact angles were calculated using the sessile drop technique (Kruss MobileDrop II, Kruss, Germany). This instrument deposits 2 µL volumes from a standard dropper. The contact angle on the surface was measured using a camera and prism inside the device. The image was analysed using Kruss SW23 (DSA2) (Kruss, Germany) analysis software, using the Young-Laplace bubble fit method to obtain the angle between the 'bubble' and the surface interface

Surface free energy and components were determined using the van Oss and Good (van Oss et al., 1986; van Oss, 1995) calculation [26,27]. Slate et al. [16] have previously described the equations for both surface free energies and hydrophobicity [16]. For all the hydrophobicity and surface fee energy assays, the surfaces were prepared as above ($n = 3$).

2.7. Microbial adhesion to hydrocarbons (MATH) assay

The MATH assay was carried out in accordance to an adapted method described by Bellon-Fontaine et al. [28]. To enable an insight into the effects the blood components might have on the bacteria, a blood plasma conditioning film had to be used since the cellular material in the whole blood meant that this assay would not perform ($n = 3$).

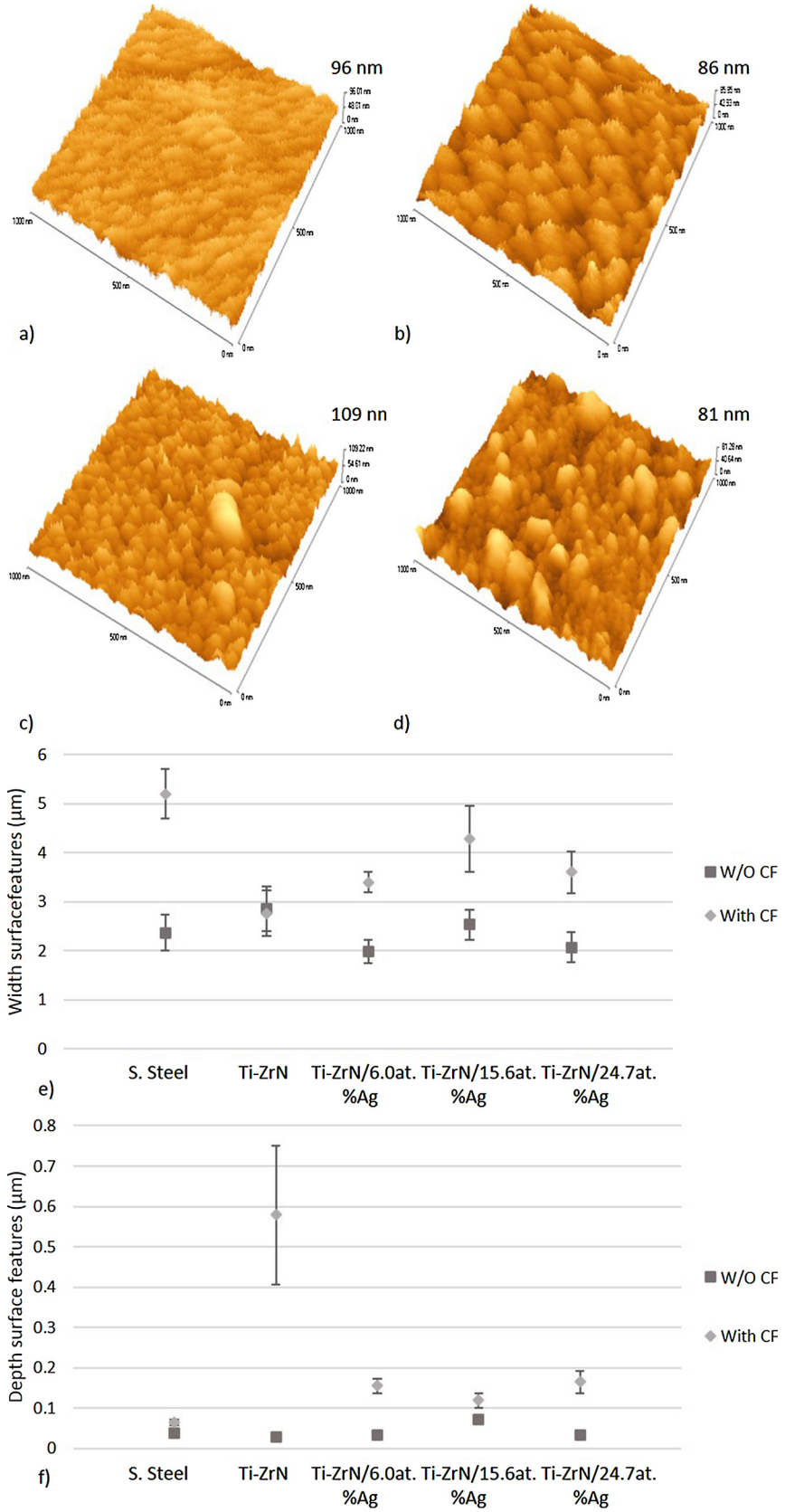

Fig. 1. AFM images demonstrating the topography of a) Ti-ZrN, b) Ti-ZrN/6.0 at.%Ag c) Ti-ZrN/15.6 at.%Ag and d) Ti-ZrN/24.7 at.%Ag and the ranges of the e) width and f) depth of the surface features following the addition of a blood conditioning film (n = 15).

2.8. Nitro tetrazolium violet assay (NTV) assay

This method was derived from Barnes et al and modified to suit the application [29]. Cells were prepared as per bacterial preparation. Colonies were counted and a dilution containing 5.0×10^5 CFU/mL was prepared [30]. The cells and blood conditioning film were mixed to ensure that the number of cells was equal on both the non-conditioned and the conditioned surfaces. The suspensions (10 µL), with or without was deposited onto each coupon and put into a class (II) cabinet for one hour to dry. Following drying, 25 mL of molten BHI agar (Oxoid, Basingstoke, UK) at a temperature of 50 °C was poured over the coupons and once set, incubated for 24 h at 37 °C. Following incubation, the surface of each plate was flooded with two mL of 0.3% filter sterilised NTV (Sigma-Aldrich, USA) and incubated for six hours at room temperature. Colonies were visible on the coupons as dark violet regions ($n = 6$).

2.9. ISO 22196:2011 standard

The ISO 22196:2011 standard for testing antimicrobial surfaces was adapted [31]. Bacterial suspensions were prepared in the presence or absence of blood conditioning film (as above) and 16 µL of the cell suspension was applied to a 10 mm x 10 mm coupon. A piece of Parafilm®, 1 cm x 1 cm, sterilised with 70% ethanol and dried, then was placed using sterile forceps upon the surface to reduce evaporation and create contact across the whole surface. The samples were stored in Petri dishes. The number of bacterial cells applied to the surfaces was 10^4 CFU/mL. Samples were tested immediately at T0 and after T24 h at room humidity (dry) and also following incubation under humid conditions, which was created by placing four Petri dishes into a large sterile container and surrounding them with absorbent paper saturated in sterile distilled water. After incubation, coupons were placed into 10 mL of BHI broth. The coupon and suspension were vortexed for 30 s to remove any unattached bacteria from the surfaces. The bacterial suspensions were plated onto agar and following serial dilutions, were incubated overnight at 37 °C for 24 h. The following day the cfu/mL was calculated. ($n = 3$).

2.10. Differential staining/retention assay

Retention assays were adapted in accordance with Wickens et al. [15]. Bacterial cultures were washed and standardised as above to ensure that the number of cells was equal on both the non-conditioned and the conditioned surfaces. Sterile test coupons were attached inside a sterilised glass Petri dish using double-sided adhesive tape. Fifteen millilitres of the bacterial suspension was added and the lid was placed on the Petri dish. The coupons were incubated at 37 °C for 1 h without agitation. Coupons were removed using sterile forceps and gently rinsed with 5 mL of sterile dH_2O. Surfaces were dried for 1 h in a class II microbiological flow cabinet, before being stained with a combined mixture of 10 µL 4′,6-diamidino-2-phenylindole (DAPI) (Sigma, UK) and 10 µL Rhodamine (Sigma, UK). The surfaces were imaged under fluorescence and the percentage coverage was measured. Sterile test coupons were attached inside a sterilised glass Petri dish using double-sided adhesive tape and the 30 mL sheep's blood and cell suspension mixture was poured over the coupons ($n = 30$).

2.11. Statistical analysis

For statistical tests two tailed T-tests with two-sample homoscedastic variance was used and the results were reported as the mean ± standard error. Differences in statistical data were considered to be significant when $p \leq 0.05$.

3. Results

3.1. Surface analysis

White light interferometry demonstrated that the average width of the surface features ranged between 2.85 µm (Ti-ZrN) and 1.98 µm (Ti-ZrN/6.0at%Ag). The addition of silver significantly increased the size of the surface feature widths. The depths of the surface features were smaller than the widths, with the largest average depth value of 0.07 µm observed on the Ti-ZrN/15.6at%Ag, which was significantly higher than the other surfaces in the absence of blood (Fig. 1). Ti-ZrN displayed the lowest average depth value of 0.03 µm, as did Ti-ZrN/6.0at%Ag (0.03 µm) and Ti-ZrN/24.7at%Ag (0.03 µm). Stainless steel produced an average depth value of 0.04 µm; it was observed that all surfaces showed similar average levels of surface depth.

White light interferometry of the surfaces following the addition of a blood conditioning film demonstrated that the blood layer affected the surface topography. Following conditioning with the blood, the surface features of the stainless steel were no longer visible and from the two-dimensional map it appeared that the shape of the red blood cells was visible on the surface. On the blood-coated samples, the addition of the conditioning film resulted in a significant difference in the depth of the surface features. This was particularly noticeable on the Ti-ZrN coating (0.03 µm without conditioning film and 0.58 µm with conditioning film). Again, the greatest depth observed on the Ti-ZrN coating (0.58 µm) was significantly increased compared to any of the other conditioning film coated surfaces. Although the blood-coated samples displayed a significantly rougher surface topography, the depth values were not significant in relation to the size of a microbial cell, 1 µm–2 µm (Fig. 1).

The hydrophobicity of the coatings was determined by obtaining the contact angles using two polar solvents (water and formamide) and one non-polar solvent (1-bromonaphthalene) to calculate the ΔG_{iwi} (the quantitative measure for hydrophobicity/hydrophilicity). The results (Table 1) showed that all five surfaces (without a blood conditioning film present) possessed negative ΔG_{iwi} values, indicating that the surfaces were all hydrophobic, with Ti-ZrN being the least hydrophobic at ΔG_{iwi} -39.63 mJ m^{-2}. Of the Ti-ZrN/Ag coatings, the sample containing 15.6 at.% Ag was the most hydrophobic, at ΔG_{iwi} -77.22 mJ m^{-2}. All the surfaces tested were significantly different to one another, except for Ti-ZrN/6.0at%Ag and Ti-ZrN/15.6at%Ag. However, in the presence of a blood conditioning film, all of the test surfaces demonstrated a change in hydrophobicity status, becoming hydrophilic (Table 1). Stainless steel became the most hydrophilic (ΔG_{iwi} 43.02 mJ m^{-2}) followed by the Ti-ZrN surface (ΔG_{iwi} 20.58 mJ m^{-2}). Of the Ti-ZrN silver containing surfaces, Ti-ZrN/15.6at%Ag was the most hydrophilic at ΔG_{iwi} 16.21 mJ m^{-2}; differences between of Ti-ZrN/6.0at%Ag and Ti-ZrN/24.7at%Ag were insignificant, producing hydrophilic values of 8.53 mJ m^{-2} and 8.76 mJ m^{-2}, respectively. The surface free energy (γ_s) of the coatings was calculated from the contact angles and demonstrated the overall surface energy (Table 1). The results indicated no clear trend between the five surfaces (with or without a blood conditioning film) with the γ_s being between 35.27 mJ m^{-2} and 43.31 mJ m^{-2} without and between 39.04 mJ m^{-2} and 44.23 mJ m^{-2} with the conditioning film. Liftshitz van der Waals forces (γ_s^{LW}) demonstrated lower values on the Ti-ZrN/6.0at%Ag (33.86 mJ m^{-2}) and Ti-ZrN/15.6at%Ag (38.21 mJ m^{-2}) coatings compared to the stainless steel (38.30 mJ m^{-2}) and Ti-ZrN (40.53 mJ m^{-2}). The Ti-ZrN/24.7at%Ag coating possessed the highest γ_s^{LW} of the co-deposited silver coated surfaces (38.77 mJ m^{-2}). However, the Lifshitz van der Waals forces were significantly ($p < 0.05$) lower for the silver containing coatings than the Ti-ZrN. In the presence of a blood conditioning film, the Lifshitz van der Waals forces displayed no significant difference between any of the surfaces. The highest values were recorded for Ti-ZrN (36.89 mJ m^{-2}) and Ti-ZrN/15.6at%Ag (36.72 mJ m^{-2}), followed by stainless steel (36.14 mJ m^{-2}), Ti-ZrN/24.7at%Ag (36.0 mJ m^{-2})

Table 1
Changes in the surface physicochemistry following the addition to the surfaces of a blood conditioning film ($n = 3$).

	Without Blood					With Blood				
	S. Steel	Ti-ZrN	Ti-ZrN/ 6.0 %Ag	Ti-ZrN/ 15.6 %Ag	Ti-ZrN/ 24.7 %Ag	S. Steel	Ti-ZrN	Ti-ZrN/ 6.0 % Ag	Ti-ZrN/ 15.6 % Ag	Ti-ZrN/ 24.7 %Ag
ΔG_{iwi}	−60.83 ± 5.6	−39.63 ± 6.4	−69.98 ± 4.0	−77.22 ± 4.4	−52.98 ± 3.4	43.02 ± 16.7	20.58 ± 8.0	8.53 ± 4.1	16.21 ± 5.5	8.76 ± 2.3
γ_s	39.60 ± 0.4	43.31 ± 0.7	35.27 ± 0.8	39.12 ± 0.4	42.42 ± 0.6	39.90 ± 1.0	44.23 ± 2.3	39.51 ± 1.0	39.16 ± 1.3	39.04 ± 1.5
γ_s^{LW}	38.30 ± 0.3	40.53 ± 0.3	33.86 ± 0.9	38.21 ± 0.5	38.77 ± 0.4	36.14 ± 0.3	36.89 ± 0.4	35.71 ± 0.9	36.72 ± 0.2	35.95 ± 0.1
γ_s^{AB}	1.29 ± 0.3	2.77 ± 0.6	1.41 ± 0.3	0.91 ± 0.2	3.61 ± 0.4	3.76 ± 0.7	7.34 ± 2.5	3.80 ± 1.1	2.44 ± 1.2	3.09 ± 1.5
γ_s^+	0.18 ± 0.1	0.23 ± 0.1	0.62 ± 0.2	0.32 ± 0.1	2.29 ± 0.5	0.14 ± 0.1	0.54 ± 0.3	0.15 ± 0.1	0.10 ± 0.1	0.25 ± 0.2
γ_s^-	5.05 ± 1.4	11.45 ± 2.1	1.85 ± 0.6	1.63 ± 0.6	3.49 ± 0.9	58.32 ± 10	41.63 ± 5.6	32.47 ± 2.3	37.09 ± 3.2	28.90 ± 4.8

and Ti-ZrN/6.0at%Ag (35.71 mJ m^{-2}). The Acid-Base interactions (γ_s^{AB}) of the five surfaces demonstrated that on the surfaces tested in the absence of a blood conditioning film, the Ti-ZrN coating (2.77 mJ m^{-2}) had a significantly higher value than that of stainless steel (1.29 mJ m^{-2}), whilst Ti-ZrN/24.7at%Ag had the highest Acid-Base value of the samples tested (3.61 mJ m^{-2}). The addition of a blood conditioning film to the samples lead to an overall increase in Acid-Base values, the Ti-ZrN surface had the greatest value (7.34 mJ m^{-2}) followed by Ti-ZrN/6.0at%Ag (3.80 mJ m^{-2}) and then stainless steel (3.76 mJ m^{-2}). The positive charge (γ_s^+) of the five surfaces found that stainless steel demonstrated the lowest positive charge (0.18 mJ m^{-2}) whilst Ti-ZrN/24.7at%Ag had the highest value (2.29 mJ m^{-2}). The addition of increasing silver concentration resulted in a trend where there was also an increase in positive charge; Ti-ZrN/6.0at%Ag (0.62 mJ m^{-2}) was greater than the non-silver coated Ti-ZrN (0.23 mJ m^{-2}) and Ti-ZrN/24.7at%Ag demonstrated the greatest positive charge (2.29 mJ m^{-2}), whilst Ti-ZrN/15.6at%Ag was an outlier to this trend (0.32 mJ m^{-2}). In the presence of a blood conditioning film, only Ti-ZrN demonstrated an increase, 0.54 mJ m^{-2}, whilst all other surfaces demonstrated a decrease in γ_s^+ energy, with the Ti-ZrN/6.0%Ag and Ti-ZrN/24.7%Ag decreases being significant (0.15 mJ m^{-2} and 0.25 mJ m^{-2} respectively). Finally, the negative charge (γ_s-) values demonstrated that Ti-ZrN possessed a significantly ($p < 0.05$) higher γ_s- value (11.45 mJ m^{-2}) than all the other surfaces, suggesting that the addition of a silver impaired the electron donor ability. As the silver content increased the γ_s- values were 1.85 mJ m^{-2}, 163 mJ m^{-2} and 3.49 mJ m^{-2} for Ti-ZrN/6.0at%Ag, Ti-ZrN/15.6at%Ag and Ti-ZrN/24.7at%Ag respectively. In the presence of the blood conditioning film, the γ_s- energies of all the surfaces significantly increased, with stainless steel and Ti-ZrN demonstrating the highest values of 58.32 mJ m^{-2} and 41.63 mJ m^{-2} respectively. Surfaces with increased silver concentrations demonstrated a decreasing trend in the negative charge recorded, except for Ti-ZrN/15.6at%Ag (37.09 mJ m^{-2}). Ti-ZrN/6.0at%Ag produced a negative charge of 32.47 mJ m^{-2} compared to Ti-ZrN/24.7at %Ag (28.90 mJ m^{-2}).

3.2. Microbiological assays

The microbial adhesion to hydrocarbons (MATH) assay was used to determine the hydrophobicity status of bacterial cells in liquid suspension, complementing the surface hydrophobicity results determined *via* contact angles (Fig. 2). The MATH assay determined the hydrophobicity of bacterial cells by calculating the percentage of bacteria adhered to four hydrocarbons; the apolar n-alkanes decane and hexadecane, the acidic (Lewis-acid) solvent chloroform, and the basic (Lewis-base) solvent ethyl acetate. *S aureus* (Fig. 2a) displayed a high affinity to the apolar hydrocarbons, decane (93%) and hexadecane

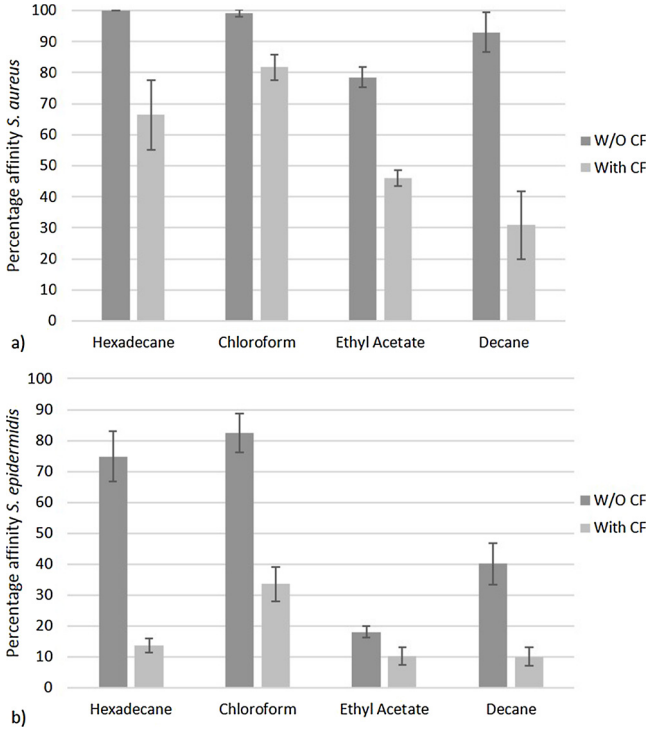

Fig. 2. Percentage affinity to hydrocarbons of bacterial cells in the presence and absence of human blood plasma a) *S aureus* b) *S. epidermidis* ($n = 3$).

(100%) which was greater than the affinity to the polar hydrocarbons, chloroform (99.1%) and ethyl acetate (78.5%), indicating that *S. aureus* was hydrophobic. Further, upon comparison of pairs containing both, one polar and one non-polar liquid with similar Lifschitz van der Waals forces, the affinity for chloroform and hexadecane was greater than the affinity for ethyl acetate and decane, indicating that *S. aureus* was an electron acceptor. However, *S. epidermidis* (Fig. 2b) displayed a lower affinity towards all four hydrocarbons tested when compared towards *S. aureus* (Fig. 2a). *S. epidermidis* demonstrated a higher affinity towards chloroform (82.4%) than hexadecane (74.8%), which is indicative of greater electron accepting and hydrophilic properties. Further, electron accepting properties were further confirmed with the lower affinity demonstrated towards the non-polar solvent decane (40.1%) over the basic solvent ethyl acetate (18%). However, when combined, the affinity for the non-polar hydrocarbons was greater than that of the combined polar hydrocarbons, indicating hydrophobic properties. Due to this, it was hard to determine the exact hydrophilic / hydrophobic

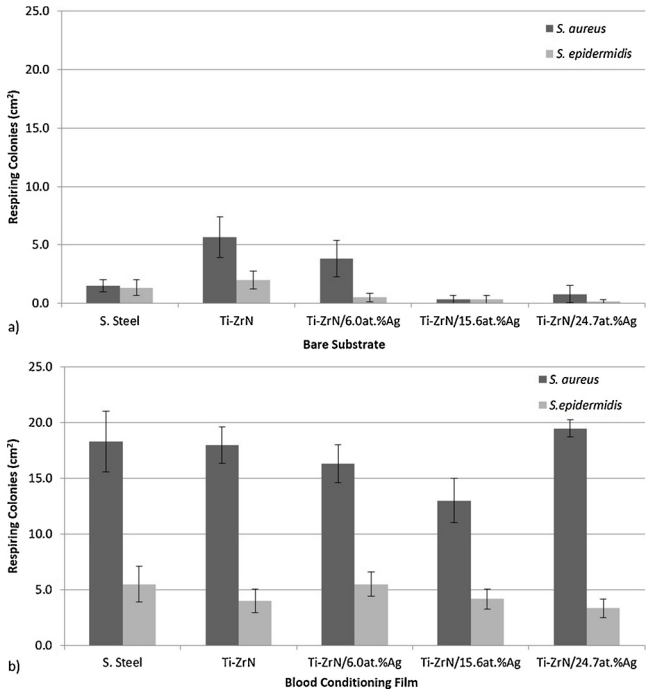

Fig. 3. NTV assay bacterial incubation onto the surfaces in the presence and absence of blood conditioning film a) bare substrate with bacteria b) substrate coated with a blood conditioning film and addition of bacteria (n = 6).

status of *S. epidermidis* as it may still be hydrophobic but with weaker hydrophobic forces than *S. aureus*.

Due to the nature of the MATH assay, it was not compatible with whole blood and instead a blood plasma conditioning film was used in order to explore the effect of a conditioning film on the bacteria. Both *S. aureus* and *S. epidermidis* displayed the highest affinity for the apolar hydrocarbon, chloroform (81.7% and 33.51%), followed by hexadecane (66.4% and 13.64%), however, the values obtained were lower than those seen in the MATH assay without blood plasma present. The results demonstrated that *S. aureus* was still more hydrophobic than *S. epidermidis*, and both *S. aureus* and *S. epidermidis* carried on demonstrating electron accepting properties in the presence of a blood conditioning film.

In order to determine the contact kill efficacy of the zirconium nitride/silver coatings, NTV assays were performed on bacterial colonies *in situ* (Fig. 3). The results demonstrated that the Ti-ZrN coatings containing silver produced an effective antimicrobial contact kill response, and all significantly ($p < 0.05$) reduced the survival of both microorganisms. However, a linear increase in silver content did not significantly increase the antimicrobial efficacy, with the most efficacious for *S. aureus* demonstrated by the Ti-ZrN/15.6at%Ag coating and for *S. epidermidis*, the Ti-ZrN/24.7at%Ag surface. The NTV assay was also conducted in the presence of a blood conditioning film and it was determined that on the Ti-ZrN/Ag surfaces, there was a reduction in contact kill efficacy. In the case of *S. aureus,* the least efficacious surface tested was the Ti-ZrN/24.7at%Ag at 20 respiring colonies per cm^2 and the most effective was Ti-ZrN/15.6at%Ag with 13 respiring colonies per cm^2. *S. epidermidis* also displayed a reduced antimicrobial effect in the presence of a conditioning film. The highest number of respiring colonies (and therefore least effective surfaces) were calculated for stainless steel and Ti-ZrN/6.0at%Ag, both producing 6 colonies per cm^2. The most efficacious surface was Ti-ZrN/24.7at%Ag, with three respiring colonies per cm^2 (Fig. 3).

To further investigate and quantify antimicrobial contact kill, an adapted British Standard ISO method (BS ISO 22,196:2011) was performed at two isolated time points, 0 h and 24 h in dry conditions (Table 2) [31]. Results showed that following the immediate recovery at T0 the coatings containing silver had an immediate effect on the bacteria. *S. aureus* at T0 was recovered in highest numbers from the Ti-ZrN coating (25.92%) followed by stainless steel (7.25%) and Ti-ZrN/6.0at%Ag (1.17%). The Ti-ZrN/15.6at%Ag coating possessed the most effective antimicrobial properties towards *S. aureus* with significant reductions ($p < 0.005$). In the case of *S. epidermidis* at T0, 75.0% and 92.92% of the total bacteria were recovered from the stainless steel and Ti-ZrN substrates, respectively. The presence of silver reduced the amount of *S. epidermidis* cells recovered; Ti-ZrN/6.0at%Ag was the most effective, with 17.08% recovery. This was followed by Ti-ZrN/24.7at%Ag with 34.92% recovery and Ti-ZrN/15.6at%Ag where 35.50% bacteria of the bacteria was recovered.

Following 24 h of contact in dry conditions, a significant reduction ($p < 0.05$) of bacteria occurred on all the surfaces. No cells were recovered from any of the silver containing surfaces demonstrating an effective contact kill over time. On the stainless steel, 0.02% of the *S. epidermidis* cells were recovered, whilst no viable *S. aureus* were recovered. Further, *S. aureus* displayed a preference for Ti-ZrN, a 0.08% recovery was observed, whereas no viable *S. epidermidis* cells were recovered. When repeated with humid conditions (data not shown), recovery of bacteria was observed only from stainless steel (*S. aureus*: 0.01%; *S. epidermidis*: 3.24%) and the Ti-ZrN (*S. aureus*: 0.09%; *S. epidermidis*: 1.03%).

The BS ISO 22196 method was then carried out in the presence of a blood conditioning film [31]. At T0, results suggested that no immediate antimicrobial activity was demonstrated, with the bacteria recovered for each surface displaying no significant difference ($p > 0.05$) in numbers from any of the surfaces. Therefore, it can be suggested that it was the blood conditioning film that was inhibiting the previously seen antimicrobial activity of the surfaces. In the presence of the blood conditioning film, the Ti-ZrN/15.6at%Ag recovered the least microorganisms for *S. aureus* (0.25%) and the second least for *S. epidermidis* (0.28%). After 24 h exposure to the surfaces, there was a decreasing trend throughout the surfaces in terms of recovered microorganisms. Following 24 h, in contrast to T0, *S. aureus* was recovered in lower numbers than *S. epidermidis*, whilst on the Ti-ZrN substrate less cells were recovered than that of the stainless steel. The Ti-ZrN/Ag coated substrates demonstrated similar levels of percentage recovery, with Ti-ZrN/15.6at%Ag and Ti-ZrN/24.7at%Ag demonstrating similar levels of antimicrobial activity, both giving a percentage recovery value of 0.02%. In the presence of a blood conditioning film, *S. epidermidis* demonstrated greater resilience over time, when compared to *S. aureus*. The Ti-ZrN/15.6at%Ag coating demonstrated the most effective antimicrobial effects, suggesting that the amount of silver did not solely contribute to the efficacy of the surfaces.

Finally, retention assays were performed on all the surfaces (Fig. 4). The retention assays determined that without the presence of a conditioning film, both *S. aureus* (0.09 - 0.20%) and *S. epidermidis* (0.07 – 0.10) displayed very low levels of coverage, with slightly higher, but not significantly, coverage by *S. aureus* but was no significant. However, when the assays were repeated with a blood conditioning film they demonstrated different results. *S. aureus* appeared to have significantly reduced coverage on all the surfaces, with the greatest coverage being less than 0.03%. However, *S. epidermidis* demonstrated a mix of increase and decrease in the coverage of the surfaces. The stainless steel, Ti-ZrN and Ti-ZrN/6.0at%Ag surfaces all demonstrated a significant increase in *S. epidermidis* coverage, with the Ti-ZrN having the greatest increase to a coverage of 0.51% but also having the greatest range. The other two silver containing surfaces, Ti-ZrN/15.6at%Ag and Ti-ZrN/24.7at%Ag, both demonstrated decreased *S. epidermidis* coverage with the highest coverage reaching 0.01%.

Retention assays of the blood conditioning films were also performed. The conditioning film demonstrated much greater coverage than the bacteria, having almost 20% coverage on the Ti-ZrN/15.6at%Ag. The coverage was slightly different on the surfaces depending on

Table 2
BS ISO 22196 method with and without a blood conditioning film displaying the percentage recovery of *S. aureus* at T0 and T24 and *S. epidermidis* at T0 and T24 ($n = 3$).

	S. aureus				*S. epidermidis*			
	With conditioning film		Without conditioning film		With conditioning film		Without conditioning film	
	T0		T24		T0		T24	
S. Steel	7.25 ± 2.1	0.35 ± 0.06	75.0 ± 13.6	0.31 ± 0.02	0.00 ± 0.0	0.04 ± 0.02	0.92 ± 0.6	0.00 ± 0.01
Ti-ZrN	25.9 ± 6.1	0.32 ± 0.04	92.9 ± 18.6	0.27 ± 0.01	0.08 ± 0.1	0.03 ± 0.01	0.00 ± 0.0	0.07 ± 0.01
Ti-ZrN/6.0 at.% Ag	1.17 ± 0.7	0.33 ± 0.02	17.1 ± 6.32	0.28 ± 0.03	0.00 ± 0.0	0.02 ± 0.01	0.00 ± 0.0	0.05 ± 0.01
Ti-ZrN/15.6 at.% Ag	0.08 ± 0.1	0.25 ± 0.04	35.5 ± 7.31	0.28 ± 0.01	0.00 ± 0.0	0.01 ± 0.01	0.00 ± 0.0	0.04 ± 0.01
Ti-ZrN/24.7 at.% Ag	0.42 ± 0.2	0.31 ± 0.03	34.9 ± 9.93	0.35 ± 0.02	0.00 ± 0.0	0.03 ± 0.01	0.00 ± 0.0	0.02 ± 0.01

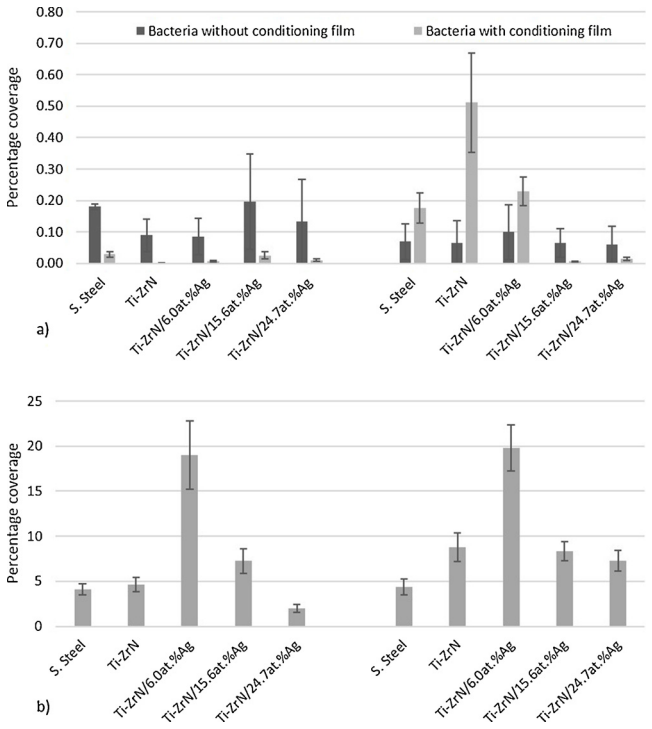

Fig. 4. Percentage coverage following differential staining of the surfaces to determine the amount of bacteria or conditioning film retained on the surface a) *S. aureus* (left hand side) and *S. epidermidis* (right hand side). The percentage coverage of bacteria are represented by dark grey columns and bacteria in the presence of a blood conditioning film in light grey conditioning films b) conditioning films alone retained on the surfaces following retention assays (n = 30).

which bacteria the assay was performed with. There were no significant differences in the coverage of Ti-ZrN/6.0at%Ag and Ti-ZrN/15.6at%Ag surfaces depending on which bacteria was used. However, on the Ti-ZrN and Ti-ZrN/24.7at%Ag, coverage when mixed with *S. aureus* (4.67% and 2.00%) was significantly less than when mixed with *S. epidermidis* (8.78% and 7.27%).

4. Discussion

The complex microtopography of surfaces are vital parameters to consider when evaluating antimicrobial efficacy of surface coatings, especially when the surface features are around the micron range (similar to bacterial cell dimensions) [32,33]. An increase in surface roughness can cause a greater degree of microbial retention due to an increase in contact surface area [34–37]. Further, physicochemical properties of the surfaces have been attributed to affecting initial bacterial attachment to surfaces [34–37]. However, once conditioning films have developed on the surface, the surface topography and/or physicochemistry may become altered and this can lead to variation in interactions of the bacteria and the surface. In order to observe the effect of a conditioning film on the antimicrobial activity of surface coatings and the retention of bacteria, all the assays were performed with or without the presence of a conditioning film [32,33,38–40].

White light interferometry results demonstrated that the addition of silver led to a reduction in surface feature widths, but had little to no effect on surface feature depths. Upon addition of a blood conditioning film, it was demonstrated that the distinct 316 L surface features were lost and that small circular pits were displayed across the surface, which resembled the outline of red blood cells. It is proposed that the blood may create an underlying air pocket after drying, which could lead to variances in microbial retention [41].

The physicochemistry of the surfaces appeared to be influenced by the surface roughness and the blood conditioning film. All the surfaces were hydrophobic prior to the application of a blood conditioning film, but all became hydrophilic after application. These results suggest that it may be possible to influence conditioning film properties by altering the surface characteristics, which could be used in order to produce a more hydrophilic surface with greater anti-adhesive properties [42–44]. This could be ideal for external fixation pins, potentially leading to a reduction in bacterial retention on the surface and combined with the antimicrobial efficacy of the coatings could dramatically reduce the risk of infection [44].

The changes to the surfaces physicochemistry due to the blood conditioning film may be the most vital aspect, as whilst there is much debate on the topic, hydrophobic bacteria preferentially adhere to hydrophobic surfaces and that hydrophobic surfaces in general can increase microbial retention [45,46]. The results of the MATH assays, which demonstrated that both *S. aureus* and *S. epidermidis* were hydrophobic, could indicate that pre-coating a pin surface with a blood plasma conditioning film may reduce the ability of these organisms to adhere to the surface. Further, both organisms were electron donors and all the surfaces demonstrated significant increases in their base energy, which represents their ability donating capability, potentially further reducing the ability of these organisms to adhere to a pre-coated surface [18,45,46].

The NTV assay replicated a situation where the fixation pin would have been pre-conditioned and the adsorbed material affected the hygienic status of the pin outside of the body. When performed in the presence of a dried blood conditioning film, the antimicrobial properties of the silver-coated surfaces appeared to be reduced against both organisms. This decrease in antimicrobial efficacy suggested that the dry blood affected the antimicrobial properties of the surface, which may be due to silver requiring a moist surface to exhibit an antimicrobial effect on the cells through the conditioning film [47].

The surface contact kill was evaluated after immediate application and following 24 h contact time *via* the BS ISO 22196 method [31]. Immediately and without a conditioning film, *S. aureus* had no viable cells recovered from the silver surfaces whilst *S. epidermidis* still had

viable cells, whilst both species had either no or very few cells recovered on the silver containing surfaces after 24 h. This indicated that *S. aureus* was more susceptible to the presence of silver, whilst *S. epidermidis* had greater resistance to the contact kill effect. When the assay was performed with a blood conditioning film however, both species demonstrated little/no cell recovery immediately after. This indicated that the presence of a moist environment potentially increased initial antimicrobial effects. However, both control surfaces also demonstrated the same effect, therefore the blood plasma conditioning film could be responsible for the affected cell recovery. After 24 h, both species demonstrated low recovery of viable cells, but this was greater than what was recovered immediately after application. These results suggest that when using Ti-ZrN/Ag surfaces, the antimicrobial effect was greatest upon initial contact and increased in a time dependent manner. The BS ISO 22196 results support work already described in the literature where silver requires moisture to display antimicrobial properties from a surface, as the presence of a blood conditioning film enhanced the antimicrobial activity of the surface in question [31,48–50].

The retention assays demonstrated that the addition of a conditioning film influenced the ability of the bacteria to adhere to the surfaces. The conditioning film caused a reduction in the coverage of *S. aureus* across all the surfaces. However, the conditioning film appeared to encourage *S. epidermidis* adherence to some surfaces. The differences in the effect that the conditioning film had on retention is likely due to differences in the bacterial physicochemistry, as it may be that the conditioning film creates conditions that are more suitable for *S. epidermidis* adherence. However, an increase in silver content of the surfaces demonstrated a reduction in bacterial adherence, the silver content could influence both the bacteria and/or the conditioning film, leading to a reduction in bacterial retention. Silver-coated materials have previously shown a reduction in the retention of bacteria to their surfaces [51–54]. Most studies focus entirely on this reduction in bacterial retention being due to the antimicrobial effect elicited by the silver. However, the addition of silver to a substrate can result in changes to surface topography and this could result in alteration to adsorption of the conditioning film and therefore the retention of bacteria [24,55]. Thus, such surfaces may provide in part a solution for deterring the rate of bacterial infection. In support of this work, Slate et al., (2018) had also previously evaluated the viability of Ti-ZrN/Ag coatings for potential applications and cytotoxicity testing revealed that the Ti-ZrN/Ag coatings, did not possess a detrimental effect towards a human monocyte cell line U937 [16].

5. Conclusion

The addition of a conditioning film was undertook to replicate organic constituents that would adhere to a biomaterial surface in order to evaluate the effect on the surface properties and antimicrobial efficacy of the Ti-ZrN/Ag coatings. Following the addition of a blood conditioning film to the Ti-ZrN/Ag surfaces, it was demonstrated that both the surface properties and antimicrobial properties were both affected. More specifically, the addition of a conditioning film reduced antimicrobial efficacy of the Ti-ZrN/Ag coatings but on the high silver content surfaces also reduced adhesion. The results from this study suggest that altering the Ti-ZrN/Ag surface properties *via* the introduction of a conditioning film, lead to an alteration in the surface characteristics, resulting in a more hydrophilic surface with greater anti-adhesive properties. In order to further evaluate these surfaces for potential use in external fixation pins, the underpinning antimicrobial mechanisms will be explored. Further, standardised cleaning assays used in external fixation pin care and inoculation cycles to test long term efficacy of the surfaces will be incorporated into any future study conducted, to replicate external pin-tract fixation and exposure times in patients.

Competing interests

The authors declare no competing financial interests.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author, on reasonable request.

Author contributions

D. J. Wickens and J. Wilson-Nieuwenhuis were involved in the experimental protocols. A.J. Slate and J. Wilson-Nieuwenhuis wrote the manuscript. N. Dempsey-Hibbert was involved in data analysis. P. Kelly and G. West oversaw the production of the coatings. C. E. Banks, J. Verran and K.A. Whitehead wrote the manuscript and conceived the concept and ideas presented in this paper.

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