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ANTIMICROBIAL SURFACES TO COMBAT MICROBIAL AND ORGANIC FOULING IN THE FOOD INDUSTRY

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ABSTRACT

The fouling and contamination of surfaces used in the food industry by microorganisms and organic material is a long established problem. Antimicrobial and/or anti-adhesive surfaces may be improved in order to target key areas that become fouled in the food industries. A range of materials were screened to identify which surface properties (topography, chemistry, physicochemistry) most affected bacterial retention under static conditions and which surfaces were also the most antimicrobial. Magnetron sputtered Ti, Ag, TiN and TiN/19.04at%Ag coatings were tested. The results demonstrated that surface chemistry and wettability influenced *Staphylococcus aureus* retention to the surfaces. Surfaces that contained silver were antimicrobial. In the presence of horse blood, less *S. aureus* were retained on the TiN and TiN/19.04at%Ag coatings. This work is now leading to the development of a new generation of more chemically and tribologically advanced, metal compound coatings.

INTRODUCTION

Fouling in the food industry can be a major problem in terms of organic material and microbial retention. Following first contact with a food, organic material becomes adsorbed onto the surface. It is known that physicochemical and thermodynamic properties of the surface influence this initial attachment of organic material and then cells (Ma et al., 2008). Adhesion of microbial cells to the conditioned surface may then be affected by surface chemistry and subsequent microbial retention by surface topography (Whitehead et al., 2005: 2007). It is important to know what organic material has conditioned the surface, since it has been suggested that contamination with subsequent microorganisms are dependent on interactions with the organic material rather than the underlying substratum (Changani et al., 1997; Whitehead et al., 2015a; 2015b). Under certain situations, breaking of this linking organic material layer may result in microorganisms being released into the process stream resulting in contamination of downstream areas of industrial processes (Bansal and Chen, 2006).

There are a number of factors which can be used to reduce biofouling in industrial settings. An area of interest is the design and use of modified surfaces in processing plants. In order to design antifouling surfaces it is important to determine the effect of the surface properties on organic material and cell retention so that the substratum properties may be adapted. For example, it has been suggested that reducing the surface roughness and wettability is likely to lower the tendency of the proteins to adsorb onto the surface (Bansal and Chen, 2006). However, this may or may not be of benefit since although it has been suggested that the presence of an organic material layer may increase the number of microorganisms attached to a surface (Speers and Gilmour, 1985). It has also been shown that organic material films such as aqueous cod extract significantly decrease bacterial attachment by a factor of 10–100 (Bernbom et al., 2006; Pillai et al., 2009). Thus, it may be that a 'super clean' surface may not always be the most desirable.

With regards to antimicrobial surfaces, it is important to understand the interactions between the surface properties and the organic material layer since the surface needs to maintain its antimicrobial activity in the presence of fouling. However, it has been demonstrated that coatings developed to reduce biofouling of engineered surfaces do not always perform as expected based on their native properties (Fisher et al., 2014; Navabpour et al., 2014). One reason is that novel engineered surfaces rarely have a uniform chemistry. Once molecules and microorganisms overcome the surface physicochemistry to attach to a surface, they may become adhered and retained to a relatively small number of highly adhesive chemical sites which are available due to the heterogeneity of the coated surface; these chemical sites may control the overall response of the system to initial bacterial deposition (Ma et al., 2008). Further, once *in situ,* the coatings may not be robust enough to withstand

industrial conditions (Fisher et al., 2014; Navabpour et al., 2014).

The aim of this work was to determine the effects of surface properties on bacterial retention and antimicrobial activity of single phase Ti and Ag metal coatings, and the metal compounds TiN and TiN/19.04at%Ag. These results will be used to develop more efficient antimicrobial and anti-adhesive surfaces.

EXPERIMENTAL TECHNIQUES

Magnetron sputtering

In order to produce the substrata, prior to deposition, substrates (10 mm × 10 mm stainless steel samples n= 20) were cleaned with methanol (BDH, UK). To produce the coatings for the study the magnetron sputtering, the method was followed, according to Kelly, et al., (2009). The sputtering rig used was a Teer Coatings UDP 350 magnetron sputtering system, in a closed field unbalanced magnetic configuration. The chamber was pumped down until a vacuum below $1.1x10^{-3}$ Pa was achieved. Argon gas (99.9 % purity) was introduced into the chamber using a mass flow controller (MKS Instruments, UK) to increase the chamber pressure to 0.4 Pa. The substrates were sputter cleaned by applying -550V DC to the substrate holder for 10 minutes.

Titanium and silver coatings

The pure metal coatings were deposited using a 99.9% Ag target or a 99.5% Ti target of 150 mm diameter (Teer Coatings, UK). Ag and Ti coatings were deposited using DC mode (Advanced Energy MDX) magnetron sputtering. An average power of 500 W was applied to the Ag or Ti targets at an operating pressure of 0.36 Pa with an Ar flow of 5 standard cubic cm per min (sccm). Due to the different sputtering rates of each metal, the deposition time was 3 min (Ag) and 15 min (Ti).

Titanium nitride/silver coatings

Throughout the sputtering procedure the Ti target was driven at 1.5kW in pulsed DC mode (Advanced Energy MDX with SPARC-LE pulse unit) at a frequency of 20 kHz and a duty of 90%. The Ag target was driven at a power of 120 W to provide the 19% Ag concentration in the deposited film. Although a coating of 19% silver was selected for use, concentrations of Ag in the coatings can be made in a variety of ranges. Deposition took place in Ar-N_2 atmospheres at 2.4×10^{-3} mbar, with optical emission monitoring used to control the reactive sputtering process (Kelly et al., 2009). To improve adhesion a pure titanium interlayer was deposited prior to the nitride coating. Deposition times were set at 30 minutes. The samples were attached to the magnetron substrate holder (cylindrical) using kapton tape (Agar Scientific, UK). The substrate holder was placed in the magnetron sputtering chamber with the samples facing away from the targets. Shuttering was used on one side of the chamber to reduce the buildup of titanium nitride on the silver target. Following deposition the chamber was left for 40 minutes to cool before venting to minimise coating stress due to sudden temperature fluctuations.

Atomic force microscopy

Images and topography data were obtained using an AFM (Quesant Instruments, CA, USA) operated in contact mode using silicon nitride tips with a force constant of 0:12 N/m (n = 3).

Wettability measurements

Contact angle measurements were made at room temperature using the sessile drop technique. Five microlitres of high performance liquid chromatography grade water (BDH, UK) were deposited onto a horizontal sample using a syringe and the contact angles measured using a Kruss goniometer and data analysis system (KRUSS GMBH, Germany). Five measurements were taken on three different newly prepared samples, so for each liquid n = 15 per material.

Microorganisms

Stock cultures of *Staphylococcus aureus* NCTC 3048 were prepared by overnight incubation in nutrient broth (Oxoid, UK) at 37 ºC. Following incubation each inoculated broth solution was centrifuged for 10 minutes at 3500 rotations per minute (RPM). Excess broth was then decanted from the cell pellet formed and washed by re-suspension in sterile distilled water using a vortex mixer. Cells were re-suspended and diluted with sterile distilled water until an optical density (OD) reading of 1.0 was reached at 540 nm on the spectrophotometer. The resultant inoculum had colony forming units (CFU) corresponding to approximately 10^9 CFU/mL the numbers of which were confirmed using serial dilutions.

Tetrozolium violet assay

Using aseptic technique, the cells were diluted in sterile water until a concentration of 10^5 CFU/mL was reached. The number of CFU/mL was checked using serial dilutions. Ten microlitres of 10^5 cells of *S. aureus* inoculum was applied to the coated surfaces and spread using a sterile pipette tip. The coupons were then placed in a microbiological class II flow hood for between 15-30 minutes, until completely dry. Using sterile forceps, the coated coupons were placed in triplicate onto a sterile Petri dish, surface facing upwards. Using aseptic technique, 25 ml of molten nutrient agar was poured over the coupons. Each test was performed in duplicate. The plates were left to set before being incubated overnight at 37 ºC. Two millilitres of tetrozolium violet (1g/L Sigma, UK) was applied to the agar surface, ensuring complete coverage of the agar. The

tetrozolium violet stain was left to develop for 6 hours. Colonies on the coupon stained violet were counted and recorded.

Retention assays

Retention assays were carried out as previously described (Whitehead et al., 2007). Three replicate test pieces were placed horizontally in a glass Petri dish, to which 25 ml of cell suspension were added and incubated for 1 h without agitation. Test pieces were removed, rinsed once for 5 s with sterile distilled water and *S. aureus* cells were dried in a microbiological class 2 hood. The retained cells were stained for 2 min using 0.03% acridine orange in 2% glacial acetic acid (Sigma, UK), rinsed and air dried. Acridine orange was used as a stain for visualisation only (n = 60).

Differential staining

The novel, combined coated TiN and TiN/19.04at%Ag surfaces only were used in these assays, since these would be the surfaces most likely to be trialed in an industrial setting. Sterile horse blood (HB; TCS Biosciences) was diluted to a 10 % solution using sterile distilled water. *S. aureus* inoculum was prepared to an OD of 1.0 and 12.5 ml of inoculum was mixed with 12.5 ml of 10 % HB solution using a vortex mixer. TiN and TiN/19.04at%Ag coatings were placed in triplicate into a sterile Petri dish. The 25 ml inoculum-HB solution was then poured over the surfaces ensuring complete emersion and left without agitation for 1 hour at room temperature. Using sterile forceps, the coupons were removed from the solution and rinsed using sterile distilled water (using a driplock bottle held at 45º). Coupons were dried in a class II microbiological hood for 1 hour. Seventy microlitres of Rhodamine B (0.1 mg/L) was mixed with 70 µl of 4',6-diamidino-2-phenylindole (DAPI) (0.1 mg/L) in a ratio of 1:1 (Whitehead et al., 2009). Ten microlitres of Rhodamine B-DAPI mix was pipetted onto the coupons and spread using a sterile pipette tip. Each variant (TiN and TiN/19.04at%Ag) was performed in triplicate and then the experiment was duplicated. Samples were dried in a class II microbiological hood in the dark. Samples were stored in the dark until visualising using an epifluorescent microscope.

Epifluorescent microscopy

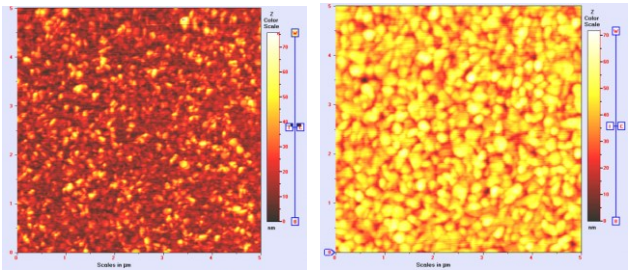
The coupon surfaces were viewed using epifluorescent microscopy with the following filters (DAPI 330–380 nm, acridine orange 510-560 nm and rhodamine 590–650 nm) (Nikon Eclipse E600, UK). The microscope had a F view-II black and white digital camera attachment (Soft Imaging System, UK) in order to visualise the surfaces. The percentage coverage of cells was calculated and viewed using Cell F software (Olympus, UK).

Statistical analysis

Statistical analysis was carried out using Microsoft Excel. A two tailed T-test was used to determine variance within the data. The results are reported as the mean +/- standard error. Variance seen within the data was considered significant if $p < 0.05$.

RESULTS

A range of coatings were screened to identify which surface properties (topography, chemistry, physicochemistry) most affected bacterial retention and which surfaces were also the most antimicrobial. Atomic force microscopy was used to determine the surface features of the coatings, and it was found that the coatings had different surface features, for example the Ag coating had more rounded, and larger surface features (0.2 – 0.4 µm) than the Ti coating (0.1 – 0.2 µm (Fig.1). The TiN surfaces had rounded features of size 0.01 µm – 0.1 µm whereas the TiN/19.04at%Ag surface had more irregularly spaced, rounded surface features that ranged from 0.01 µm to 0.2 µm.



a) b)

Fig. 1 AFM images of the a) Ti b) Ag coatings demonstrating the different shapes of the surface features. Image size 5 µm x 5 µm. Z height of a = 80 nm, b = 72 nm.

Despite the differences in surface topography, the R_a values of the coatings demonstrated that only the TiN coating had a significantly greater R_a value than the other surfaces. This may be due to a surface irregularity. The wettability of the surfaces was more varied whereby the TiN surface was the most wettable (27.3º), whilst the Ag surface was the least (81.5º).

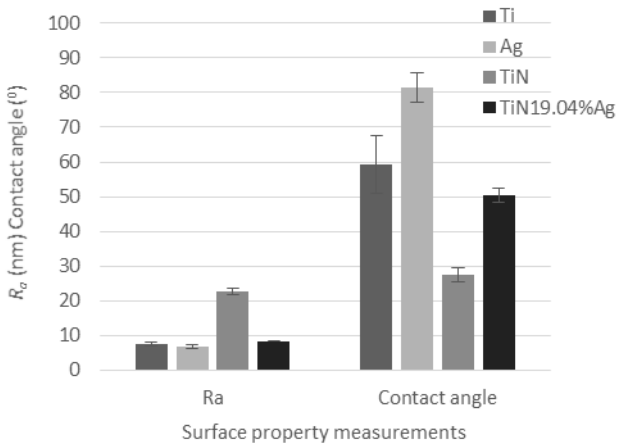


Fig. 2 R_a and water contact angles of the coatings demonstrating that although there was only a small difference in the R_a values, the coating were more varied in terms of their wettabilities (n = 5).

Retention assays were used in order to determine if the surface properties demonstrated an effect on *S. aureus* retention (Fig. 3). It was found that although the R_a values or the topography of the coatings did not demonstrate an effect on *S. aureus* retention, the more wettable, TiN containing coatings retained significantly more bacteria.

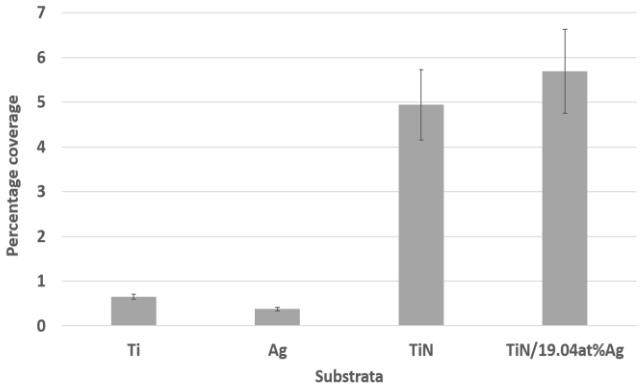
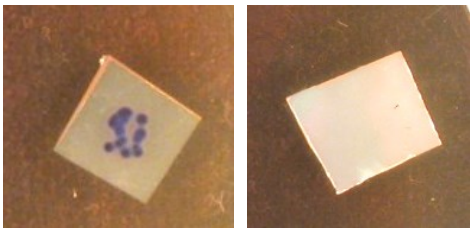
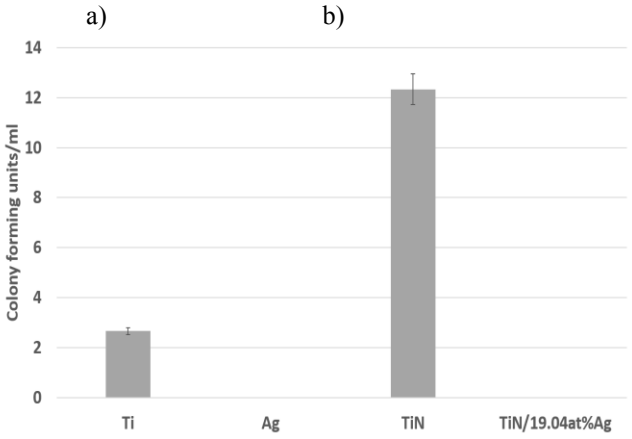


Fig. 3 The number of bacteria retained on the coatings demonstrating that more were retained on the TiN (4.94 %) and the TiN/19.04at%Ag (5.69%) coatings.

In order to determine the antimicrobial activity of the surfaces, tetrozolium violet assays were carried out (Fig. 4). The titanium coating was demonstrated to have less antimicrobial properties than the Ag containing coatings. However, the Ti coatings was demonstrated to be 6x more antimicrobial than the TiN coating. The TiN/19.04at%Ag coating also did not result in the recovery of *S. aureus*.





c)

Fig. 4 Tetrozolium violet assays demonstrated that *S. aureus* l colonies could grow on the a) Ti coating, they did not grow on the b) Ag coating. When quantified, c) coatings that contained silver demonstrated that *S. aureus* was not recovered from the surfaces.

Retention assays were also carried out on the TiN and TiN/19.04at%Ag coatings in the presence of a horse blood in order to determine the effect the organic material had on *S. aureus* retention. It was demonstrated that, in the presence of horse blood, the percentage coverage of the *S. aureus* on either surface was significantly lower than when the bacteria were used in the retention assays alone (Fig. 5).

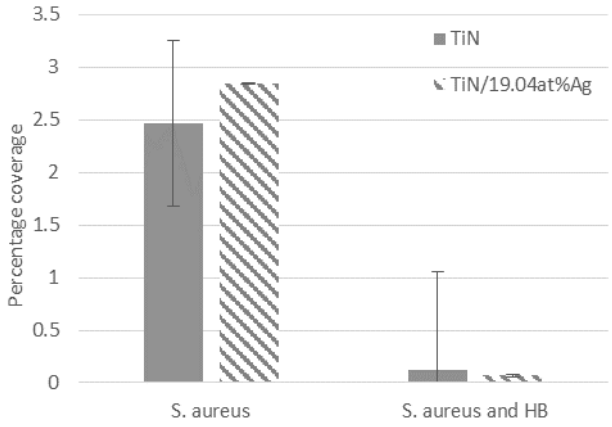


Fig. 5 In the presence of a horse blood the number of *S. aureus* retained on the coatings was significantly reduced (p<0.05) HB = horse blood.

DISCUSSION

Biofouling in the food industry can cause major issues. Much work has been carried out to determine the effect of surface properties on bacterial retention, however findings are often conflicting. This is due in part to the different methods, microorganisms and surface properties examined. This work initially aimed to determine the effects of surface properties on *S. aureus* retention and antimicrobial activity using single phase metal coatings. Progressing from single phase metal coatings to compounds, these effects were again

examined for the TiN and TiN/19.04at%Ag coatings to determine if any relationship could be made between the surface properties of the pure metals and the compound surfaces and their effects on *S. aureus* retention. Finally, the surfaces that would be most likely to be trialed in an industrial setting, the TiN and TiN/19.04at%Ag coatings were tested to determine their effect on *S. aureus* retention in the presence of horse blood.

There has been a range of studies which have suggested that surface properties such as physicochemistry, chemistry or roughness may or may not affect biofouling (Ma et al., 2008; Puckett et al., 2010; Vermeltfoort et al., 2004; Whitehead et al., 2005: 2007). This work showed that although the coatings demonstrated results with similar R_a values, the shapes of the topographies of the surfaces varied. This has been verified previously and it has been suggested that the use of R values alone is not enough to described surface topographies (Whitehead et al., 2005). Further, in this instance, surface topography was not found to affect microbial retention and this may also be due to the difficulties associated with using only R_a values to describe the surface topography. Increased surface roughness has been demonstrated to increase bacterial retention (Edwards and Rutenberg, 2001; Faille et al., 2000; Medilanski et al., 2002; Vermeltfoort et al., 2004), or decrease microbial retention (Puckett et al., 2010). Others have been in agreement with our findings where it has been found that surface roughness does not play any part in microbial retention (Barnes et al., 1999; Husmark and Ronner, 1993; Jeyachadran et al., 2007; Vanhaecke et al., 1990).

Surface chemistry has also been shown to affect microbial retention. The results from this work found that the chemistry of the alloyed coatings resulted in an increase in bacterial retention. Silver concentrations in TiNAg coatings have been shown to influence surface characteristics, such as nanotopography (surface features, grain size) and physicochemistry (Whitehead et al., 2010). In agreement with our findings, Ma et al., (2008) demonstrated that increased adhesion of bacteria and particles to a hydrophilic layer was due to the surface resulting in a small number of chemically different sites that created 'stickier' areas on the surface. The heterogeneity of the TiN and TiN/Ag coatings used in this work may have resulted in an increase in *S. aureus* retention. Aheran et al., (2005) also demonstrated that greater numbers of bacteria attached to ion beam assisted deposited silver surfaces. Jeychadran et al., (2007) further demonstrated that more bacteria were observed on a TiN film, than a Ti film. It has been suggested that this change in activity is due to the presence of the mixed oxidation state of Ti which would be as Ti^{4+} in TiO_2 and Ti^{3+} in TiN and oxynitride components. This would result in the bacteria being able to mediate differences in retention through the charge transfer interactions (Jeyachadran et al., 2007). Despite these surface properties, there is potential use for these surfaces since, when compared to a fine polished stainless steel and TiN coated surfaces, TiN/8.6 at.% Ag coatings have been shown to have marked anti-listerial properties (Skovager et al., 2013).

The TiN and TiN/19.04at%Ag coatings were more wettable and retained significantly more *S. aureus* than the single phase metal coatings. There is controversy in this area regarding whether hydrophobic or hydrophilic surfaces exert the greatest effect on bacterial retention. It has been shown that substratum hydrophobicity may be a major determinant in bacterial retention (An and Friedman, 1998; Dahlback et al., 2004), or that surface wettability has no influence on bacterial retention (Bos et al., 2000; Mediaswanti et al., 2012). It would seem that there is an interplay between the surface parameters that determines the amount of bacterial retention that will occur. Further, there has been suggestions that the surface properties that affect bacterial retention are species specific and thus methods to control fouled areas should be examined on an individual basis (Whitehead et al., 2015c).

The antimicrobial efficacy of the coatings against *S. aureus* was demonstrated using the tetrozolium violet assay. Antimicrobial activity was determined in all the Ag containing coatings. There has been demonstrated to be a significantly reduced amount of viable *P. aeruginosa* and *S. aureus* cells on surfaces with a range of silver contents in TiN/Ag coatings when compared to TiN coatings (Kelly et al., 2009), the degree of reduction being dependent on the silver concentration (Kelly et al., 2010). The Ti coating alone also demonstrated better antimicrobial activity than the TiN coating, and a antimicrobial effect of Ti has been demonstrated previously (Berry et al., 1992; Bundy et al., 1980). However, since the TiN type coatings were shown to retain more *S. aureus*, further work needs to be carried out to determine at what concentrations of biofouling the antimicrobial action of the coating becomes ineffective. Further work is being carried out to determine if the properties of the coatings can be altered to lower bacterial retention, and to increase the antimicrobial effect and hence induce a better antimicrobial response.

The results on the combined surfaces using the horse blood demonstrated that the presence of the organic material film lowered the number of *S. aureus* retained on the surfaces. Although an organic material layer has been suggested to increase bacterial retention to surfaces (Moore et al., 2007), it has also been demonstrated that the presence of organic material films may be beneficial in the food environment since the presence of BSA or fish extract have been shown to decrease bacterial retention on surfaces (Bernbom et al., 2009; Whitehead et al., 2008).

In previous work, we have deposited hard, wear resistant metal nitride coatings onto steels to reduce the formation of pits and scratches that could harbour microbes. These are currently being developed into coatings with antimicrobial properties. In other work, we are aiming to produce a more anti-adhesive surfaces using surface modification techniques.

CONCLUSIONS

1. R_a demonstrated no effect on *S. aureus* retention, whereas surface wettability had a greater influence.
2. The presence of a horse blood reduced *S. aureus* retention on the combined metal surfaces.
3. Information from this work will be used to produce new combinations of antimicrobial coatings that retain the required surface properties (e.g. hardness), but also reduce bacterial retention.

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