Staphylococcus aureus adhesion to different treated titanium surfaces

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Staphylococcus aureus is a major pathogen, associated with medical-device related infections. Converting biomaterial surfaces into non-interactive surfaces requires a specific surface/interface design. One approach is to polish the surface, and a second is to coat the surface with an antimicrobial or protein resistant coating. This study showed that polishing a titanium surface or coating titanium with various treatments that decreased the surface's coefficient of friction, had no significant effect on minimising *S. aureus* adhesion to these surfaces under static conditions in comparison to standard medical grade titanium. The cell promoting coating, TAST, was found to increase the *S. aureus* density on its surface as expected. The only coating that significantly decreased the density of adhering *S. aureus* was the titanium surface coated with sodium hyaluronate. Thus such a coating could have potential use as a coating for ostoesynthesis, orthopaedic or dental implants.

1. Introduction

Once biomaterial implants are implanted they are coated immediately with host plasma constituents, including extracellular matrix (ECM) [1], and eventually host cells. It is well known that if host cells, such as fibroblasts arrive at the biomaterial/ECM surface and secure bonds are established, bacteria are confronted by a living, integrated cellular surface. Such an integrated viable cell layer with a functional host defence mechanism can resist colonisation from bacteria such as *Staphylococcus aureus* [2]. The ability of *S. aureus* to adhere to the ECM and plasma proteins deposited on biomaterials is a significant factor in the pathogenesis of medical-device related infections since they form biofilms [3, 4]. Biofilm formation is a two-step process that requires the adhesion of bacteria to a surface followed by bacteria cell–cell adhesion, forming multiple layers of the bacteria [5]. Once a biofilm has formed, it can be very difficult to clinically treat because the bacteria in the interior of the biofilm are protected from phagocytosis and antibiotics [6]. The surface properties of medical implants, including topography and chemistry are important in the promotion or inhibition of cell and bacterial adhesion [7, 8]. Converting biomaterial surfaces into non-interactive surfaces requires a specific surface/interface design. Different surface treatments have been used to modify the topography and surface chemistry of materials such as titanium [7–9]. One approach is to polish the surface [8], and a second is to coat the surface with an antimicrobial or protein resistant coating [10–12]. This study describes the visualisation and quantification of *S. aureus* adhering to a variety of different treated/coated titanium surfaces.

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2. Materials and methods

2.1. Materials – substrates

Table I lists all the surfaces used in this study. The samples from Supplier A were made out of implant quality titanium grade 4, meeting ASTM F67 implant material specification, cut from bar, deburred, tumbled with ceramics, cleaned and gold anodised, then coated with various surfaces treatments (exception TSS). The THY and TAST polymer films were applied by dip coating, and the nitrogen implantation on TIG, was only applied to one side causing a change in the optical properties of the anodised film. TLF surfaces were not gold anodised. The samples from Supplier B were also made out of implant quality titanium grade 4, meeting ASTM F67 implant material specification, punched from sheet (TS) or cut from bar, deburred, tumbled with ceramics and cleaned. The TS samples were then gold anodised, whilst the others (TC, TE and TM) were polished using one of the methods below, before being gold anodised. The electropolished surfaces were produced by immersing the samples in a liquid (electrolyte) and applying an electric current. The chemical polishing was accomplished by immersing the samples in a liquid chemical without applying an electric current. Finally the mechanically polished surfaces were produced using diamond paste on the sample surfaces.

2.2. Surface characterisation

The surface topography of each sample (Table I) was quantitatively measured by laser profilometry (UBM Messtechnik GmbH, Germany). The surfaces were also imaged with a Hitachi S-4700 scanning electron

TABLE I List of the surfaces used in this study, including surface label and description of the surface treatment

Label	Description
TSS	Unalloyed Ti, gold anodised, Supplier A
TS	Unalloyed Ti, gold anodised, Supplier B
TLF	Low friction grey anodised Ti, Supplier A
TIG	Nitrogen ion implanted TSS
THY	TSS grafted with sodium hyaluronate
TAST	TSS with polymer cell promotion
TC	Chemically polished Ti, gold anodised, Supplier B
TE	Electropolished Ti, gold anodised, Supplier B
TM	Mechanically polished Ti, gold anodised, Supplier B

microscope (SEM), using the secondary electron detection mode at an acceleration voltage of 4 kV and an emission current of 40 μA.

2.3. Bacteria culturing and visualisation

Staphylococcus aureus 8325-4 was grown in brain heart infusion broth (BHI) to an $OD_{600} \sim 1.0$ at 37 °C in a shaking water bath, and used to inoculate 1 mL pre-warmed BHI in four well plates containing the different surfaces to a starting OD_{600} of 0.05, and incubated stationary at 37 °C for 1 h. To visualise *S. aureus* adherence to the surfaces with an SEM (exception TC, TE and TM), adherent bacteria were fixed with glutaraldehyde, post-stained with 1% OsO_4, dehydrated, critical point dried, coated with Au/Pd and visualised with an SEM using a backscattered electron detector at an acceleration voltage of 5 kV and emission current of 40 μA [13]. To quantify the density of *S. aureus* adhering, bacteria were stained with fluorescent redox dye, 5-Cyano-2, 3-ditolyl tetrazolium chloride [14] for 1 h, and visualised with a Zeiss Axioplan 2 Epifluorescence microscope fitted with an Axiocam camera. The density of live bacteria adhering to the surface observed in each image were counted using KS400 software, and analysed statistically using a one-way ANOVA with Tukey test.

3. Results

The R_a and R_q roughness parameters are shown in Table II. Differences in roughness were observed between the samples, with TS, THY, TIG, TLF and TAST showing comparable roughness, TSS and TC were smoother, whilst TE and TM were the smoothest. SEM images of the surface topographies confirmed the roughness parameter results (Fig. 1, surfaces clearly seen behind the bacteria).

Scanning electron microscope images of the coated surfaces showed that *S. aureus* adhered to all the surfaces prepared (Fig. 1), with the exception of the THY surface (Fig. 1(c)). Fluorescence microscopy confirmed the SEM imaging, where significantly less *S. aureus* were counted on the THY surface in comparison to the other coated surfaces (Fig. 2(a)). The amount of adhesion was much higher for the TSS and TAST surfaces. Significantly more *S. aureus* adhered to the TC surface than to the TS (control) and other polished titanium surfaces (TE and TM), as seen in Fig. 3. The density of bacteria on TS, TE

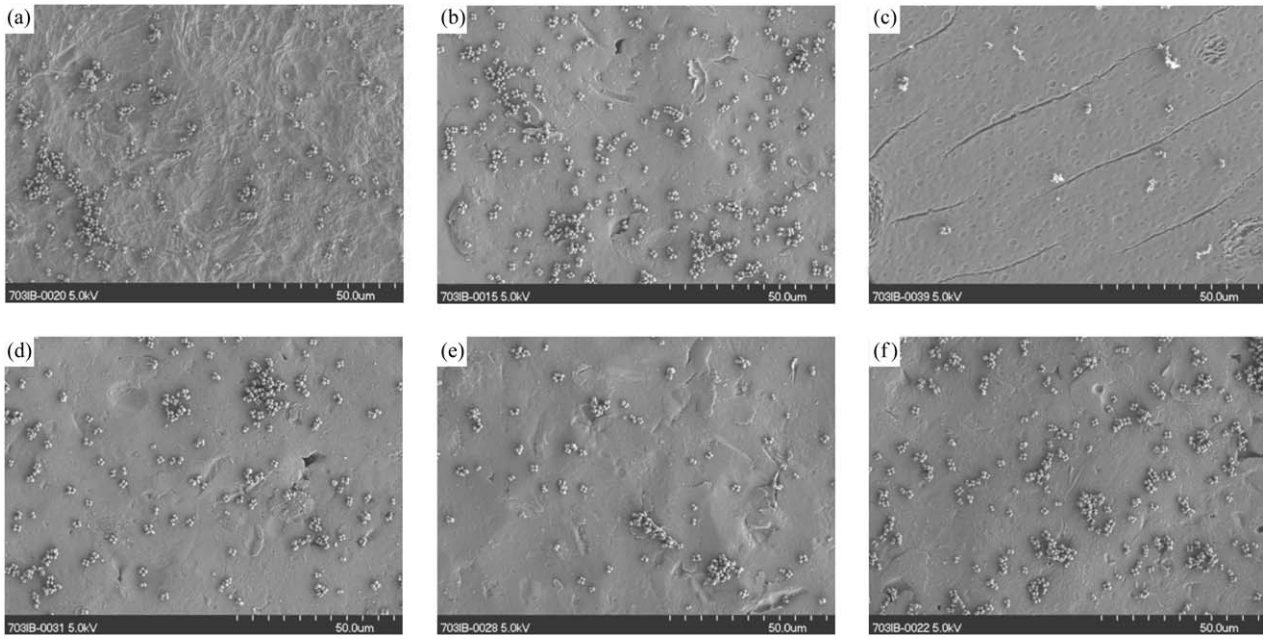

Figure 1 SEM images of *S. aureus* adhered to the standard titanium surfaces and coated surfaces after 1 h culturing. (a) TS, (b) TSS, (c) THY, (d) TIG, (e) TLF, (f) TAST. Very few bacteria are seen on the THY surface compared to the other surfaces, where small clumps of bacteria are observed all over the rough surfaces.

TABLE II Surface roughness parameters for the different treated titanium surfaces. R_a is the arithmetic mean and R_q the root mean square

Test surface	TS	TSS	THY	TIG	TLF	TAST	TC	TE	TM
R_a	1.15	0.83	1.09	1.05	1.14	1.09	0.67	0.18	0.15
R_q	1.45	1.08	1.35	1.31	1.42	1.46	0.85	0.23	0.2

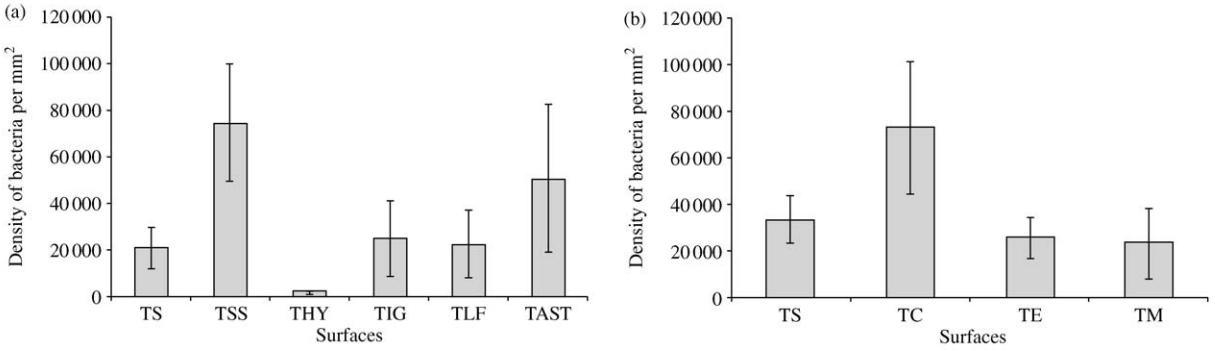

Figure 2 Graphs showing the density of *S. aureus* adhering to the different surfaces. (a) TS, TSS and coatings, (b) TS and polished surfaces. The densities of bacteria vary between the two graphs due to different bacteria cultures being analysed.

and TM were comparable (Fig. 2(b)), despite differences in surface roughness (Table II).

4. Discussion

Over the last decade, systemic antibiotics have not provided an effective treatment against infections associated with implants [15–18]. Hence, various studies have modified implant surfaces in an attempt to decrease infections [12, 18, 19]. The adherence of eukaryotic cells and ECM proteins to modified surfaces has received much more attention than bacterial adherence [7, 8, 20].

The ideology behind the coatings (THY, TIG and TLF) and the polished surfaces (TC, TE and TM), was to decrease the amount of cell and bacterial adhesion, whereas the TAST coating was formulated to enhance eukaryotic cell adhesion. With the exception of THY, no major differences were observed in *S. aureus* adhesion to the different coated samples. This was expected as a hydrophilic coating such as hyaluronan is known to have antiadhesive properties [21]. Pavesio *et al.* [21] and Cassinelli *et al.* [22] reported on how coating polymeric medical devices, e.g. intraocular lenses, stents and catheters, with a hyaluronan coating decreased fibroblast and *Staphylococcus epidermidis* adhesion. Pavesio *et al.* [21] also looked at the adhesion of plaque-forming bacteria to hyaluronan coated titanium implants, and found that the coated titanium remained clean and shiny, indicating a lack of plaque accumulation. On the THY surface used in this study (Fig. 1(c)), the density of *S. aureus* was minimal compared to TS and TSS (Fig. 1(a) and (b)), the control surfaces, thus suggesting that a THY coating has potential for inhibiting bacterial adhesion to metal and polymer implants.

An unexpected result was the fact that differences in topography and bacterial adhesion were found between the two manufactured medical implant quality anodised titanium surfaces (TSS and TS), despite that they should have a very similar surface topography and chemistry (Table II; Fig. 1(a) and (b)). This may be due to the fact that the surfaces were produced at different manufacturing sites, using slightly different methods. Considering that the TIG surface is a TSS surface implanted with nitrogen ions, the density of *S. aureus* adhering to this surface was lower than on untreated TSS. Therefore, it is possible that the nitrogen ions were affecting the resistivity and chemical topography of TSS, as when titanium-oxy-nitride (TiNOX®) with a specific resistivity is applied to stainless steel [12]. A TiNOX® coating minimises adhesion of *S. epidermidis*, *Streptococcus mutans* and *Pseudomonas aeruginosa* to coated stainless steel [12]. To date, no information has been published on cell or bacterial adhesion to surfaces with low friction coefficients, such as the TLF surface.

A significant difference was observed on the density of *S. aureus* adhering to the TC surface compared to the others, but no differences were seen between the TS, TE and TM surfaces (Fig. 2). This was unexpected, as it was hypothesised that smooth surfaces such as TC, TE and TM would decrease the amount of bacterial adhesion, as observed by some publication with eukaryotic cells [7, 8, 20]. However, despite the smoothness of the TC surface in comparison to the TS surface ($R_a = 0.67$ and 1.15, respectively), more bacteria adhered to TC surface. This is contradictory to previous studies, which found more adherence to rough surfaces than smooth [23, 24]. This can be explained by the fact that bacteria colonising a groove or crack are more protected from both

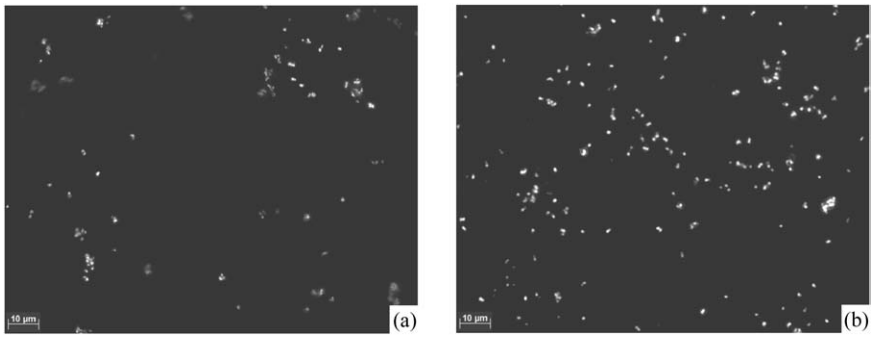

Figure 3 Examples of fluorescence microscopy images of *S. aureus* adhering to (a) TS and (b) TC. The white dots represent the live bacteria, which were seen red in the original images. Note the differences in the density of bacteria between the two surfaces. Scale bar represent 10 µm.

mechanical disturbances and from the host's immune system [25]. In their review paper, Bos *et al*. [25] cited that surface roughness appears to be a minor factor in the initial adhesion of bacteria to a surface, and that bacteria rarely show a preference to adhering to scratches or grooves. This suggests that the surface chemistry, such as surface energy, has more influence on bacterial adhesion than topography in the case of these surfaces [7, 26]. For example, Tsibouklis *et al*. [26] found that a smooth surface with low-surface energy could inhibit bacterial adhesion.

One aspect that is not taken into consideration in this *in vitro* study, is the effect host cells and the host immune system, would have in limiting the colonisation of these surfaces (coated and polished) by *S. aureus*.

5. Conclusions

In this *in vitro* study, polishing or coating the surfaces (exception sodium hyaluronate coated surface) did not have a significant effect on minimising *S. aureus* adhesion to these surfaces. The study confirmed that the TAST surface could promote bacterial adhesion, as well as the cell adhesion it is designed to promote. Coating titanium (TSS) with sodium hyaluronate, significantly decreased the density of *S. aureus* adhering to the surfaces, and could have potential use in osteosynthesis, orthopaedics or dental applications.

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