Antibacterial properties of biomedical surfaces containing micrometric silver islands

R Pérez-Tanoira1,*, C Pérez-Jorge1, J L Endrino2, E Gómez-Barrena3, D Horwat4, J F Pierson4, J Esteban1

1Department of Clinical Microbiology, IIS-Foundation Jiménez Diaz, Madrid, Spain
2 Instituto de Ciencia de Materiales de Madrid, ICMM. Madrid, Spain
3 Department of Traumatology and Orthopaedic, IIS-Foundation Jiménez Diaz, Madrid, Spain
4 Institut Jean Lamour, Ecole des Mines de Nancy, Nancy, France

*E-mail: rptanoira@fjd.es

Abstract. A set of Cu-Mn-O and Ag-Cu-Mn-O films were sputter-deposited onto polished Ti-6Al-4V coupons and the microbiological adherence of Staphylococcus sp. was studied in these biomedical surfaces modified using advanced ternary and quaternary oxides that incorporated micrometric silver islands. The as-deposited ternary and quaternary compounds were amorphous. Upon air annealing the Ag-Cu-Mn-O films, silver-oxygen bonds in the compound destabilize, resulting in the segregation of metallic silver in the form of micrometric layered silver islands with high specific area dispersed at the surface of the remaining oxide. Silver is well known to have a natural biocidal character and its presence in the surface forming large micrometric escalonated islands is, in principle, predicted to enhance the antimicrobial properties of biomedical surfaces. Microbial adhesion tests were performed in triplicates using collection strains of Staphylococcus aureus and Staphylococcus epidermidis. Preliminary results indicate that both strains showed decreased adherence to modified materials, S. epidermidis showed higher adherence these materials than S. aureus, however, there was no statistically significant differences between Cu-Mn-O and Ag-Cu-Mn-O containing silver islands.

1. Introduction

Prosthetic joint implants are used with increased frequency to improve our quality of life. In 2004, 265,441 total hip arthroplasties and 496,018 total knee arthroplasties were performed in U.S. alone. Of these, an estimate of 1.23% total hip arthroplasties and 1.21% total knee arthroplasties were treated for infection. About 8% of hip arthroplasties and 15% of knee arthroplasties revision surgeries were a direct result of infection [1].

The infections due to prosthetic joint implantation have severe consequences not only for patients but also for society because of long hospital stays, long and expensive treatments, and multiple surgeries with severe clinical and economic consequences [2].

Joint prosthetic infection costs about $50,000 U.S. dollars per episode while the associated mortality rate may be as high as 2.5%. In addition, if the infection persists into the deep tissue, amputation may also be required [1].

Although they are less common than infections related to catheters, infections associated with surgical implants are generally more difficult to manage because they require a longer
period of antibiotic therapy and repeated surgical procedures [3]. The diagnosis and treatment of prosthetic osteoarticular infections are further complicated by the development of a bacterial biofilm, where the bacteria have changed their phenotypes to an extremely resistant sessile form of life [2,4]. This biofilm is responsible for many chronic infections and prevents proper integration of the implant to the surrounding tissue [1].

Bacterial adhesion to a material surface is the first step in biofilm development. It can be described as a two-phase process including an initial, instantaneous, and reversible physical phase (phase one) and a time-dependent and irreversible molecular and cellular phase (phase two). On the first phase, the planktonic bacteria move to or are moved to a material surface through and by the effects of physical forces, such as Brownian motion, van der Waals attraction forces, gravitational forces, the effect of surface electrostatic charge, and hydrophobic interactions. In the second phase of adhesion, molecular reactions between bacterial surface structures and substrate surfaces become predominant. This implies a firm adhesion of bacteria to a surface by the bridging function of bacterial surface polymeric structures, which include capsules, fimbriae or pili, and slime. In fact, the functional part of these structures should be the adhesins that are parts of these structures, especially when the substrates are host tissues [5].

The adherence process depends largely on the surface and near-surface atomic structure and composition of implanted biomaterials [6]. The factors influencing bacteria adherence to a biomaterial surface include chemical compositions of the material, surface charge, hydrophobicity, and simply surface roughness or physical configuration. Also, their surface energy, empty binding sites, hydrophobic or hydrophilic characteristics can be quickly altered by the adsorption or binding of serum proteins and the formation of biofilms [5]. The number of adherent bacteria that bind to a metal surface is dependent upon the strain of the microorganism and the type of metal [5,6].

The ability to form a biofilm affords at least two properties: the adherence of cells to a surface and multiplication of bacteria to form multilayered cell clusters [7]. Thus, it can be argued that the prevention of bacterial adhesion without drugs may be one of the best ways to reduce orthopedic implant infection [1]. It would be desirable to develop biomedical coatings for implants which are repellent to bacteria to minimize the colonization of the implant surface with circulating planktonic bacteria.

Silver nanoparticles have been shown to possess an unsurpassed antimicrobial spectrum, with efficacy against 150 different pathogens. Silver ions bind strongly to electron donor groups on sulfur-, oxygen- or nitrogen-containing enzymes. These ions displace other cations (e.g., Ca²⁺) important for enzyme function. In addition, nanocrystalline silver also provides broad-spectrum fungicidal action and only small amounts of silver ion concentrations are required for microbiidal activity [8]. Cu²⁺ and Ag⁺ ions are small enough to disrupt bacterial cell membranes and gain entry in order to disrupt enzyme function. Indirect effects through changes in the surrounding charge environment may also impact on the effectiveness of nanoparticulate metals against microorganisms [9]. These changes can also affect adherence, a process where differences in physical properties could have a high effect in the results.

The reactive sputtering process is well known for its ability to synthesize metastable phases in thin film forms. It is suitable for the deposition of silver-cooper oxides films with various stoichiometries. Since the as-deposited films are amorphous, air annealing has been performed to crystallize the coatings. Annealed films are composed of silver islands exhibited a highly faceted morphology [10]. The annealing of amorphous Ag–Cu–Mn–O films has proven to be an original method to synthesize micrometric silver islands on an oxide surface. The observed high specific area of silver islands seems very promising for applications such as antibacterial coatings. The purpose of this study is to determine whether silver islands can reduce the adhesion of S. aureus and S. epidermis bacteria.

2. Experimental

2.1 Materials and methods
The ternary (and quaternary) films were deposited onto Ti-6Al-4V substrate by co-sputtering of manganese and copper (and Ag₆₀Cu₄₀) targets in a reactive Ar–O₂ reactive mixture. The currents
applied to the Mn and the Ag/Cu targets were adjusted to deposit films with an approximate atomic ratio (Ag+Cu)/Mn of 2.9. The as-deposited films were X-ray amorphous and air annealing in the 350–550 °C range of the Ag-Cu-Mn-O films did not allow the crystallization of the delafossite (Ag$_2$CuMnO$_4$) phase. On the other hand, such treatments induced the formation of visible silver islands located on the surface of the film, as can be observed in the photograph of Fig. 2. The morphological properties of the silver containing films were characterized using a profilometer instrument (Veeco Dektak 150) and Vision 3-D analysis software. This technique allowed obtaining a three-dimensional representative image (0.5 mm x 0.5 mm) of the surface of the prepared silver islands.

2.2 Staphylococcal adhesion experiments

Ti-6Al-4V coupons coated with Ag-Cu-Mn-O films containing islands of silver located on the surface of the film (Fig. 1c and Fig. 2) were compared against controls of uncoated Ti-6Al-4V coupons (Fig. 1a) and Ti-6Al-4V coupons coated with an amorphous Cu-Mn-O film (Fig. 1b).

Staphylococci (Staphylococcus aureus and coagulase-negative Staphylococcus species) are the most frequent infective agents, followed by streptococci, enterococci, gram-negative bacilli, and Propionibacterium acnes [4]. S. aureus and S. epidermidis were selected for testing of bacterial adhesion. This reflects the potential of the soluble planktonic bacteria to stick to the implant surface and to colonize it. In this study the adhesion is tested using collection strains, which are reproducible and available for all investigators.

Staphylococcal adhesion experiments were performed as described by Kinnari et al. Briefly the biofilm-forming collection strains S. aureus 15981 [11] and S. epidermidis ATCC 35984 were cultured overnight in tryptic soy broth (bioMérieux, Marcy l’Etoile, France) at +37°C. After culture, bacteria were harvested by 10-min centrifugation at 3500 x g at room temperature. Supernatant was discarded and the pellet was washed three times with sterile phosphate buffered saline (PBS). Bacteria were then suspended and diluted in PBS to 10$^8$ colony-forming units (CFU)/mL. The biomaterial discs were placed into the bacterial suspension and incubated during 90 min at +37°C. Afterwards, the biomaterial plates were rinsed three times with sterile PBS to remove any nonadherent bacteria [12].

Dried plates were stained for 15 min with Backlight© Live/Dead stain. On each plate, 8 fields were viewed and photographed with Nikon Coolpix 8400 (Nikon, Melville, NY) under a fluorescence microscope at 40x magnification (Figure 5 and figure 6). All experiments were performed in triplicates. The number of microphotographs studied was 24 per each material and
bacterium. The surface area covered with adhered bacteria was calculated using the ImageJ software (National Institute of Health, Bethesda, MD)

3. Results and discussion
3.1 Thin film morphology
X-ray diffraction (XRD) patterns (not shown) revealed that the as-deposited coatings were amorphous and there were only peaks for crystalline fcc-Ag peaks in the case of the annealed Ag-Cu-Mn-O film due to the formation of the silver islands. 3D perfilometer plot of the annealed Ag-Cu-Mn-O coating showed that about one fourth of the surface is covered by the Ag islands (Fig. 3). Each individual silver island appears to be composed of about hundred silver rounded agglomerates with a thickness of over 1μm, which is larger than the as-deposited film thickness (which was about 0.75 μm). Silver agglomerates around the perimeter of the islands had higher thickness than agglomerate grains within the island. The overall increase in surface determined by the surface index, calculated using Vision program (Veeco), was 1.066. This means that the overall increase in the lateral surface area was only about 0.66%. Observation of one individual silver island at higher magnification is shown in Fig. 4, together with the surface area and surface index calculations for this region. The slight increase in surface index close to 1.01 indicates that the surface of the silver islands is very flat and they contained no faceted morphology.

Figure 3. 3-D plot of a perfilometer surface scan (500μm x 500μm) of annealed Ag-Cu-Mn-O film deposited onto a Ti6Al4V. Silver islands on the surface are formed due to annealing at 550°C for 2 hours
3.2 Bacterial adhesion tests

The adherence results, represented in the table 1 and in the figure 5, indicate that both strains showed lowered adherence to modified surface materials.

Table 1: Percentage of Ti-6Al-4V surface, Ti-6Al-4V with Ag-Cu-Mn-O surface and Ti-6Al-4V with Cu-Mn-O surface covered by S. aureus or S. epidermidis.

| Bacteria   | Material          | Percentage of biomaterial surface covered (Mean ± Std Dev) |
|------------|-------------------|------------------------------------------------------------|
| S. aureus  | Ti-6Al-4V         | 3.1167 (+0.6651)                                          |
|            | Ag-Cu-Mn-O        | 1.6750 (+0.3698)                                          |
|            | Cu-Mn-O           | 1.7583 (+0.3374)                                          |
| S. epidermidis | Ti-6Al-4V      | 5.0542 (+1.2779)                                          |
|            | Ag-Cu-Mn-O        | 3.2833 (+1.6056)                                          |
|            | Cu-Mn-O           | 2.9208 (+1.4788)                                          |

Figure 4. Coloured contour plot and lateral area analysis of profilometer surface scan of one silver island.

Figure 5. Mean percentage of biomaterial surface covered with each bacteria and for each material.
Figure 6. Fluorescence microscope images of *Staphylococcus aureus* stained using live-dead strain attached to the Ti-6Al-4V coated with annealed Ag-Cu-Mn-O. The white bar represents 10 μm length.

Figure 7. Fluorescence microscope images of *Staphylococcus epidermidis* stained using live-dead strain attached to the Ti-6Al-4V coated with annealed Ag-Cu-Mn-O. The white bar represents 10 μm length.
The surface of area covered by adhered bacteria was adjusted to the surface available for bacterial cells in case of the discs with silver islands. These islands represent an increase of 0.1% in this surface compared with other materials due to the morphology of the silver islands. We have also detected some fluorescence of the silver islands. All calculations have been performed take into account this fact. Figures 6 and 7 show the fluorescence microscope images corresponding to the sample containing silver islands for S. aureus and S. epidermidis, respectively. These images of the adhered bacteria show different taint when they are on the top of islands of silver. Moreover, the dead bacteria which are tainted in orange-red appear to describe the morphology of smaller silver grains within the islands, as described in Fig. 3 and 4.

3.3 Statistical analysis

For the statistical study was using non-parametric test. Mann-Whitney/Wilconxon test was using for two samples and Kruskal-Wallis test was using for more than two samples.

The differences between Ti-6Al-4V surface and Ti-6Al-4V with Ag-Cu-Mn-O were statically significant for S. aureus (p<0.0001, test of Kruskal-Wallis) and S. epidermidis (p=0.0005, test of Kruskal-Wallis).

The differences between Ti-6Al-4V surface and Ti-6Al-4V with Cu-Mn-O were statically significant for S. aureus (p<0.0001, Test of Kruskal-Wallis) and S. epidermidis (p<0.0001, test of Kruskal-Wallis).

There were no statistical differences between Ti-6Al-4V with Cu-Mn-O and Ti-6Al-4V containing silver islands for S. aureus (p=0.3188, Test of Kruskal-Wallis) and S. epidermidis (p=0.5493, test of Kruskal-Wallis), even when surface was adjusted to the higher exposed surface area of the silver-containing compounds. Such increase of surface was calculated to be 0.1% higher than other materials.

When we compared the adhesion of both strains, the difference between the adhesion of S. aureus and S. epidermidis was statistically significant for Ti-6Al-4V (p<0.001, test of Kruskal-Wallis), for Ti-6Al-4V with Ag-Cu-Mn-O (p=0.0014, test of Kruskal-Wallis) and for Ti-6Al-4V with Cu-Mn-O (p=0.0109, test of Kruskal-Wallis)

2. Conclusions and future work

Biomedical Ti-6Al-4V plates were successfully coated using amorphous Ag-Cu-Mn-O or Cu-Mn-O films deposited using the reactive magnetron sputtering technique. Films composed of Ag-Cu-Mn-O were thermally treated and upon annealing silver islands that contained smaller silver agglomerates were formed at the film surface. Coated plated had a decreased bacterial adherence of collection strains of S. aureus and S. epidermidis in comparison to uncoated plates. However, differences were not detected between both surface coatings with these strains. S. epidermidis showed higher adherence for all materials than S. aureus.

This study evaluated the rough adherence of collection strains. These strains have lower genetic load than clinical strains isolated of patients because they are laboratory-adapted strains which loss genes due to several passages on culture medium. Because this fact, it must be interesting to prove these materials with strains isolated of patients, which can behave in a different way than collection strains due to their different pathogenic properties. Further experiments are currently being performed to evaluate such adherence using clinical strains isolated from patients with prosthetic joint infections (which can show different behavior than laboratory-adapted strains). These experiments also study the antibacterial effect of silver through evaluation of percentages of live and dead bacteria present in the surfaces using the live/dead strain.

Future studies must analyze the effect of silver islands on biofilm development, which needs longer incubation times and where silver elution can have a prolonged effect on sessile cells, and effect that cannot be detected in adhesion experiments, where only immediate interactions between bacteria and the surface can be studied.
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