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Decrease of Staphylococcal adhesion on surgical stainless steel after Si ion implantation

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ABSTRACT

316LVM austenitic stainless steel is often the material of choice on temporal musculoskeletal implants and surgical tools as it combines good mechanical properties and acceptable corrosion resistance to the physiologic media, being additionally relatively inexpensive.

This study has aimed at improving the resistance to bacterial colonization of this surgical stainless steel, without compromising its biocompatibility and resistance. To achieve this aim, the effect of Si ion implantation on 316LVM has been studied. First, the effect of the ion implantation parameters (50 keV; fluence: 2.5–5 $\times$ 10^{16} ions/cm^2; angle of incidence: 45–90°) has been assessed in terms of depth profiling of chemical composition by XPS and nano-topography evaluation by AFM. The *in vitro* biocompatibility of the alloy has been evaluated with human mesenchymal stem cells. Finally, bacterial adhesion of *Staphylococcus epidermidis* and *Staphylococcus aureus* on these surfaces has been assessed.

Reduction of bacterial adhesion on Si implanted 316LVM is dependent on the implantation conditions as well as the features of the bacterial strains, offering a promising implantable biomaterial in terms of biocompatibility, mechanical properties and resistance to bacterial colonization. The effects of surface composition and nano-topography on bacterial adhesion, directly related to ion implantation conditions, are also discussed.

Introduction

External fixation devices and surgical implant procedures have become extremely valuable and common to restore the function of joints, limbs and fractured bone segments. Nevertheless, even a currently low risk of infection has to be considered very relevant for its serious consequences. Clinical practice has shown that systemic administration of antibiotics is often ineffective once bacteria form biofilms, and thus implant removal and replacement represent sometimes the only options to eradicate infections [1]. These drastic interventions have major consequences, in terms of socioeconomic costs due to revision surgeries and prolonged hospitalization, not to mention patient suffering and discomfort.

In the strategy for the prevention of surgery related infections, much has been done to improve the operating standards, minimize the possibility of contamination during surgery, reduce the establishment of infection by peri-operative antibiotic prophylaxis, and confine pathogenic strains by patient isolation. Nevertheless, little advancements in terms of decreased infection rates are being expected in return of these types of efforts. As a consequence, increasing attention has progressively been focused on the epidemiology and the pathogenesis of the infections, especially those associated with implant materials [2]. Furthermore, with the rise in bacterial resistance to antibiotics, the need for materials that

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reduce or inhibit bacterial adhesion and colonization is significant [3].

Accidental contamination, which might develop into the establishment of a clinically relevant infection, typically takes place at the tissue biomaterial interface, in the context of the "race to the surface" of extracellular matrix proteins, host cells and bacteria [4]. Bacterial adhesion represents an initial crucial step in this process. The most convenient way to interfere with the early phases of microbial adhesion is a modification of the chemistry and/or the micro/nano-topology of the outer layer of the implant [2,3]. One of such surface treatment techniques that can modify the chemistry and nano-topography in a controlled way is ion implantation [5]. Actually, ion implantation has been applied as a surface treatment of implants to achieve antimicrobial properties [6,7]. Ag and Cu ions have been implanted into titanium alloys and stainless steel [8–10], F ions into titanium [11], SiF_3^+ into stainless steel [12,13] and Cu ions into polyethylene [14] with promising results.

Although it has been reported that under otherwise identical experimental conditions the rate of infection for stainless steel implants is significantly greater than that for titanium implants [15], they are extensively used due to its corrosion resistance, excellent mechanical properties and relatively low cost. 316LVM (low carbon, vacuum melted—to improve purity) is in fact the most commonly used alloy among the several types of stainless steels available for implant use [16]. Recently it has been shown that a larger dose of Si ion implantation (10^{17} ions/cm^2; 80 keV) renders the surface with lower bacterial adhesion ability, characterized by a lower number of attached bacteria in dynamic experiments and smaller adhesion rates, without compromising the excellent *in vitro* behaviour of bone cell precursors, although a decrease in the *in vitro* corrosion resistance at the earlier stages of immersion in simulated human fluids was observed [17].

The objective of the present work was to optimize the parameters of Si^+ ion implantation on surgical stainless steel and to assess their influence on the biological response, with special emphasis on the bacterial activity. Possible mechanisms behind the beneficial role of implantation are analysed.

Material and methods

Medical grade AISI 316LVM stainless steel discs with a diameter of 20 mm and a thickness of 2 mm were supplied by the implant manufacturer (Surgival SL, Valencia, Spain) with the following chemical composition (wt%): 17.48% Cr, 14.31%Ni, 2.87%Mo, 1.62%Mn, 0.53%Si, 0.024%C, 0.067%Cu, 0.061%N, 0.001%S, 0.016%P, and Fe balance. Surface preparation consisted of grinding with SiC papers and polishing with diamond paste. A final finishing with alumina powder of 0.5 μm in size was applied to obtain a mirror-like surface. The samples were then sonicated in 70% acetone, in distilled and deionized water and in ethanol, before storing them in vacuum till ion implantation treatment.

Si ion implantation was performed in a 1090 Danfysik high current ion implanter (Jyllinge, Denmark) with Si sputtering targets as precursors for Si^+ ions. An acceleration voltage of 50 keV with doses of 5×10^{16} and 2.5×10^{16} ions/cm^2 were used, with an angle of ion incidence *vs.* the rotating sample surface of 90° and 45°, respectively, referenced as Si-I and Si-II. A non-perpendicular angle of incidence, *e.g. 45°*, typically reduces the range of ion penetration (shorter nominal projectile range) and increases the sputtering yield. Thus under otherwise identical experimental conditions the expected maximum concentration peak for 90° implantation would be deeper than for 45°; and the resulting surface roughness larger. Unimplanted samples, subjected to the same sample preparation conditions were used as control.

The resultant ion implanted surfaces were characterized by X-ray diffraction (XRD), X-ray photoelectron spectroscopy (XPS), atomic force microscopy (AFM), and magnetic force microscopy (MFM).

XRD was carried out with a Bruker AXS D8 diffractometer in grazing incidence condition with a beam incidence angle of 1° and 2θ scans between 30 and 100° with a step size of 0.03°. For the XRD data refinement the version 4.0 of the Rietveld analysis program TOPAS (Bruker AXS) was used. The instrument functions were empirically parameterized from the profile shape analysis of a corundum sample measured under grazing incidence at the same conditions.

The XPS analysis was performed using a *K*-alpha equipment (Thermo Scientific, UK) working with an excitation source of Al-*K*α radiation (1486.6 eV), under a chamber pressure of 10^{-9} Torr. Photoelectron detector was placed at a normal position (90° take-off angle) with regard to the sample. Depth profiling was made by sputtering the sample surface with Ar^+ ions. Ion beam was placed 30° to the normal. After each sputtering cycle (5 s), the depth profiling analysis was done by recording XPS spectra (50 eV pass energy) of the elements detected in the samples.

For AFM analysis a Nanoscope IIIa Atomic Force Microscope (Digital Instruments, Santa Barbara, CA, USA) was used working in contact mode. The average roughness (R_a) and the root-mean-squared roughness (R_q) were derived from at least three 1×1 μm^2 images.

Combined AFM and MFM imaging were performed using a microscope from Nanotec Electrónica S.L. in dynamic mode [18]. Nanosensors standard MFM probes with a force constant of 3 N/m and a resonance frequency of 75 kHz were used in these experiments [19].

In order to assess bacterial adhesion, different strains of bacteria were used. *Staphylococcus aureus* ATCC29213 (*S. aureus*), *Staphylococcus epidermidis* ATCC35984 (*S. epidermidis4*) and *S. epidermidis* HAM892 (*S. epidermidis2*) were stored at −80 °C in porous beads (Microbank, Pro-Lab Diagnostics, Toronto, Canada). *S. epidermidis2* is a negative extracellular polysaccharide substance producer (EPS-negative) mutant derived by acriflavine mutagenesis from *S. epidermidis4* (EPS-positive) that was kindly provided by L. Baldassarri from the Laboratorio di Ultrastrutture, Istituto Superiore di Sanita, Rome, Italy. From the frozen stock, blood agar plates were inoculated and incubated at 37 °C to obtain cultures. From these cultures, tubes of 4 ml of Trypticase Soy Broth (TSB) (BBL, Becton Dickinson, Franklin Lakes, NJ, USA) were inoculated for 10 h at 37 °C and then 25 μl of this pre-culture was used again to inoculate 50 ml of TSB at 37 °C for 14 h. This period of time was sufficient to guarantee that all strains were at the beginning of the stationary growth phase. The bacteria were then harvested by centrifugation for 5 min at 1000 g (Sorvall TC6, Dulont, Newtown, PA, USA) and washed three times with PBS. Finally, the bacteria were re-suspended in PBS at a concentration of 3×10^8 bacteria/ml. Bacterial adhesion and bacterial retention experiments were carried out at 37 °C in a parallel plate flow chamber previously described [20]. Bacterial adhesion was assessed during 5 h and then two liquid–air interfaces were flowed through the adhesion chamber channel to check the bacterial retention to the surface. Initial adhesion rates (j_0) and the total bacterial coating of the surfaces at the end of each experiment (n_f) were plotted.

In vitro biocompatibility assays were performed using purified human bone marrow-derived mesenchymal stem cells (hMSCs) ($CD105^+$, $CD29^+$, $CD44^+$, $CD14^-$, $CD34^-$, $CD45^-$) purchased from Cambrex Bio Science (Verviers, Belgium), which were expanded in growth medium (Cambrex Bio Science). Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere. All the samples were routinely sterilized under UV light in a laminar flow hood for 12 h on each side and stored until use. To assess the

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