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Microbial adhesion to zirconium alloys

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Abstract

We present data and analyses concerning the adhesion of clinically relevant *Staphylococcus aureus, Staphylococcus epidermidis*, and *Pseudomonas aeruginosa* (bacteria) and *Candida albicans* (yeast) to Zircaloy-2 (Zry-2) and Zircadyne-705 (Zr705) surfaces. These zirconium-based materials are similar to those now being used in total hip and knee replacements. Here we study clinical strains of microbes under shaken and stationary exposure conditions, and their ability to adhere to Zr surfaces having different oxide thicknesses. We use X-ray photoelectron spectroscopy (XPS), scanning electron microscopy (SEM), viable counts, endotoxin assays, and statistical analysis methods, and demonstrate a predictive model for microbial adhesion based on XPS data.

Keywords: Microbial adhesion; Zirconium alloys; Endotoxin; Yeast; Gram negative; Gram positive; Oxidation; XPS

1. Introduction

Zirconium-based materials, similar to those now being used in total hip replacements (THR) and total knee replacements (TKR) [1–4], are the focus of this study. The oxide layer that forms on the surface of zirconium and its alloys is known to be chemically stable in many environments, with good mechanical strength and excellent wear and corrosion resistance. The question is whether the chemical properties of this oxide layer make these materials compatible for use in biomedical applications. Zirconia (ZrO_2, zirconium oxide) ceramic ball heads are commonly found in THR device designs because of good wear and biocompatibility properties [5], and it might be presumed that oxidized Zr would behave similarly. However, these ceramic materials are stabilized into the tetragonal phase by the addition of other oxides such as yttria, but the oxides grown on zirconium alloys are predominantly in the monoclinic phase and are substoichiometric (ZrO_x, $x < 2$).

Although it appears that oxidized Zr alloys exhibit superior wear behavior [1–4], there is little direct evidence that their oxide layers are biocompatible. For example, Olmedo et al. [6] studied the dissemination of zirconium in rats and detected intracellular aggregates of zirconium particles in peritoneum, liver, lung and spleen. They reported that zirconium dissemination is active throughout the body and particles target vital organs. Thus, it is vitally important that we investigate the biocompatibility of Zr-based materials that are being introduced into the human body in the form of prosthetics. Our findings to date [7,8] indicate that understanding and controlling the adhesion of microbial species on these surfaces is not straightforward, and that synergistic effects dominate.

The present study builds on and expands our growing body of knowledge and experience with microbial adhesion on zirconium alloy surfaces. We present data and analyses concerning the propensity of clinically relevant *Staphylococcus aureus, Staphylococcus epidermidis*, and *Pseudomonas aeruginosa* (bacteria) and *Candida albicans* (yeast) to adhere to Zircaloy-2 (Zry-2) and Zircadyne-705 (Zr705) surfaces. Here we study clinical strains of microbes under shaken and stationary exposure conditions, and different surface oxide thicknesses. The choice of microbes includes those found in hospitals and both Gram-positive and

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-negative genera. We use X-ray photoelectron spectroscopy (XPS), scanning electron microscopy (SEM), viable counts, endotoxin assays, and statistical analysis methods, and discuss trends that are both experimentally and statistically significant.

2. Experimental details

The alloys Zr705 (nominally 2.5% Nb, balance Zr + Hf) and Zry-2 (nominally 1.4% Sn, Hf depleted, balance Zr) were received from Wah Chang (Albany, OR) in the form of sheet stock (approximately 1.0 mm thick) and cut into nominally rectangular coupons. For viable counts and endotoxin assay experiments the coupons were approximately 20 mm × 48 mm, whereas those for XPS and SEM were smaller (approximately 5 mm × 20 mm). Each substrate was polished on one side using diamond pastes followed by a 0.05 μm alumina suspension. The samples were then ultrasonically cleaned, degreased with acetone, and placed in Petri dishes until needed. These samples, that were not deliberately oxidized, are referred to as metallic.

For the deliberately oxidized samples, a convection furnace was used. Polished and cleaned samples from the Petri dishes were placed in the pre-heated furnace. Then, after pre-specified lengths of time, the samples were removed from the hot furnace and allowed to cool in air at a relative humidity of approximately 50%. Annealing temperatures were in the range 500–600 °C, and more details about this oxidation procedure are available elsewhere [7–10]. Standard X-ray diffraction (XRD) measurements were performed on oxidized substrates, to verify that our oxidation procedure predominantly yields the expected monoclinic form of zirconium oxide on the surfaces [8]. All of the oxide layers that we use are in the thickness range before the transition region in the growth kinetics, where the oxides turn from black to beige and white [10].

Clinical strains of *S. aureus, S. epidermidis, P. aeruginosa* and *C. albicans* were incubated overnight at 37 °C in tryptic soy broth (TSB). The four microbes were tested separately and as a mixture in eight-flask sets. The eight flasks each contained 250 ml of TSB, 0.5 ml of microbes (or 0.13 ml of each in the mixture), and two sample coupons. A stationary four-flask set and a separate shaken (175 rpm) four-flask set were incubated at 37 °C for 3 days and then the broths were carefully removed.

A metal coupon was aseptically removed from each flask and rinsed for one min in separate 250 ml beakers of sterile saline (0.85 wt.%/vol.) solution. Each rinsed coupon was then put into a tube of sterile saline and vortexed for one min, allowed to sit for approximately five min, and then vortexed for an additional min. The second coupon in each flask was aseptically rinsed in a corresponding beaker of sterile water and placed on sterile bibulous paper in a sterile glass Petri dish for surface analysis. The latter procedure avoided the use of saline solution as a rinsing agent, since this would contaminate the surfaces studied by XPS and microscopy. The microbes in the saline tubes were diluted and plated using the spread plate technique. Total dilutions of 2×10^1 to 2×10^7 in sterile saline were used and plated on tryptic soy agar (TSA). The plates were incubated at 37 °C overnight. Viable counts were reported as the number of microbes per ml.

The XPS analysis was performed in fixed analyzer transmission mode under high vacuum conditions (pressures often below 8×10^{-9} Torr) using a Kratos ES-300 electron spectrometer with a dual anode X-ray source. For all measurements, the aluminum source was used for the primary survey and detail scans, however the magnesium anode was sometimes used to verify the identity of some Auger features. The X-ray source was operated at 12 kV with emission currents in the range 7–10 mA, and the samples were approximately 1 cm away from the source. Samples were mounted onto the end of a probe inserted through a load-lock sample transfer flange. Focused and rastered argon ion sputtering (2.5 keV) was performed to remove surface contamination and to depth profile. Ultra-high purity argon at a pressure of 2×10^{-5} Torr was used, which corresponds to a sputtering rate of a gold standard of approximately 0.63 nm/min. Microscopic imaging of some of the surfaces was performed with optical and SEM techniques.

The Limulus Amebocyte Lysate (LAL) gel clot assay (BioWhittaker, Walkersville, MD) was used to detect Gram negative bacterial endotoxin. The endotoxin is a portion of the lipopolysaccharide of the outer membrane of the Gram negative cell wall. In most Gram negative bacteria, the endotoxin is exposed upon cell lysis. The lysate contains amebocytes (leukocytes) from the horseshoe crab (*Limulus polyphenus*). Bacterial endotoxin catalyzes a reaction that produces coagulase, an enzyme which hydrolyzes coagulogen (a protein in the amebocyte lysate) resulting in a detectable clot [11]. The LAL gel clot assay is validated by the FDA as an end product endotoxin test [12].

For LAL studies, clinical microbes were incubated with the metal coupons under stationary or shaken conditions and the metal coupons were washed in saline as previously described. The microbes were removed from the saline by five min centrifugation in a microfuge, and the supernatants were stored at 5 °C. All extraneous endotoxin on the glassware was presumably destroyed by autoclaving and baking the glassware at 180 °C for four hr. The water, lysate and plastic-ware used in the assays were endotoxin-free. The test amebocyte lysate was prepared by adding 1.8 ml of endotoxin-free water to the 16-test vial of lyophilized lysate. The endotoxin positive control was 10 ng of *E. coli* endotoxin standard hydrated with 5.0 ml of endotoxin-free water. This standard had a potency of 20 EU/ml and was diluted with endotoxin-free water to 1 EU/ml for use as a positive control. The negative control was endotoxin-free water.

An endotoxin test assay tube contained 0.1 ml of test sample (positive control, negative control or experimental) and 0.1 ml of lysate, and was incubated for one hr in a 37 °C heating block. The positive controls were the 1 EU/ml endotoxin diluted with endotoxin-free water by a series of five 1:2 dilutions to 1:32 and lysate was added to each dilution tube. The experimental samples were diluted to 1:32 by five series of 1:2 dilutions using endotoxin-free water. Lysate was added to each diluted sample. A positive reaction was a clot with the endpoint being the positive tube containing the most dilute sample.