Published in final edited form as:

Microsc Res Tech. 2010 January ; 73(1): 71–76. doi:10.1002/jemt.20757.

Regenerating Titanium Ventricular Assist Device Surfaces after Gold/ Palladium Coating for Scanning Electron Microscopy

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Abstract

Titanium is one of the most commonly used materials for implantable devices in human s. Scanning electron microscopy (SEM) serves as an important tool for imaging titanium surfaces and analyzing cells and other organic matter adhering to titanium implants. However, high-vacuum SEM imaging of a non-conductive sample requires a conductive coating on the surface. A gold/ palladium coating is commonly used and to date no method has been described to ‘clean’ such gold/ palladium covered surfaces for repeated experiments without etching the titanium itself. This constitutes a major problem with titanium based implantable devices which are very expensive and thus in short supply. Our objective was to devise a protocol to regenerate titanium surfaces after SEM analysis.

In a series of experiments, titanium samples from implantable cardiac assist devices were coated with fibronectin, seeded with cells and then coated with gold/palladium for SEM analysis. X-ray photoelectron spectroscopy spectra were obtained before and after five different cleaning protocols. Treatment with aqua regia (a 1:3 solution of concentrated nitric and hydrochloric acid), with or without ozonolysis, followed by sonication in soap solution and sonication in deionized water, allowed regenerating titanium surfaces to their original state. Atomic force microscopy confirmed that the established protocol did not alter the titanium microstructure. The protocol described herein is applicable to almost all titanium surfaces used in biomedical sciences and because of its short exposure time to aqua regia, will likely work for many titanium alloys as well.

Keywords

Titanium; Ventricular Assist Device; Scanning Electron Microscopy; Biomaterials

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Introduction

Metals and alloys are the most commonly used materials for engineering surgically implantable devices in humans. Titanium in particular shows properties which render it a very suitable implant based on its durability and the passively formed oxide film on the metallic surface (Breithaupt-Faloppa and others, 2008). Accordingly, titanium is now used extensively as a biomaterial in the fields of orthopedic surgery (Long and Rack, 1998), plastic surgery (Vinzencz and others, 2008), dental surgery (Arcuri and others, 2007) and cardiovascular surgery (Guidoin and others, 2000). Furthermore, titanium is the building block for engineering mechanical cardiac assist devices which can be implanted into patients with end stage heart failure in order to support the heart pump blood through the circulatory system. At our institution at Duke University Medical Center, we have implanted several hundred of these mechanical assist devices in the last ten years.

Since titanium is not translucent, light microscopy cannot be utilized to image cells and other organic matter adhering to titanium implants. Instead, Scanning Electron Microscopy (SEM) has been established as an indispensable tool (Al-Nawas and others, 2001), (Guidoin and others, 2000). However, when titanium biomaterials have been prepared for SEM analysis by gold/ palladium coating, they cannot easily be cleaned and regenerated into their original state for further experiments. A review of the literature fails to describe a methodology for cleaning off gold/ palladium without destroying the titanium surface itself. Whereas Takahashi et al. (Takahashi and Fukuzaki, 2008) compared the abilities of titanium and stainless steel to be cleaned of bovine serum albumin contamination, and both Parashos et al (Parashos and others, 2004) and Van Eldik et al (Van Eldik and others, 2004) developed methods for the removal of organic matter from nickel-titanium dental instruments, nobody has described a method for cleaning titanium of organic material and Au/Pd coating without etching the titanium surface. This constitutes a problem when special titanium surfaces or titanium implantable devices are very expensive, hard to produce or in short supply and therefore need to be used repeatedly for experiments.

At Duke University Medical Center, we are investigating the feasibility to seed cells onto the inner titanium surfaces of present generation cardiac mechanical assist devices in an effort to engineer a more biocompatible blood/pump interface. The HeartMateII left ventricular assist device (Thoratec Corporation, Pleasanton, CA) surface is made up of sintered titanium microspheres (50-75 μm) covering the inside housing. In order to further improve the biocompatibility of these mechanical assist devices, we are seeding the titanium microspheres with bone marrow derived mesenchymal stem cells. We routinely employ SEM to assess cell seeding density and morphology on the sintered titanium surface of HeartMate II devices. However, it is unclear how to clean and 'recycle' the surfaces for further experiments. We have therefore established a reliable and consistent method for regenerating them. Whereas we have originally developed our method for the sintered titanium surface of ventricular assist devices, our technique can be applied to almost any titanium surface or implantable titanium device.

Materials and Methods

Sample pieces of the sintered titanium surface found in the HeartMateII left ventricular assist device were obtained from Thoratec Corporation, Pleasanton, CA, and used in the experiments described herein.

In preparation for imaging with our scanning electron microscope, Philips XL30 ESEM TMP (FEI Company, Hillsboro, OR), samples were dehydrated in an ethanol series, dried in a Pelco CPD2 critical point dryer (Ted Pella Inc., Redding, CA), and coated with Au/Pd (60/40%) in a Hummer 6.2 sputter coater (Anatech Ltd, Springfield, VA). Au/Pd was sputtered from a single alloy source to a film thickness of 6-7 nm. Of note, the resulting film is enrichment in Pd due to the preferential sputtering of Pd over Au due to differences in surface binding energies and recoil densities in the Au/Pd alloy (Betz, 1980).

A series of experiments was devised (see Table 1) to find the optimal method of regenerating the titanium surface after Au/Pd coating. The success of the various cleaning protocols was assessed by evaluating the surface chemistry of the titanium samples before and after each experiment with X-ray photoelectron spectroscopy (XPS). XPS was performed on a Kratos Axis Ultra instrument (Chestnut Ridge, NY). Samples were attached to the sample holder via copper tape. They were then inserted into the sample transfer chamber and allowed to reach a pressure of 5.0×10^{-7} torr. Following this, the samples were transferred to the sample analysis chamber where the measurements were performed. Spectra were obtained at $\sim 2.0 \times 10^{-8}$ torr. A monochromated Al X-ray source at a power of 15 kV and an emission of 10 mA was used to obtain scans. The lens mode used was hybrid, i.e. magnetic and electrostatic. The slot aperture was used for these experiments and a resolution of 160 eV was used for survey scans. Scans were performed with the charge neutralizer on to counteract any sample charging effects and were taken over the range of 5 - 1200 eV with a step eV of 1 and a dwell time of 200 msec. Each scan constitutes an average of 2 individual scans.

The atomic percentages were obtained by determining the area under the primary peaks for the element under consideration. The areas were determined for the C1s at 284.5 eV, O1s at 529.7 eV, N1s at 400.9 eV, Au4f at 84.1 eV, Pd3d at 335.1 eV, Ti2p at 453.8 and the sensitivity factors were C=0.278, O=0.78, N=0.477, Au=6.25, Pd=5.356, Ti=2.001. A linear background subtraction was performed. Analysis was achieved using CasaXPS software.

As a control experiment, a sample piece of bare sintered titanium surface (Thoratec Corporation, Pleasanton, CA) was analyzed by XPS. This XPS spectrum served as a baseline (Table 1, control, Figure 1a). The titanium surface was then coated with Au/ Pd as described above and imaged with SEM using standard high vacuum imaging at 25kV (Figure 1b). Following, five sintered titanium surfaces (Thoratec Corporation, Pleasanton, CA) were precoated with bovine fibronectin (10 μ g/ml) at room temperature for a duration of 12 hours and seeded with 1×10^6 porcine mesenchymal stem cells by incubation in 6 well plates for 24 hours at 37°C (Figure 2a). All samples were fixed in 3.7% formaldehyde (Ricca Chemical Company, Arlington, TX) overnight before sputter coating as described above.

Porcine mesenchymal stem cells were obtained from bone marrow of Yorkshire swine by isolating mononuclear cells by density gradient centrifugation according to our established protocol (Werner and others, 2003). Cells were then washed with Dulbecco's Phosphate Buffered Saline (D-PBS), (Gibco, Invitrogen Corporation, Carlsbad, CA) suspended in 10% dimethylsulfoxide (DMSO), (Sigma-Aldrich, St. Louis, MO), 90% Fetal Bovine Serum (FBS), (HyClone, Thermo Fisher Scientific, Logan, UT) and plated onto fibronectin coated chamber slides in Endothelial Cell Basal Media-2 (EBM-2), (Lonza, Clonetics, Walkersville, MD) with EGM-2 Single Quots (Lonza, Clonetics, Walkersville, MD) and 10% FBS (HyClone, Thermo Fisher Scientific, Logan, UT). Cells were incubated at 37°C in a water-jacketed incubator at 5% CO₂ for 7 days when nonadherent cells were removed. Cultures were then incubated an additional day before cells were harvested with 0.25% Trypsin/EDTA (Lonza, Clonetics, Walkersville, MD) and replated into T25 flasks in media. Passage 3 and 4 cells were used for our experiments.

The first of the samples coated with biologic material and Au/Pd was submerged in aqua regia (1:3 solution of concentrated nitric and hydrochloric acid) for 5 min at ambient temperature without stirring. No attempts were made to control the temperature; however, the temperature did reach ~ 30°C upon mixing the acids together and the Ti pieces were immediately immersed into that solution. This was followed by sonication (Fisher Scientific Solid State/Ultrasonic FS-14, Pittsburgh, PA) in deionized water (DI H₂O) for 5 min and the Ti pieces were then dried with (N₂) nitrogen gas. XPS spectra were obtained before and after cleaning.

The second coated sample was also submerged in aqua regia for 5 min, followed by sonication in DI H₂O for 5 min. It was then treated by ozonolysis (UV-ozone cleaner, UVO-Cleaner Model No. 42, Jelight Company Inc., Irvine, CA) for 45 min.

The third sample was subjected to aqua regia, sonication and ozonolysis and then further sonicated in water saturated with a soap solution (Contrex labware detergent, Decon Labs Inc., King of Prussia, PA). Finally, the sample was sonicated in DI H₂O for 1 hour and dried with nitrogen gas.

The fourth sample was processed like the third but without the ozonolysis step. The fifth sample processed like the third sample but without submersion in aqua regia. For all of the samples, XPS spectra were obtained before and after cleaning.

In order to determine whether aqua regia exposure would significantly alter the surface structure of titanium microspheres by etching the titanium, we used atomic force microscopy (AFM) to image titanium samples before and after aqua regia treatment. AFM images were obtained in contact mode using a Digital Instruments Dimension 3100 AFM (Veeco Instruments Inc., Plainview, NY) and a Veeco Probes DNP cantilever. Images were acquired at a scan rate of 2 Hz using 512 samples/line and 512 lines. Each image was second order flattened. The surface area evaluated was 930 µm² on average per titanium sample.

Results

The SEM image shown in Figure 1b shows the microspheres which make up the sintered titanium-oxide surface of the HeartMateII left ventricular assist device (Thoratec Corporation, Pleasanton, CA). XPS reveals the chemical composition of the titanium oxide microspheres to contain 12.56% Ti and 37.49% O₂ (Table 1, control, untreated titanium). As can be seen in the SEM image (Figure 1b), there are small organic contaminations from the environment found on the surface, which are also reflected in the XPS spectrum by the carbon atom and small nitrogen atom peaks (Figure 1a).

The same sintered titanium oxide surface is then lined with a confluent layer of cells on fibronectin-precoated microspheres, which can be seen using SEM (Figure 2b). The XPS spectrum of this surface (Figure 2a and Table 1, experiment 1) clearly indicates the gold and palladium peaks secondary to the surface modification. After aqua regia and sonication, XPS indicates that the Au/ Pd is mostly gone. However, biological material is still present based upon the high nitrogen content (12.57%) and the absence of a significant Ti peak (only 2.498%) (Table 1, experiment 1).

Another sample is further treated by ozonolysis in order to break down crosslinked protein. Despite this additional step, XPS reveals that there is still a significant amount of nitrogen (11.93%) indicative of a 'dirty' sample (Table 1, experiment 2).

Sonication in soap solution followed by sonication in DI water is added to the protocol in the third experiment. After these steps, the sample is devoid of significant amounts of nitrogen and carbon, indicating a clean surface (Table 1, experiment 3). In fact, the thus cleaned surface has a lower nitrogen (0.8377%) and carbon percentage (23.36%) than the bare, unmodified titanium surface (2.324% and 37.49%) (Figure 3a and Table 1, control: untreated titanium). (These nitrogen and carbon percentages of the bare unmodified Ti reflect the adventitious adsorption of organics from the environment.) This fact is substantiated by the SEM and AFM images after cleaning (Figure 3b,c,d), which show almost no contaminations.

The question then arose whether the ozonolysis step is necessary to remove all organic matter. In the fourth experiment, the full protocol of the third experiment is modified by the omission of ozonolysis. XPS indicates that such an omission makes no difference (Table 1, experiment 4)- ozonolysis is thus an unnecessary step.

In the fifth experiment, the full protocol of the third experiment is modified by the omission of aqua regia treatment. After cleaning, XPS reveals that significant gold (13.72%) and palladium (3.428%) peaks remain (Table 1, experiment 5), emphasizing the importance of aqua regia treatment.

Imaging of the titanium microsphere surface structure with atomic force microscopy before (Figure 1c,d) and after aqua regia treatment (Figure 3c,d) failed to show any significant difference in the surface roughness (the root mean square for the control is 101nm and for the aqua regia exposed sample 108nm). Therefore, aqua regia application according to our protocol does not etch titanium surfaces.

Discussion

Scanning electron microscopy is an excellent tool to image the surface of titanium samples, especially when assessing organic structures such as cells adherent to its surface. Thus far no method has been described on how to regenerate a titanium surface for repeat experiments after it had been coated with gold/ palladium for SEM analysis. We are reporting a technique (treatment with aqua regia, +/- ozonolysis, followed by sonication in soap solution, followed by sonication in deionized water) that allows for cleaning off the gold/ palladium coating as well as all organic matter without destroying the titanium microspheres of our ventricular assist device titanium surfaces.

Our method was validated with X-ray photoelectron spectroscopy before and after treatment of titanium samples. Aqua regia has a low etch rate for Ti as described by Williams et. al (Kirt R. Williams, 2003) and we used atomic force microscopy to establish that the surface roughness was unaltered by our cleaning protocol (due to the short exposure time of the samples). Our method is applicable to any kind of titanium surface used in biomedical sciences. Furthermore, we anticipate that this cleaning technique will be appropriate for cleaning alloy materials, which have Ti coupled to their structure. Alloys may be less stable to aqua regia treatment than compound pure Ti, rendering it more difficult to remove the Au/Pd coating needed for routine SEM analysis, without altering the material on a micro or nano scale. Whereas this has not yet been thoroughly tested, we believe that the short exposure time with aqua regia and thus least aggressive methodology described herein will be gentle enough to regenerate a wide variety of titanium alloy surfaces.

Acknowledgments

We would like to thank Dr. David Farrar and Dr. Karen Masterson from Thoratec Corporation, Pleasanton, CA for graciously providing HeartMateII surface samples and expertise and the NIH (EB001037 to SLC) for funding.

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Figure 1.
Untreated Ti. (a) XPS (b) SEM (c,d) AFM

Figure 2.
Before cleaning. (a) XPS (b) SEM

Figure 3.

Sample in experiment 4 underwent: aqua regia + H₂O sonication + ozonolysis + soap sonication + H₂O sonication. (a) XPS following treatment. (b) SEM following treatment (c,d) AFM following treatment

Table 1

Cleaning steps for each experiment and XPS results before and after each cleaning procedure.

Experiment		XPS Spectrum	Image	N (%)	Ti (%)	Pd (%)	Au (%)
Control	Untreated Ti	1a	1b, 1c, 1d	2.32	12.56	0	0
1	Before cleaning	2a	2b	8.19	0	9.393	9.121
	After cleaning: aqua regia + H ₂ O sonication	Not shown	Not shown	12.57	2.498	0.02857	0.04717
2	Before cleaning	Not shown	Not shown	8.993	0	12.93	11.9
	After cleaning: aqua regia + H ₂ O sonication + ozonolysis	Not shown	Not shown	11.93	9.385	0.03929	0.07404
3	Before cleaning	Not shown	Not shown	6.192	0	13.25	11.75
	After cleaning: aqua regia + H ₂ O sonication + ozonolysis + soap sonication + H ₂ O sonication	3a	3b, 3c, 3d	0.8377	18.19	0.1356	0.1145
4	Before cleaning	Not shown	Not shown	12.37	0	7.545	7.183
	After cleaning: aqua regia + H ₂ O sonication + soap sonication + H ₂ O sonication	Not shown	Not shown	1.015	18.04	0.1585	0.09485
5	Before cleaning	Not shown	Not shown	11.25	0	10.99	10.39
	After cleaning: H ₂ O sonication + ozonolysis + soap sonication + H ₂ O sonication	Not shown	Not shown	2.032	0	3.428	13.72