

# Traces of biomolecules as footprints on titanium surfaces

Sannakaisa Virtanen<sup>1,2</sup>, Antti Soininen<sup>3</sup>, Veli-Matti Tiainen<sup>3</sup>, Danijel Besic<sup>4</sup>, Miha Pukl<sup>2</sup>, Teemu Kinnari<sup>5,6</sup>, Jari Salo<sup>7</sup>, Rihard Trebse<sup>8</sup>, Andrej Trampuz<sup>4</sup>, Yrjö T Konttinen<sup>2,9,10</sup>

<sup>1</sup>Institute of Materials Science, University of Erlangen-Nürnberg, Germany; <sup>2</sup>Department of Medicine, Helsinki and Uusimaa Hospital District; <sup>3</sup>Anders Langenskiöld Research Laboratory, ORTON Research Institute; <sup>4</sup>Department of Infectious Diseases, University Hospital Basel, Switzerland; <sup>5</sup>Department of Orthopaedic Surgery, Fundación "Jiménez Díaz" Hospital, Madrid, Spain; <sup>6</sup>Department of ENT, Helsinki and Uusimaa Hospital District; <sup>7</sup>Department of Orthopaedic Surgery, Helsinki and Uusimaa Hospital District; <sup>8</sup>Orthopaedic Hospital Valdoitra, Slovenia; <sup>9</sup>ORTON Orthopaedic Hospital of the Invalid Foundation; <sup>10</sup>COXA Hospital for Joint Replacement

It was hypothesized that *Staphylococcus aureus* and/or host cells leave a footprint on the surface of the biomaterial upon infection and implantation. Preliminary experiments indicated that logarithmic phase *Staphylococcus aureus* is particularly sticky and that both host and bacterial molecules regulate adhesion. After regular implant cleaning (by sonication and sterilization) X-ray photoelectron spectroscopy (XPS) disclosed O/Ti ratios > 2 indicating that in addition to TiO<sub>2</sub> also other (organic) oxygen compounds are present, in particular after an implant-related infection (7.6 vs. 4.1 atomic % on exposed and "as received" samples, respectively). This was confirmed by a relatively high carbon (60.2 vs. 49.3 at. %) and nitrogen (9.8 vs. 4.7 at. %) content. Analysis of XPS binding energies (eV) disclosed complete oxidation of titanium to TiO<sub>2</sub> on the substrate surface (Ti 2p spectra). C-C and C-H carbon contamination and C-O, C=O (carbonyl) and COOH (carboxyl) adsorbants were present (C1s spectra). The O1s peaks varied significantly between the bacterial and mammalian cell exposed

and the "as received" samples, whereas the N1s peaks were similar in all samples. The XPS analysis strongly suggests the presence of organic compounds other than normal substrate contamination on exposed samples. Field-emission scanning electron microscope (FE-SEM) disclosed approximately 50 nm-sized particles on exposed samples but not on "as received" samples. These findings suggest that both staphylococcal and mammalian cells leave footprints on biomaterial surface, which can be detected even after regular cleaning. Such footprints can be used to analyze biofilm formation and characteristics and to develop a "golden standard" to reveal implant infections even in culture negative cases. Finally, novel patent pending non-stick diamond-like carbon polymer hybrid (DLC-p-h) coatings with polytetrafluoroethylene (PTFE) or polydimethylpolysiloxane (PDMS) as additive polymers (DLC-PTFE-h and DLC-PDMS-h, respectively) could be used to prevent implant-related infections so that antibiotic and one- or two-stage implant exchange can be avoided.

## 1. Introduction

From clinical point of view, infection of implants constitutes a devastating complication. Incidence of prosthetic joint infection is up to 5 %. Development of a reliable method, a golden standard, to study bacterial (or host cell) attachment in retrospect could enable evaluation of various diagnostic methods for their specificity and sensitivity.

Adhesion of biomolecules (e.g., proteins, bacteria) and cells on biomaterial surfaces is important for the biomaterial behavior (1). It has been proposed that poor bacterial attachment combined with a good host cell attachment could help to avoid implant-related infections ("Race for the surface" hypothesis). Implant surface will determine how well the bacteria adhere (initial step) and form biofilms (final step). Bacteria are first attracted to implant surface by non-

specific (electrostatic, hydrophobic) or specific (binding to e.g. fibronectin, fibrinogen or collagen) interactions. Later, in one or a few days, they form a mature biofilm, which is protected by extracellular polymeric substance against phagocytosis, complement and antibiotics. At this stage, antibiotics may only increase bacterial resistance (2).

We hypothesized that adhesion and biofilm formation leave a trace on the surface of biomaterials. This trace will in the text below be referred to as a "footprint". The aim of this first pilot study was therefore to clarify if bacteria alone in vitro or together with host cells in vivo leave a footprint, which is invisible for the eye but still detectable with special techniques. Ability to detect reliably such footprints would have an advantage over microbial cultures of living organisms as false negative results caused by an ongoing antibiotic treatment can be avoided as footprints withstand antibiotics and remain stable even after living microbes have disappeared. From practical point of view it is of interest to determine if a typical cleaning procedure erases the footprints if e.g. the head in a modular hip is not found in storage and can not be replaced with a new one but the cleaned and sterilized old head has to be reused.

In the present study, we therefore used surface analytical techniques common in materials science to characterize biomaterial surfaces, after exposure to bacteria and/or living cells. The surface chemistry was studied by X-ray photoelectron spectroscopy, whereas field-emission scanning electron microscope was used to obtain high-resolution images of the surface morphology.

## 2. Materials and methods

A sheet cutter was used to produce 2 mm x 30 mm x 9.5 mm samples from 2 mm-thick titanium foil (Ti 99.6+%). Samples were polished to root mean square (RMS) roughness 80 nm with Struers RotoPol-25/RotoForce-4 polishing unit. Grinding paper grit 120 and grit 1200 were used and the final polishing surface was done using 6 µm diamond paste. Finally, samples were cleaned with acetone and ethyl alcohol in an ultrasonic cleaner. Similarly treated plain and DLC coated surgical steel (AISI 316L) plates were used as reference samples for bacterial growth and adhesion. The high quality diamond-like carbon (DLC) coatings were prepared with the filtered pulsed arc discharge (FPAD) method. In FPAD deposition system the

carbon plasma pulse is created by igniting a vacuum arc between the anode and the cathode, and thus discharging the capacitor bank connected in series to the anode and cathode. The arc sustained by a discharge current generates the plasma, which is steered to the sample with the aid of a solenoid-generated magnetic field (3).

*Staphylococcus aureus* 113 was cultured for 7 hours to the logarithmic or 24 hours to the stationary phase of growth. 4 ml supernatant was then cultured on biomaterial plates without shaking at +37°C for 18 or 24 hours. Log phase staphylococci adhered much better and in subsequent experiments 4 x 10<sup>6</sup> cfu/ml log phase staphylococci were used (see below). After the experiment the plates were sealed in a glass test tube containing 4 ml of physiological saline containing 0.15 % EDTA and 0.1 % Triton-X and subjected to vortexing at max. speed (7 Minivortexer) for 45 seconds, sonicated for 3 minutes at 40 ± 5 kHz using 0.1-1.5 W/cm<sup>2</sup> power density in an ultrasonic bath (Labsonic 2000, Bender & Hobein, Zürich, Switzerland) and vortexed once more for 45 seconds. 50 µl of sonicate was diluted to 10-3, 10-4 and 10-5 and 50 µl of each was inoculated on agar plates. After the procedure, the plates were still sterilized in 80 % ethanol and gently dried with blotting paper in air. After this treatment the surface, which appeared totally clean upon inspection, was subjected to XPS and FE-SEM analysis.

Ti implant-related infection was caused in 5 mice by injection of 40 µl physiological saline containing 10<sup>6</sup> cfu *Staphylococcus aureus* through a subcutaneously implanted catheter. Infected implants were removed 7 days later. After the experiments the plates were cleaned as above.

XPS and FE-SEM. X-ray photoelectron spectroscopy spectra were measured with PHI 5600 spectrometer using AlK $\alpha$  radiation as the excitation source. The take-off angle of the emitted photoelectrons was 45° with respect to the surface normal. SEM images were acquired with a Hitachi S 4800 field emission scanning electron microscopy.

## 3. Results and discussion

Logarithmic phase *Staphylococcus aureus* was much more "sticky" than the same strain in the stationary phase of growth (figure 1). This is a new observation and indicates that the same microbial strain uses qualitatively or quantitatively different adhesins depend-

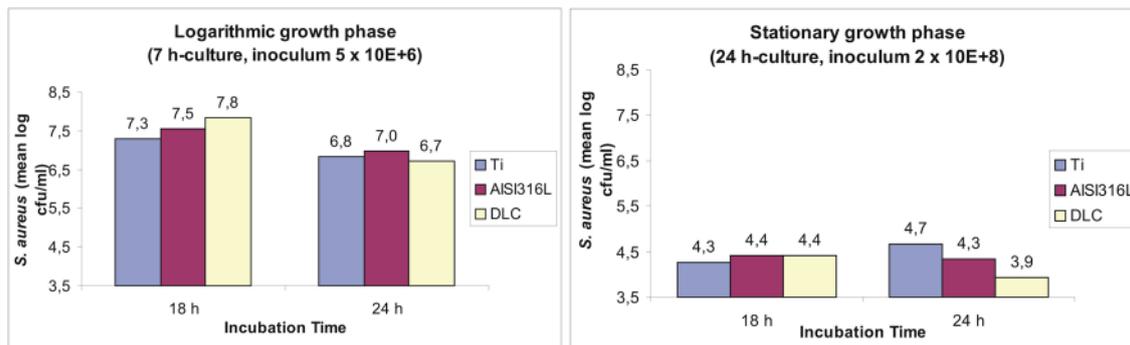


Figure 1: Comparison of the number of staphylococci on titanium (Ti), surgical steel (AISI 316L) and diamond-like carbon (DLC) (from left to right) when cultures were performed using logarithmic (left panel) or stationary phase (right panel) bacteria. Even though the static phase bacteria were initially added in higher numbers ("handicap";  $2 \times 10^8$  cfu vs.  $5 \times 10^6$  cfu), the numbers of bacteria recovered at 18 and 24 hours were much lower when static phase bacteria were used. Different materials did not differ in this respect.

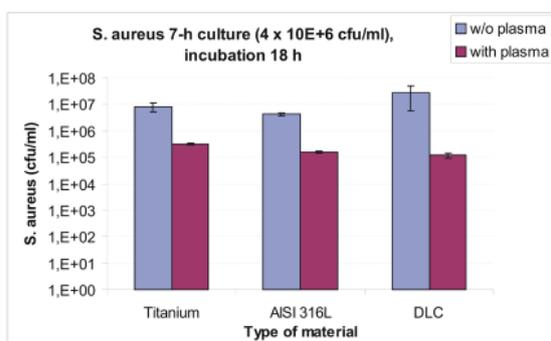


Figure 2: The effect of plasma pre-incubation on the bacterial adhesion on Ti, AISI 316L and DLC surfaces.

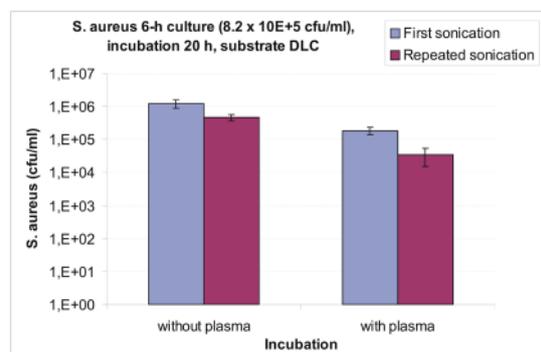


Figure 3: The results of the first and second sonication of DLC samples.

ing on its phase of growth. As the adhesion properties were different, probably also the footprints are different. Unfortunately, in earlier scientific reports on bio-material-microbe interactions only the bacterial numbers have been reported, not their phase of growth. This makes it impossible to perform reliable head-on comparisons between different studies. On the basis of these experiments, only adherent staphylococci in their logarithmic phase of growth were used in the subsequent experiments.

### 3.1. Effect of type of material on bacterial adhesiveness

For all materials tested in the previous experiment, preincubation of them in blood plasma decreased adhesion (figures 2 and 3).

### 3.2. Development of a sticky biofilm

To demonstrate biofilm maturation, sonication was

used to assess when the non-specific and reversible adherence becomes specific and (under physiological conditions practically) irreversible. As a sign of such maturation and biofilm formation between 18 and 24 hours (2), also the second sonication always yielded bacteria (figure 3) and some bacteria still detached after 9th round of sonication (data not shown).

Sonication has been widely used to detach and subsequently enumerate bacteria recovered from infected implants. The present study demonstrates that this method does not work in a reproducible and reliable manner when applied to metallic or polymeric implant materials at frequencies, which allow detachment of intact whole bacteria for subsequent counting. This contrasts with the results obtained using microbes cultured on glass Petri dishes. As in implant-related chronic infections biofilm is always formed, it can also be concluded that relatively sticky footprints, containing rests of bacteria and extracellular polymer-

ic substance, are also always formed. This bacteria can not be totally detached from such biofilms, at least not without using high frequencies and energies in sonication, which disrupts and destroys them.

### 3.3. Surface chemistry

Surface chemistry of the plates used for in vitro bacterial cultures and/or in vivo implant-related infection studies was studied using XPS survey and high resolution scans of the different elements (table 1). The oxygen signal mostly stems from surface TiO<sub>2</sub> as is typical for such samples (4). As the O/Ti ratio is larger than 2, it can be concluded that also other oxygen species are present on the surface. The strong carbon signal indicates the presence of a layer of carbon contamination on the surface. It has been reported in the literature that the carbon concentration can vary between 10-80 % depending on the treatment and storage history of the sample (4). Small amounts of nitrogen are often found as surface contaminants. A significant increase in the amount of carbon and nitrogen was detected on the surface of the sample exposed to in vivo implant-related infection. This indicates presence of organic remnants on the surface of apparently "clean" samples. This can explain the small intensities of the Ti and O signals as they may be covered by an organic layer, which attenuates the signals stemming from TiO<sub>2</sub>. Moreover, the O/Ti ratio was higher in the in vivo than in the in vitro samples suggesting that a higher fraction of the oxygen signal stems from bonds other than the TiO<sub>2</sub> bond.

The XPS high resolution spectra of the different elements provided additional information on the surface chemistry. Table 2 shows the binding energies of different relevant species. As this represents a pilot study, peak convolution to determine the concentrations of the different chemical states of the elements was not carried out as significant differences can be observed in a qualitative comparison of the spectra as described below.

The Ti2p spectra were identical for all samples (data not shown). Only contributions from Ti(IV) (at  $\approx 459$  eV) were observed, indicating a complete oxidation of the surface into TiO<sub>2</sub>. No contribution was seen from the metallic Ti(0) substrate, most probably due to the autoclaving of the samples which leads to a slight thickening of the oxide film as compared with the native air-formed passive film on Ti surface.

The C1s spectrum (Figure 4) showed contributions from carbon in different chemical states. The

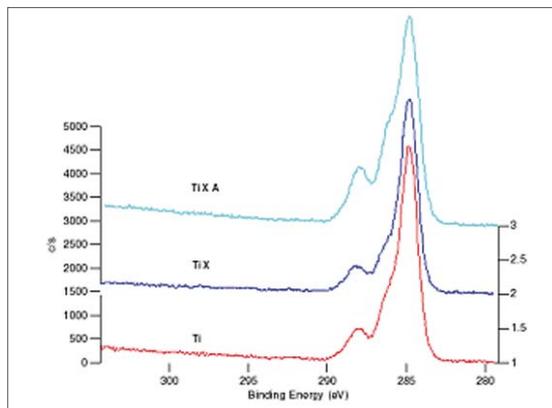


Figure 4: C1s spectra for the as-received (Ti), staphylococcus (TiX) and staphylococcus and animal-exposed (TiX A) samples. C/S = counts per second, binding energy in eV.

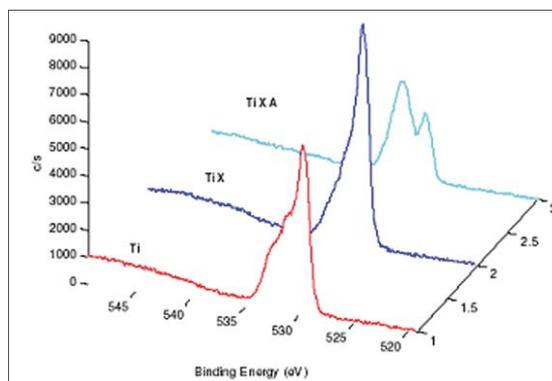


Figure 5: O1s spectra for the as-received (Ti), staphylococcus (TiX) and staphylococcus and animal-exposed (TiX A) samples.

major contribution at 285 eV (C-C, C-H) is typically assigned to surface contamination. The carbon peaks at higher binding energies are often ascribed to the presence of organic adsorbants (C-O, C=O, COOH) on the surface. Between the three samples, small differences exist in the relative intensity of the different adsorbants within the carbon peak.

The O1s peaks (figure 5) were very different in these three samples, in particular between the sample analyzed as received (Ti) and the one used in the experimental mouse infection study (TiX A). An exact assessment of the surface chemistry is not possible, since the binding energies of different oxygen bonds overlap, or are very near to each other (530.2 - 539.6 eV O<sub>2</sub>- in TiO<sub>2</sub>; 531.3 - 532.2 eV in OH- and C=O; 532.5 - 533.4 eV in H<sub>2</sub>O and C-O; see also table

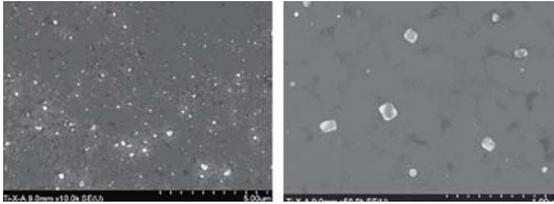


Figure 6: SEM images of the footprints on Ti sample exposed to animal tissues, lower magnification to the left and higher magnification to the right.

2). Nevertheless, the results clearly indicate a higher fraction of oxygen in other chemical states than O<sub>2</sub>- (in TiO<sub>2</sub>) in the animal-exposed sample. Considering the fact that for this sample also significantly higher nitrogen and carbon concentrations were found (see table 1), it is probable that the higher non-oxide contribution to the oxygen signal stems from organically bound oxygen.

The binding energy of the N1s peak was identical for all samples: the peak at  $\approx 400$  eV can be assigned to organic amines (399 - 402 eV in NH<sub>4</sub><sup>+</sup> and organic amines). However, the results show that this peak is also observed in the as-received Ti sample and typically a small amount of nitrogen with a binding energy around 400 eV is often observed on Ti surface. Therefore, the exact origin of this nitrogen is unknown. Only in case of compound formation (e.g., titanium

nitride or TiN), significantly different binding energy (396.2 - 397.0 eV) is found for the N1s peak on Ti.

To enable a detailed identification of the species present on the surface, spectra from standards of different biomolecules are now being measured. Moreover, other techniques such as time-of-flight secondary ion mass spectrometry (ToF-SIMS) or Fourier transform infrared spectroscopy (FTIR) spectroscopy will be used in the future to provide molecular information on the surface chemistry and could be helpful in the identification of the organic surface remnants. Nevertheless, the present results clearly demonstrate that the surface chemistry of the Ti sample previously exposed to Staphylococci in vitro or in animal tissues carries significant traces of the biological exposure, even after cleaning procedures. In the future, experiments will be performed using samples with only gentle cleaning to further enhance the footprint detection.

### 3.4. Surface morphology.

Figure 6 shows SEM images of the surface of the sample exposed to the experimental implant-infection. The most significant observation was the presence of very small (ca. 50 nm side length) "particles" on the surface – such particles were not found on the surface of the as-received sample. The nature of these particles could not yet be determined; further work is planned to study the composition of these small bio-induced

Table 1. Elemental concentrations of the surfaces of the two samples: Ti (as-received) and Ti-A (sample exposed to the animal infection study). The atomic concentrations were determined from XPS survey.

Element	Ti	O	C	N	Ca	P	O/Ti
Ti as received	8.7	36.1	49.3	4.7	1.1	-	4.1
Ti exposed to staphylococci in vitro	11.7	39.1	41.8	3.8	1.2	2.5	3.3
Ti implanted and infected	3.4	26.1	60.2	9.8	0.48	-	7.6

Table 2. Binding energies of different elements (5).

Ti 2p		O1s	
458.8 - 459.2 eV	Ti(IV)	530.2 - 539.6 eV	O <sub>2</sub> - (in TiO <sub>2</sub> )
453.5 - 454.2 eV	Ti(0)	531.3 - 532.2 eV	OH-, C=O
C1s		N1s	
285 eV	C-C, C-H	396.2 - 397.0 eV	Nitride
286.4 - 286.7 eV	C-O	399 - 402 eV	NH <sub>4</sub> <sup>+</sup> , organic amines
287.8 - 288.0 eV	C=O	407 - 408 eV	Nitrate (NO <sub>3</sub> -)
288.5 - 289.3 eV	COO		

nanoparticles on the Ti surface. It therefore remains a speculation, if these particles are remnants of cell-adhesion points. Cell remnants have been observed previously in a SEM study of surfaces after cell detachment experiments by jet impingement (6). A direct comparison of the results of the literature regarding cell remnants and our study is not possible, since the experimental approach is very different (cell culture test in the lab vs. animal exposure; jet impingement vs. sonication) and as the cell attachment and consequently also cell detachment depend on the substrate, cell type, time of cell exposure, and other experimental parameters.

#### 4. Conclusions

The results of this preliminary surface analytical study on Ti samples exposed to an *in vivo* experiment clearly indicate that remnants of mammalian or bacterial cell biomolecules can be found on the sample surface, even after regular sample cleaning (sonicating) procedures. These traces are apparent as changes in the surface chemistry (increased nitrogen and carbon concentrations, increased amount of organically bound oxygen on the surface) as observed using highly surface-sensitive XPS. Moreover, a high-resolution SEM indicates the presence of scattered particles on the surface, which may represent cell or adhesion point remnants. Further work utilizing these methods and time-of-flight-secondary ion mass spectrometry (ToF-SIMS) will aim at identification of biological remnants on the implant surface. Moreover, to chemically analyse the small nanoparticles on the surface, surface sensitive Scanning Auger Microscopy (SAM) with a high lateral resolution will be employed. These tools can also be used to test new anti-soiling biomaterials, such as diamond like carbon polymer hybrid (DLC-PTFE-h when polytetrafluoroethylene, commonly known under the trade name Teflon®, or DLC-PDMS-h when polydimethylsiloxane silicone rubber is used as the additive polymer) coatings, as they may not allow colonization of and biofilm formation on the implant surface (7). If this strategy succeeds, it can be used as primary prophylaxis against implant-related infections and can replace *i.v.* antibiotics and one- or two-stage implant exchange.

#### Literature

1. Brunette DM: Principles of cell behavior on titanium surfaces and their application to implanted devices. In *Titanium in Medicine*, Eds. Brunette DN, Tengvall P, Textor M, Thomsen P. Springer, p. 486, 2001.
2. Kinnari TJ: The effect of albumin coating on the properties of tympanostomy tubes. Academic dissertation, Thesis, University of Helsinki, 2004.
3. Anttila A, Lappalainen R, Tiainen VM, Hakovirta M: Superior attachment of high-quality hydrogen-free amorphous diamond films to solid materials. *Adv Mater* 1997;9:1161-1164.
4. Lausmaa J: Surface spectroscopic characterization of titanium implant materials. *J Elect Spectrosc Rel Phenom* 1996;81:343.
5. Vörös J, Wieland M, Ruiz-Taylor L, Textor M, Brunette DM: Characterization of titanium surfaces. In *Titanium in Medicine*, Eds. Brunette DN, Tengvall P, Textor M, Thomsen P. Springer, p. 87, 2001.
6. Richards DG, Gwynn I, Bundy KJ, Rahn BA: Microjet impingement followed by scanning electron microscopy as a qualitative technique to compare cellular adhesion to various biomaterials. *Cell Biol Int* 1995;19:1075.
7. Anttila A, Tiainen VM, Kiuru M, Alakoski E, Arstila K: Preparation of diamond-like carbon polymer hybrid films using filtered pulsed arc discharge method. *Surface Engineering* 2003;19: 425-428.