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A novel soft tissue model for biomaterial-associated infection and inflammation – Bacteriological, morphological and molecular observations

Sara Svensson [a, b], Margarita Trobos [a, b], Maria Hoffman [a, b], Birgitta Norlindh [a, b], Sarunas Petronis [a, c], Jukka Lausmaa [a, c], Felicia Suska [a, b], Peter Thomsen [a, b, *]

[a] BIOMATCELL VINN Excellence Center of Biomaterials and Cell Therapy, Box 412, 405 30 Gothenburg, Sweden
[b] Department of Biomaterials, Sahlgrenska Academy at University of Gothenburg, Box 412, 405 30 Gothenburg, Sweden
[c] SP Technical Research Institute of Sweden, Box 857, 501 15 Borås, Sweden

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ABSTRACT

Infection constitutes a major risk for implant failure, but the reasons why biomaterial sites are more vulnerable than normal tissue are not fully elucidated. In this study, a soft tissue infection model was developed, allowing the analysis of cellular and molecular responses in each of the sub-compartments of the implant–tissue interface (on the implant surface, in the surrounding exudate and in the tissue). Smooth and nanostructured titanium disks with or without noble metal chemistry (silver, gold, palladium), and sham sites, were inoculated with *Staphylococcus epidermidis* and analysed with respect to number of viable bacteria, number, viability and gene expression of host cells, and using different morphological techniques after 4 h, 24 h and 72 h. Non-infected rats were controls. Results showed a transient inflammatory response at control sites, whereas bacterial administration resulted in higher recruitment of inflammatory cells (mainly polymorphonuclear), higher, continuous cell death and higher gene expression of tumour necrosis factor-alpha, interleukin-6, interleukin-8, Toll-like receptor 2 and elastase. At all time points, *S. epidermidis* was predominantly located in the interface zone, extra- and intracellularly, and lower levels were detected on the implants compared with surrounding exudate. This model allows detailed analysis of early events in inflammation and infection associated to biomaterials *in vivo* leading to insights into host defence mechanisms in biomaterial-associated infections.

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1. Introduction

Infection has been recognised as one of the main risks for the failure of medical devices. These events are often detrimental for the patient and frequently lead to surgery, long-term antibiotic treatment and confer a major individual and societal burden and cost [1,2]. There are various routes via which bacteria may enter the implant site [3,4]. One major risk for a totally internal implanted device is during the surgical procedure [3,4]. For example, bacteria from the deeper layer of the patient's skin [5], the operating theatre [6,7] or on the implant itself may contaminate the surgical wound [4]. *Staphylococcus aureus* and *Staphylococcus epidermidis* are bacterial species commonly found at biomaterial-associated infection sites and both have the ability to form a protective biofilm when adhering to a material surface [8]. The biofilm protects the bacteria from phagocytic uptake, bactericidal and opsonising antibodies as well as antibiotic treatment [9,10]. However, a biomaterial already covered with viable, functional host cells will be less vulnerable to bacterial colonisation, leaving the bacteria more accessible for the immune system and antimicrobial agents [10]. The race for the surface between microorganisms and host cells is therefore of great importance for the future success of an implant.

Several questions regarding the initial events at the biomaterial–tissue interface, especially in the presence of bacteria, remain to be answered. Which cells and signals predominate at different stages during the course of infection? Does the inflammatory response to infectious stimuli differ in the presence of a biomaterial? What role do the surface properties, such as chemical

* Corresponding author. Department of Biomaterials, Sahlgrenska Academy at University of Gothenburg, Box 412, 405 30 Gothenburg, Sweden. Fax: +46 317862950.
E-mail address: peter.thomsen@biomaterials.gu.se (P. Thomsen).

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and topographical characteristics, play in the complex *in vivo* environment? Many of the answers to these questions are important in order to provide improved diagnostic tools, as well as for the development of infection control strategies [3].

One major difficulty when assessing the early (within hours and days) interfacial and tissue response to pathogens is that there is a lack of relevant models [3]. Many materials with claimed antimicrobial properties have been proposed, but their efficacy has predominantly been demonstrated *in vitro* with tissue cells and bacterial cells separately [11–13]. A few *in vitro* studies have employed more complex experimental microenvironments by combining as many as two eukaryotic cell types with bacteria [14–16]. Different *in vivo* models have been used, often focussing on infection around implants in bone, frequently with different routes of antibiotic administration, where the primary outcome is whether or not the animals become infected [17,18]. However, there is also a need to investigate the early phases of biomaterial-associated infections, both on the implant and in the surrounding exudate and tissue.

Surfaces with a nanostructured noble metal-coating consisting of small amounts of metallic silver (Ag), gold (Au) and palladium (Pd) have the ability to reduce catheter-related infections in the clinic [19,20] and have been shown to reduce bacterial adhesion and colonisation *in vitro* when applied on titanium (Ti) [21]. In addition, the specific combination of noble metals on silicone has previously been shown to be important for the inflammatory events *in vivo* [22]. The viability of *S. epidermidis* was affected by nanostructures in the 35–40 nm size range *in vitro* [16]. On the other hand, the role of the surface chemistry versus nanotopography of noble metal coated surfaces for the early bacterial colonisation and inflammation is not fully understood.

The aim of the present study was to develop a simple model enabling systematic studies of the details on the interactions between the host cells, bacteria and biomaterial surface in soft tissues *in vivo*. A second aim was to use this model to study the early inflammatory response and colonisation of *S. epidermidis* in association with Ti substrates with (i) a nanostructured noble metal coating (Ag, Au and Pd) (nNoble), (ii) the same surface but coated with Ti (nTi) (thereby keeping similar nanostructure but masking the underlying surface chemistry), and as a reference, (iii) a smooth, electropolished Ti surface (sTi).

2. Materials and methods

2.1. Implants

Titanium disks (Ti grade 2) with a diameter of 9.0 mm and a thickness of 2.2 mm were manufactured by turning (Hagal Machinery AB, Mölndal, Sweden). The disks were provided with a threaded hole (diameter 1.4 mm, depth 2.5 mm) on the rim of the implant in order to mount them on a sample holder during an electropolishing process. The electropolishing was performed in a perchloric-acid-based electrolyte, which was cooled to −30 °C and vigorously stirred. The samples were mounted as anodes at 22.5 V constant potential for 5 min and eight disks were treated at a time, to make mirror-like implant surfaces [23]. Approximately two thirds of the total number of electropolished disks were then coated with the noble metals Ag, Au and Pd in an electroless plating wet chemical process (Bactiguard AB, Sweden). Subsequently, approximately half the coated samples were treated with mild oxygen plasma (150 W, 20 s, TePla 300 PC microwave plasma system, PVA TePla AG, Wettenberg, Germany) and then further coated with a 5 nm thick layer of Ti (deposition rate 0.5 nm/s, FHR MS150 magnetron ion sputtering system (FHR Anlagenbau GmbH, Ottendorf-Okrilla, Germany) in order to shield the noble metal chemistry but preserve the nano/sub-micron topography. Three different implant surfaces were used in the study: electropolished Ti disks (smooth Ti; sTi), noble metal coated electropolished Ti disks (nanostructured noble metal coated Ti; nNoble) and Ti-covered noble metal coated electropolished Ti disks (nanostructured Ti; nTi), as illustrated schematically in Fig. 1. All the disks were individually packed and sterilised using ethylene oxide (Synergy Health, Venlo, The Netherlands).

2.2. Material characterisation

The Ti disks were characterised with respect to surface chemistry and topography. Scanning electron microscopy (SEM) was used to visualise the type, lateral dimensions and surface coverage of the topographical features present on the differently prepared surfaces. The SEM analysis was performed using a Zeiss Supra 40VP system (Carl Zeiss AG, Oberkochen, Germany) equipped with a field-emission electron gun. The imaging was carried out in a secondary electron detection mode to generate mainly topographical contrast. The recorded images were further processed by ImageJ software (National Institutes of Health, Bethesda, USA) for quantitative image analysis.

Atomic force microscopy (AFM) was used to characterise the nanoscale surface roughness of the Ti disks, using a Bruker Dimension 3100 SPM system with NanoScope v6.12 software (Bruker Nano Surfaces, Santa Barbara, USA) and NSG10 probes (NT-MDT Co., Russia). A $2 \times 2\ \mu m^2$ area was scanned in at least two spots on each type of Ti disk in a tapping mode. The roughness parameters were evaluated using SPIP 3D Image Processing v3.0.0.9 software (Image Metrology A/S, Hørsholm, Denmark).

The microscale surface roughness was measured using a Wyko NT 1100 optical surface profiler (Veeco Instruments Inc., Tucson, USA). The measurements were performed using a 20× magnification objective and the Vertical Scanning Interferometry (VSI) mode. The roughness parameters were evaluated using the same SPIP software as in the case of AFM measurements.

Surface chemical composition and cleanliness were characterised by X-ray photoelectron spectroscopy (XPS) and time-of-flight secondary ion mass spectroscopy (TOF-SIMS). The high surface sensitivity (1–2 nm) of TOF-SIMS was used to evaluate the extent to which the sputtered 5 nm Ti coating shielded the underlying noble metal surface chemistry by analysing the Ti disks containing noble metals before and after coverage with the Ti film and recording the signal intensities for ions containing Ag, Au and Pd. The TOF-SIMS analyses were performed with a TOF-SIMS IV instrument (IONTOF GmbH, Münster, Germany) using 25 keV Bi_3^+ primary ions, with the instrument optimised for high mass resolution ($m/\Delta m$ ~5000, beam diameter 3.5 μm) at a pulsed Bi_3^+ current of 0.1 pA.

The XPS technique was used to obtain information about the chemical composition of the outermost 2–10 nm of the implant surface after implant packaging and sterilisation procedures. XPS survey and detail spectra were recorded using a Krator AXIS Ultra^{DLD} system (Kratos Analytical, Manchester, UK) from two areas of approximately 1 mm^2 on each sample surface ($n = 2$), using a monochromatic Al X-ray source. Relative concentrations (atomic %) of the detected elements were calculated from peak intensities in the detail spectra recorded for each element recognised in the survey spectra.

2.3. Bacterial strain and inoculum preparation

A biofilm-producing strain of *S. epidermidis* (ATCC 35984; Culture Collection, University of Gothenburg, Sweden) was used as the infectious agent. One day prior to surgery, the strain was plated on Columbia horse blood agar plates (Media Department, Clinical Microbiology Lab, Sahlgrenska University Hospital, Sweden) and incubated at 37 °C over night.

An inoculum of approximately 2×10^7 CFU/mL was prepared in 0.9% saline from an OD_{546nm} of 0.25, corresponding to 10^8 CFU/mL. The concentration of *S. epidermidis* in the inoculum and the OD suspension was confirmed by colony forming unit (CFU) quantification of serially diluted suspensions on blood agar.

The *S. epidermidis* ATCC 35984 strain was tested for susceptibility to several antimicrobial agents using a commercially prepared, dehydrated panel GPALL1F

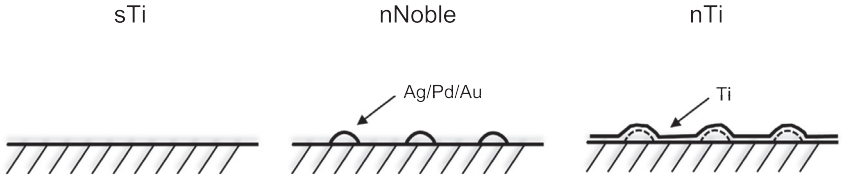

Fig. 1. Schematic drawing of the different surfaces used. Electropolished Ti (referred to as sTi) was coated with a noble metal coating, resulting in a nanostructured surface (referred to as nNoble). A 5 nm thick Ti layer was sputtered on top of the noble metal coated surfaces with the aim of hiding the noble metal chemistry but keeping the nanotopographic features of the surface (referred to as nTi).

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