CHAPTER 3

Soft tissue integration versus early biofilm formation on different dental implant materials

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Abstract

Objectives: Dental implants anchor in bone through a tight fit and osseo-integratable properties of the implant surfaces, while a protective soft tissue seal around the implants neck is needed to prevent bacterial destruction of the bone-implant interface. This tissue seal needs to form in the unsterile, oral environment. We aim to identify surface properties of dental implant materials (titanium, titanium-zirconium alloy and zirconium-oxides) that determine the outcome of this “race-for-the-surface” between human-gingival-fibroblasts and different supra-gingival bacterial strains.

Methods: Biofilms of three streptococcal species or a Staphylococcus aureus strain were grown in mono-cultures on the different implant materials in a parallel-plate-flow-chamber and their thickness evaluated using confocal-scanning-laser-microscopy. Similarly, adhesion, spreading and growth of human-gingival-fibroblasts were evaluated. Co-culture experiments with bacteria and human-gingival-fibroblasts were carried out to evaluate tissue interaction with bacterially contaminated implant surfaces. Implant surfaces were characterized by their hydrophobicity, roughness and elemental composition.

Results: Biofilm formation occurred on all implant materials, and neither roughness nor hydrophobicity had a decisive influence on biofilm formation. Zirconium-oxide attracted most biofilm. All implant materials were covered by human-gingival-fibroblasts for 80-90% of their surface areas. Human-gingival-fibroblasts lost the race-for-the-surface against all bacterial strains on nearly all implant materials, except on the smoothest titanium variants.

Significance: Smooth titanium implant surfaces provide the best opportunities for a soft tissue seal to form on bacterially contaminated implant surfaces. This conclusion could only be reached in co-culture studies and coincides with the results from the few clinical studies carried out to this end.
**Introduction**

The application of dental implants, directly anchored in the bone, can be considered as one of the major advances in modern dentistry and provides considerably more comfort for patients in the restoration of function after tooth loss than dentures or bridges. Up to now, pure titanium has been the most widespread and successfully used material for these implants because it is biocompatible, corrosion resistant, lightweight and durable, while it can be easily prepared in many different shapes and textures without affecting its biocompatibility [1]. The fate of a biomaterial implant can be envisaged as a race for the surface between bacteria and host tissue cells [2]. If bacteria win the race for the surface, the implant is mostly lost and has to be replaced, as bacteria adhere to an implant surface in their protective, biofilm mode of growth [3]. On the other hand, if tissue cells are able to integrate the implant surface, this yields the best available protection against infection [4]. Considering the relatively unsterile conditions under which dental implants are inserted and have to osseo-integrate, one can wonder how the race for the surface can ever be won by host tissue cells on a dental implant surface. Yet, early failure of dental implants occurs in “only” 8% and 5% of the implants in the maxilla and the mandible, respectively [5].

Success or failure of dental implants is directly related to the degree of integration of the implant material by surrounding soft and hard tissues [6,7] versus biofilm formation [8,9]. Osseo-integration is nowadays less of a problem than the formation of a soft tissue seal and is achieved by a tight fit of the implant in the bone and an osseo-integrative morphology and/or chemistry of the implant surface. Non-osteogenic soft tissue integration of the neck of the implant surface is necessary to create a biological seal that protects against invasion of periodontopathogens towards the bone [10]. Surface roughness and hydrophobicity, as a corollary of the chemical composition of the implant surface, are generally considered as the key-properties of dental implant surfaces for both tissue integration [11,12] and biofilm formation [13].

Early failure of dental implants is initiated by adhesion of streptococci and other initial colonizers [14] preventing the formation of a soft tissue seal. Recently *Staphylococcus aureus* has also been identified as an initial colonizer of dental implants [15]. Late peri-implantitis constitutes a totally different race for the surface, where periodontopathogens, other than supra-gingival colonizers, have to displace periodontal ligament fibroblasts from the neck of the implant surface [10], ultimately causing bone loss and possible loss of an entire implant.

The race for the surface between human gingival fibroblasts (HGF) cells and initial colonizers seems like an impossible one to win for HGF cells in the human oral cavity, as it is loaded with different microbial strains and species. Although dental implant surface properties have been investigated with respect to either biofilm formation or tissue interaction in mono-culture studies (encompassing either bacteria or tissue cells),
co-culture experiments in which bacteria and tissue cells are actually competing in one and the same experiment for an implant surface [16] have never been carried out on dental implant surfaces.

The aim of this study was to compare the outcome of the race for the surface in co-culture experiments between contaminating supra-gingival bacterial strains and HGF cells on different, currently used dental implant materials in an early failure model, used earlier to study the race for the surface on orthopedic implant materials [16]. Such a study is direly needed, as benefits of different titanium (Ti) surface modifications, as well as of the use of titanium-zirconium (TiZr) alloys and zirconia (ZrO₂), have only been evaluated in mono-culture studies with little clinical relevance or in clinical studies with highly limited statistical power [17]. The bacterial strains involved in the current study include supra-gingival strains (Streptococcus oralis J22, Streptococcus mitis BMS, Streptococcus salivarius HB and S. aureus ATCC 25923), as involved in early failure of dental implants.

Materials and methods

Implant materials

All implant materials used in this study were provided by Institut Straumann AG (Basel, Switzerland) and were received as discs (diameter 5 mm, thickness 1 mm). Three different implant materials were included in this study, comprising titanium (Ti, cold-worked, grade 4), titanium-zirconium alloy (TiZr, 15% (wt) Zr) and zirconium oxide (ZrO₂, Y-TZP). The materials were modified according to different procedures, indicated as

- **P**: mirror-polished with finally a 0.04 μm SiO₂ suspension and cleaned by Deconex solution, followed by concentrated nitric acid and water.
- **M**: ground to mimic the machined part of the implant and washed with Deconex solution, followed by concentrated nitric acid and water.
- **MA**: ground and then acid-etched with a boiling mixture of concentrated HCl and H₂SO₄ (or hot hydrofluoric acid in case of ZrO₂) and rinsed with concentrated nitric acid and water.
- **modMA**: ground and acid-etched with HCl/H₂SO₄, as described above, but rinsed with water only under N₂ protection and directly stored individually in glass containers, filled with an isotonic NaCl solution, protected by N₂ filling.

M, MA, modMA and P modifications were all applied to Ti, whereas TiZr alloy and ZrO₂ were only modified according to M and modMA (TiZr) or MA (ZrO₂). M, MA and P modified discs were individually packed in aluminum foil and sealed in plastic. All discs were sterilized in their respective packagings by γ-irradiation at 25-42 kGy.
Implant surface characterization

The hydrophobicity of the implant surfaces was determined by water contact angle measurements at room temperature with a home-made contour monitor, using the sessile drop (1-1.5 µl droplets) method. All values reported are averages over three different implant surfaces, while five droplets were placed on each disc.

The elemental surface composition of the implant surfaces was measured using an S-probe X-ray photoelectron spectrometer (Surface Science Instruments, Mountain View, CA, USA), equipped with an aluminum anode (10 kV, 22 mA) and a quartz monochromator. The direction of the photoelectron collection angle was 55 degrees with the sample surface and the electron flood gun was set at 10 eV. Survey scans were made with a 1000 µm × 250 µm spot and pass energy of 150 eV and used to calculate elemental surface compositions of the implant surfaces. The O\textsubscript{1s} photoelectron binding energy peak was also recorded with a pass energy of 50 eV and decomposed into a component at 530.1 eV due to oxygen involved in oxide bonds and other components due to, for instance oxygen involved in carbonaceous contamination. All experiments were done on two discs of each implant material, while two separate measurements were taken on each sample.

The average surface roughness of the different implant surfaces was assessed with an atomic force microscope (Nanoscope IIIa Dimension\textsuperscript{TM}3100, Bruker, Santa Barbara, CA, USA), operated in the contact mode and using a Si\textsubscript{3}N\textsubscript{4} cantilever tip (DNP from Veeco, Woodbury, NY) with a spring constant of 0.06 N/m. Analyses were done on three randomly selected places per sample disc, employing three discs for each implant material. The surface morphology of different implant surfaces was investigated using scanning electron microscopy (SEM). The specimens were fixed on SEM-stub-holders and visualized in a field emission scanning electron microscope (FE-SEM type 6301F, JEOL Ltd., Tokyo, Japan) at 2 kV and a magnification of 3000x.

Bacterial strains and culturing

For biofilm formation, *S. oralis* J22, *S. mitis* BMS, *S. salivarius* HB, and *S. aureus* ATCC 25923 were used. Strains were streaked onto blood agar plates and incubated for 24 h at 37°C. Then, a fresh colony was inoculated in 10 mL of appropriate growth medium. *S. oralis* J22, *S. mitis* BMS, *S. salivarius* HB were inoculated in Todd Hewitt Broth (THB; OXOID, Basingstoke, England) and *S. aureus* ATCC 25923 was inoculated in Tryptone Soya Broth (TSB; OXOID, Basingstoke, England) for 24 h at 37°C. Subsequently, 10 mL of the bacterial culture was inoculated into 200 mL growth medium and a second culture was grown for 16 h at 37°C. Bacteria were harvested by centrifugation at 4,000xg for 5 min at 10°C, and streptococci washed twice with sterile adhesion buffer (2 mM potassium phosphate, 50 mM potassium chloride, and 1 mM calcium chloride, pH 6.8) while *S. aureus* ATCC 25923 was washed in sterile phosphate buffered saline (PBS, 10 mM...
potassium phosphate, 0.15 M NaCl, pH 7.0). Subsequently, bacteria were sonicated on ice (3 × 10 s) in sterile buffer at 30 W (Vibra Cell model 375; Sonics and Materials, Danbury, CT, USA) in order to break bacterial aggregates. Bacteria were counted in a Bürker-Türk counting chamber and the bacterial suspension was further diluted to a concentration of 3 × 10^5 bacteria per mL for all experiments.

**Bacterial adhesion and biofilm formation**

Bacterial adhesion and biofilm formation were studied on the bottom plate of a parallel plate flow chamber (175 × 17 × 0.75 mm³). The bottom plate consisted of transparent poly (methyl methacrylate) in which six 1 mm deep inserts were prepared in the center region of the plate that could each hold one implant material disc (see Fig. 1). In this way, six different implant materials could be analyzed in one experiment, therewith reducing influence of biological variations between bacterial cultures. Poly (methyl methacrylate) bottom plates were cleaned in a 2% RBS detergent solution (Omniclean, Breda, The Netherlands) under sonication and thoroughly rinsed in demineralized water, 70% ethanol, water again and finally washed with sterile ultrapure water prior to use. The flow chamber used was equipped with heating elements and kept at 37°C throughout an experiment. Bacterial deposition was observed with a CCD camera (Basler AG, Germany) mounted on a phase-contrast microscope Leica DM2000 (Leica Microsystems Ltd., Germany) with a 40× objective lens.

Prior to each experiment, all tubes and the flow chamber were filled with suitable buffer. After removing all air bubbles from the system, a bacterial suspension in buffer was perfused through the system at a shear rate of 11 s⁻¹. Images were obtained as a function of time.

In a separate set of experiments using fluorescence microscopy after staining of adhering bacteria, it was first established that all materials including the poly (methyl methacrylate) bottom plate, initially attracted identical numbers of adhering bacteria. Hence, during final experiments phase-contrast microscopic enumeration of the number of bacteria on the transparent bottom plate could be used to control the number of initially adhering bacteria on the different implant surfaces.

As soon as the number of adhering bacteria amounted 10^3 cm⁻², flow was switched to buffer again in order to remove un-adhering bacteria from the tubes and chamber. Subsequently, modified culture medium (Dulbecco’s modified Eagle’s medium high glucose with 25 mM HEPES supplemented with 10% (v/v) fetal calf serum, 0.2 mM ascorbic acid-2-phosphate and 2% (v/v) of the appropriate bacterial growth medium, (for details see section below “Adhesion, spreading and growth of human gingival fibroblasts”) was perfused through the system at a low shear rate of 1.67 s⁻¹ for 24 h, in order to allow the adhering bacteria to grow into a biofilm.
After 24 h of growth, biofilms were stained with a vitality staining solution, containing 3.34 mM SYTO 9 and 20 mM propidium iodide (Molecular Probes Inc, USA) in PBS. Staining was done in the flow chamber for 15 min in the dark at room temperature. Next biofilms were examined with a confocal laser scanning microscope (CLSM, Leica DMRXE with a confocal TCS SP2 unit) with a water objective (HCX APO L 40.0 × 0.80 W) using 488 nm excitation and emission filters of 500 to 550 nm and 605 to 720 nm to reveal live or dead bacteria. Images were taken over the depth of a biofilm in sequential steps of 0.8 μm. Subsequently, the stacks of images acquired were analyzed for the total biofilm volume per unit area with the program “COMSTAT” [18].

**Human gingival fibroblasts and culturing**

Gingival fibroblasts were obtained from the American Type Culture Collection (HGF-1, ATCC-CRL-2014). Cells were routinely grown in monolayer cultures in Dulbecco’s modification or Eagle’s medium (DMEM) containing 25 mM HEPES and further supplemented with 10% (v/v) Fetal Bovine Serum, 0.2 mM ascorbic acid-2-phosphate at 37°C in a humidified atmosphere with 5% CO₂. At 70-80% confluence the fibroblast cultured were passaged using a Trypsin-EDTA solution (Invitrogen, Breda, The Netherlands). Thus grown cells, between passage 4-10 were used in all experiments, i.e. mono- and co-culture studies, pursuing further growth in modified culture medium in order to grow both oral supra-gingival bacteria and HGF cells simultaneously in the same growth medium.

To develop a modified culture medium allowing growth of HGFs and bacteria, bacterial medium (THB or TSB) and DMEM were combined in different ratios and growth of both bacteria and HGF cells examined. To examine HGF growth in modified culture media with different amounts of THB or TSB added, 200,000 cells, were suspended in modified culture media and seeded into T25 cell culture flasks. After incubation at 37°C in a humidified 5% CO₂ atmosphere for 48 h, cells were immuno-stained and their spreading and morphology was assessed from micrographs, obtained using fluorescence microscopy. The bacterial strains were separately inoculated from agar plates in 10 mL THB or TSB for 24 h. These cultures were used to inoculate 10³ bacteria in second 10 mL cultures of modified culture media which were incubated for 24 h (“pre-cultures”). From these pre-cultures, 0.5 mL was used to inoculate 9.5 mL of modified culture medium and grown overnight. Bacteria were counted using a Bürker-Türk counting chamber. Based on the combined results, modified culture medium comprised of DMEM with 2% THB (for *S. oralis*, *S. mitis*, *S. salivarius*) or TSB (for *S. aureus*) added, was chosen for further studies and will be denoted as “modified culture medium” in the remainder of this study.
Fig. 1 – Schematics of the parallel plate flow chamber employed, with the inserts for different samples in the bottom plate indicated

Adhesion, spreading and growth of human gingival fibroblasts
Discs of each implant material were placed in 48-wells plates and 1 mL of HGF cell suspension in modified culture medium with a concentration of 20,000 cells/mL was
added. Cells were maintained at 37°C in a humidified 5% CO₂ for 48 h. After 48 h, HGF cells were fixed in 3.7% paraformaldehyde in cytoskeleton stabilization buffer (0.1 M Pipes, 1 mM EGTA, 4% (w/v) polyethylene glycol 8000, pH 6.9). After 5 min, the fixation solution was replaced by fresh 3.7% paraformaldehyde for another 10 min. Subsequently cells were incubated in 0.5% Triton X-100 for 3 min, and stained for 30 min in 5 mL PBS with 1% albumin containing 0.4 µg/mL DAPI and 2 µg/mL of TRITC-phalloidin. Adhering cells were subsequently washed in PBS with 1% bovine serum albumin for 5 min, washed in PBS, stored in PBS and examined with Leica DMIL fluorescence microscope (Leica Microsystems Ltd, Germany) at x10 magnification. The number of adhering cells per unit area and total surface coverage of the materials by tissue cells were determined using Scion image analysis software, while the average area per spread cell was calculated from the ratio of the total surface coverage divided by the number of cells. All experiments were performed in six-fold on each implant material, comprising two sets of three independent experiments with modified culture medium, supplemented either with THB or TSB.

**Tissue cell- bacteria co-culture experiments**

Implant materials in Petri dishes were exposed to 10 µl suspensions (concentration 4 × 10⁴ bacteria/mL) of the different bacterial strains in adhesion buffer for the streptococci and in PBS for the staphylococci and incubated at 37°C, under 100% humidity for 60 min. Subsequently, the bacterial suspensions were removed by dipping three times in sterile buffer, yielding the presence of approximately 3 × 10⁴ bacteria/cm² on the implant surfaces. Subsequently, HGF cells suspended in modified culture medium, supplemented with 2% of the appropriate bacterial growth medium, were seeded on bacterial-coated implant surfaces to a density of 20,000 cells/cm². Bacteria and HGF cells were maintained at 37°C in a humidified 5% CO₂ for 48 h. After 48 h, HGF cells were fixed, stained with TRITC-phalloidin and DAPI and analyzed as described above. The outcome of the race for the surface was expressed as the reduction in surface coverage by HGF cells in the presence of adhering bacteria. All experiments were performed in triplicate on each implant material.

**Statistical analyses**

To analyze differences in the water contact angle and surface roughness of the implant surfaces, values were compared by means of independent sample t-tests at a significance level of 0.05. Biofilm formation by the different bacterial strains and HGF cells interaction with the different implant materials in mono-culture experiments were compared by means of ANOVA. ANOVA was employed with Turkey’s HSD post-hoc test using SigmaPlot version 12.0 software (Systat Software, Inc, USA). If the normality test (Shapiro-Wilk) failed, Kruskal-Wallis one way analysis of variance on ranks was applied. A value of p <
0.05 was considered to be statistically significant. Outcomes of the race for the surface between the different strains and HGF cells were compared by means of ANOVA, based on the difference between cell surface coverage in absence and presence of adhering bacteria.

Results

Surface properties of dental implant materials

Water contact angles on the implant materials included in this study varied between 2 to 92 degrees across the different implant materials (Fig. 2). Across the Ti variants, machined titanium (Ti-M) was the most hydrophobic and the protectively packaged Ti-modMA the most hydrophilic. Ti in its M variant was more hydrophobic than TiZr alloy and ZrO₂ in their M variants. Machined acid-etched (MA) variants of Ti and ZrO₂ had similar water contact angles. For TiZr-modMA not the same level of hydrophilicity was found as for Ti-modMA, despite their similar protective packaging.

Polished Ti-P surfaces were the smoothest across the different implant materials (see also Fig. 2) with an Rₐ value less than 0.007 μm, followed by Ti, ZrO₂ and TiZr in their M variants all possessing a surface roughness around 0.1 μm. Acid-etching significantly (p < 0.0001) increased the roughness of Ti-MA and Ti-modMA, as well as of ZrO₂-MA surfaces to between 0.6 and 0.8 μm, while acid-etching of TiZr in its M variant caused only a minor increase in roughness to around 0.2 μm. Morphologically, polished surfaces appeared smooth, while machined ones were patterned with unidirectional grooves and acid-etched surfaces had irregular surface patterns, with voids and open spaces at the surface (see also Fig. 2).

All surfaces contained carbon as a major constituent (see Table 1), with modMA variants of Ti and TiZr containing the lowest amounts of carbon in correspondence with their low water contact angles. The carbon content of Ti-modMA was only slightly lower than of Ti-MA however, while also acid-etching of machined ZrO₂ reduced the carbon content of its surface to levels comparable to those of modMA variants of Ti and TiZr. Considering that decomposition of the O₁s photoelectron binding energy peak indicated that on average around 26% of oxygen was involved in bonds with contaminating carbon atoms, the ratio of titanium to oxygen can be calculated to be around 2.4. Thus the oxide skin on the materials was composed predominantly of TiO₂. TiZr alloys contained 4 to 5 times higher amounts of Ti than Zr, while based on the bulk composition of the alloy a ratio of Ti/Zr around 10 would be expected. Thus the surface of the TiZr alloy must be enriched with Zr. The ratio of zirconium to oxygen was significantly (p < 0.05) higher than 2 in the ZrO₂, but after correcting for oxygen bound to carbon it reduced to 1.8.
**Biofilm formation on different dental implant materials**

Biofilms of 24 h old on the different implants materials were all highly viable, which is in line with the known high biocompatibility of the materials used for dental implants. Significant differences existed in biovolumes across the different bacterial strains and materials included (see Fig. 3a). Averaging the biovolumes of all four bacterial strains included in this study, ANOVA reveals significant differences across the implant materials, that rank according to (see Fig. 3b):

\[
\text{Ti-modMA} = 1 \quad \text{Ti-MA} = 2 \quad \text{TiZr-modMA} = 3 \quad \text{TiZr-M} < 4 \quad \text{Ti-P} < 5 \quad \text{ZrO}_2\text{-M} = 7 \quad \text{ZrO}_2\text{-MA}
\]

in which the p-values are: \(1 > 0.9999; 2 0.8211; 3 > 0.9999; 4 0.0134; 5 0.9476; 6 0.0002; 7 > 0.9999.\)

Thus it can be concluded, that ZrO\(_2\) variants attract more biofilm than comparable variants of Ti or TiZr alloys. Across the four Ti variants, on both the most hydrophilic and roughest (Ti-modMA) as well as the more hydrophobic and roughest (Ti-MA) variant, the smallest amounts of biofilm were found.

**Adhesion, spreading and growth of HGF cells on different dental implant materials**

HGF cells adhered, spread and grew to almost full confluence on all implant surfaces (see Fig. 4) with surface coverages of between 80% to 90% and ANOVA revealed no significant differences between the different materials (see Fig. 5a), irrespective of whether culture medium was supplemented with THB or TSB.
Fig. 2 - Water contact angles and surface roughness values of differently modified Ti, TiZr alloys and ZrO$_2$ (zirconia) implant surfaces involved in this study together with scanning electron micrographs of the surfaces. Error bars indicate the standard deviations over measurements on three different samples of each material. The bar denotes 10 µm. Statistical comparisons were made between differently modified surfaces within the Ti (red lines), TiZr alloy (yellow lines) and ZrO$_2$ (blue lines) group, as well as between similarly
modified surfaces of different materials (black, purple and green lines). *# significantly (p < 0.007) different from all other surfaces in the same comparison.

Table 1. Elemental surface composition (at%) of differently modified Ti, TiZr alloys and ZrO₂ implant surfaces involved in this study. Standard deviations over measurements on two different samples of each material are presented within brackets.

<table>
<thead>
<tr>
<th>Material</th>
<th>%C</th>
<th>%Ti</th>
<th>%Zr</th>
<th>%O in oxide</th>
<th>%O other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ti-P</td>
<td>51.8 (0.4)</td>
<td>11.3 (0.3)</td>
<td>—</td>
<td>27.5 (0.2)</td>
<td>9.4 (0.1)</td>
</tr>
<tr>
<td>Ti-M</td>
<td>52.4 (3.0)</td>
<td>10.7 (0.3)</td>
<td>—</td>
<td>25.4 (0.1)</td>
<td>10.5 (1.8)</td>
</tr>
<tr>
<td>Ti-MA</td>
<td>43.7 (1.4)</td>
<td>14.1 (1.4)</td>
<td>—</td>
<td>30.9 (1.5)</td>
<td>10.2 (0.1)</td>
</tr>
<tr>
<td>Ti-modMA¹,²,³</td>
<td>42.0 (1.0)</td>
<td>10.9 (2.1)</td>
<td>—</td>
<td>27.3 (2.4)</td>
<td>9.2 (1.4)</td>
</tr>
<tr>
<td>TiZr-M</td>
<td>51.0 (0.1)</td>
<td>10.2 (0.2)</td>
<td>1.9 (0.1)</td>
<td>26.5(0.7)</td>
<td>10.4 (0.9)</td>
</tr>
<tr>
<td>TiZr-modMA¹,²</td>
<td>41.2 (3.6)</td>
<td>8.8 (0.3)</td>
<td>2.2 (0.0)</td>
<td>26.1 (0.2)</td>
<td>10.0 (0.4)</td>
</tr>
<tr>
<td>ZrO₂-M</td>
<td>49.8 (2.3)</td>
<td>—</td>
<td>11.6 (1.0)</td>
<td>21.4 (2.4)</td>
<td>12.8 (0.7)</td>
</tr>
<tr>
<td>ZrO₂-MA</td>
<td>44.9 (2.5)</td>
<td>—</td>
<td>16.2 (1.9)</td>
<td>27.6 (1.7)</td>
<td>11.3 (1.1)</td>
</tr>
</tbody>
</table>

¹ contains about 5% Na and Cl.
² contains 2% to 4% N.
³ contains 2% Ca.

Interestingly, the spread area per cell was around 3900 µm² on both modMA variants, while it was between 5200 µm² and 6600 µm² on other variants of Ti and TiZr alloys, corresponding with a lower number of cells found after 48 h on other variants of Ti and TiZr alloys. ZrO₂ implant materials showed an intermediate area per spread cell of around 4650 µm², regardless whether the surface was in its M or MA variant. Accordingly cell growth was largest on both modMA variants of Ti and TiZr alloys.
The race for the surface between oral bacteria and human gingival fibroblasts on different dental implant materials

In previous sections, we have compared biofilm formation and soft tissue integration of dental implant materials as two independent processes, while in reality tissue cells and bacteria compete for estate on an implant material. Therefore, we here studied the simultaneous interaction of HGF cells and oral bacteria on the different implant materials (see also Fig. 5a).

The presence of adhering bacteria reduced the coverage by HGF cells of the implant materials after 48 h of growth on all TiZr alloys and ZrO$_2$ surfaces, regardless of the strain involved. On Ti however, bacterial presence only reduced coverage by HGF cells on Ti-MA and Ti-modMA surface, but on polished Ti-P surfaces none of the strains were able to delay the HGF cells significantly in their race for the surface. S. oralis and S. aureus were able to cause a reduction in coverage by HGF cells only on Ti-M, which neither S. mitis nor S. salivarius could achieve.

Using the reduction in coverage by adhering HGF cells as a measure for the outcome of the race for the surface, and averaging over all four bacterial strains included (Fig. 5b), ANOVA reveals significant differences across the implant material that rank according to

\[
\text{Ti-P} < \text{Ti-M} < \text{Ti-MA} = \text{Ti-modMA} = \text{TiZr-M} = \text{TiZr-modMA} = \text{ZrO}_2\text{-M} = \text{ZrO}_2\text{-MA}
\]

in which the $p$-values are: $^1 0.0004; \; ^2 0.0004; \; ^3 0.9665; \; ^4 0.8260; \; ^5 0.4266; \; ^6 0.7768; \; ^7 0.6063$.

Transformed to the surface properties of the different implant materials, this implies that HGF cells have the biggest chance to win the race for the surface on the smoothest Ti variants.

Discussion

In this study we first assessed surface properties of Ti, TiZr alloys and ZrO$_2$ implant materials after different surface modifications, including protective packaging. Protective packaging of acid-etched surfaces provided the most hydrophilic surface, both for Ti and TiZr implant materials. The hydrophilicity of modMA variants could be attributed to a combination of increased surface roughness and low carbon contamination of the surfaces due to the protective packaging. Acid-etched surfaces that were not protectively packaged (MA variants), possessed only slightly higher amounts of carbon on their surfaces, but lacked the extreme hydrophilicity as indicated by water contact angles. Clearly, this is because the surface sensitivity of contact angle measurements (2 Å) exceeds the one of X-ray photoelectron spectroscopy (10-100 Å) analyses.
**Biofilm formation and tissue interactions in mono-culture studies**

Literature is equally indecisive on the role of hydrophobicity and roughness on biofilm formation as the results of the current study: biofilm formation is small on the most hydrophilic and rough (modMA) as well as the more hydrophobic and rough (MA) Ti variants. Several *in vitro* and *in vivo* studies pointed to an influence of surface roughness and/or hydrophobicity on bacterial colonization [1,19,20]. Albrektsson and Wennerberg [21] drew a more refined conclusion and identified three distinct ranges of surface roughness among oral implants with a surface roughness up to 0.5 µm (minimally rough, machined implants), moderately rough implants with a roughness between 1.0 and 2.0 µm and rough implant surface (roughness > 2 µm). Threshold surface roughness values of around 0.2 µm have been proposed above which roughness enhances oral biofilm formation. Most studies on oral biofilm formation distinguish between rough and smooth surfaces based on the roughness being above or below say 0.8 µm [22]. However, Bollen et al. [23] suggested that a threshold roughness for clinical application was only valid in combination with the material involved. With respect to materials, our study decisively showing more biofilm formation on ZrO₂, regardless of the roughness of its surface.

The surface coverage by HGF cells after 48 h of growth was not impacted by any of the surface properties measured. Surface roughness did not appear to influence tissue cell integration, probably because, according to the classification by Albrektsson and Wennerberg [21], all our implant surfaces can be considered as smooth or “minimally rough”. This supports the use of relatively smooth parts for the soft tissue seal as in modern implant systems [20,22,28], as compared to the smoothness of osseo-integratable parts.

**Co-culture studies on soft tissue integration versus biofilm formation**

The chance for HGF cells to win the race for the surface against oral sub-gingival bacteria and form a protective seal is significantly enhanced on smooth, unstructured Ti-P and Ti-M surfaces. Probably the larger size of HGF cells as compared to bacterial dimensions provides them with a larger foothold that allows cells to successfully compete with bacteria for estate on a smooth surface, whereas on rough surfaces bacteria can anchor themselves more firmly [29] in holes and crevices. Interestingly, streptococcal surface proteins have been described to stimulate the expression of β1-integrins in periodontal fibroblasts [30] which appears especially useful on smooth Ti surfaces to create additional footholds for the cells to compete with adhering bacteria. Note that also *Shigella flexneri* has been described to enforce mucosal host cell adhesion to the basement membrane by interacting with integrin-linked kinase [31]. Evidently, bacterial adhesion forces on the rough variants [29] are too strong to allow their displacement by HGF cells, regardless of
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(a) Graphs showing biovolume (μm²/μm²) for different strains of bacteria:
- *S. oralis*
- *S. mitis*
- *S. salivarius*
- *S. aureus*

(b) Graph showing biovolume (μm²/μm²) for different treatments:
- Controls (TP, TM)
- Treatment (T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11)
- Positive controls (Z0, Z0.5, Z1, Z2, Z3, Z4, Z5, Z6, Z7, Z8, Z9)

Significance levels indicated by symbols:
- *p < 0.05
- **p < 0.01
- ***p < 0.001

Comparison between groups using Dunnett’s T3 post-hoc test.
Soft tissue integration vs. supra-gingival biofilm formation

Fig. 3 – (a) Biovolumes of S. oralis J22, S. mitis BMS, S. salivarius HB and S. aureus ATCC 25923 biofilms after 24 h of growth on the different implant surfaces were calculated from the CLSM images using dedicated software. Error bars represent standard deviations over three experiments with different samples of each material and separately cultured bacteria. Statistical comparisons were made between differently modified surfaces within the Ti (red lines), TiZr alloy (yellow lines) and ZrO₂ (blue lines) group, as well as between similarly modified surfaces of different materials (black, purple and green lines). # significantly (p < 0.05) different from all other surfaces in the same comparison. * significantly (p < 0.003) different from Ti-P. (b) Average biovolumes of S. oralis J22, S. mitis BMS, S. salivarius HB and S. aureus ATCC 25923 biofilms after 24 h of growth on the different implant surfaces. Error bars represent standard deviations over all twelve separate experiments carried out in triplicate for each of the strains. Statistical comparisons were made between differently modified surfaces within the Ti (red lines), TiZr alloy (yellow lines) and ZrO₂ (blue lines) group, as well as between similarly modified surfaces of different materials (black, purple and green lines). # significantly (p < 0.05) different from all other surfaces in the same comparison.* significantly (p < 0.02) different from Ti-M.

whether integrin expression is stimulated by bacterial presence or not. In addition, bacteria residing in crevices on a rough surface can be overgrown in a first instance by tissue cells, and then slowly form a microcolony releasing sufficient amounts of endotoxin to harm adhering tissue cells. These two reasons explain why low numbers of contaminating bacteria do not negatively affect growth of HGF cells on smooth Ti surfaces, offering them a chance to win the race for the surface under clinical conditions where Integrin receptors have indeed been found to be necessary for soft tissue integration of Ti implant surfaces by periodontal ligament fibroblasts [32].

Virtues of tissue cell-bacteria co-culture studies

Although co-culture studies involving multiple tissue cell-lines have been described [33,34], this is the first study involving a co-culture of HGF cells and oral bacteria. The use of such co-cultures provides a better mimic of the clinical situation than mono-culture studies with either bacteria or cells, but bears some danger. The exact composition of modified culture media has to be chosen such that neither the cells nor the bacteria are put at too big a disadvantage with respect to each other. The current use of a modified medium containing bovine serum on the other hand, adds a clear advantage to our study with respect to its clinical relevance, as serum possesses a similar protein composition as crevicular fluid surrounding an implant under clinical conditions. Another important issue is the number of contaminating bacteria that are allowed to adhere prior to initiating the race for the surface. Increasing the number of contaminating bacteria above 3 × 10⁴ bacteria/cm² applied here, invariably yielded HGF cell death, indicating that the ability of
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HGF cells to survive bacterial presence is not unlimited. Consequently, we have to acknowledge that the outcome of co-culture studies involving bacteria and tissue cells depends on the number of contaminating bacteria prior to the start of the race for the surface. Thus the outcome of the race for the surface will depend on a multitude of factors. However, the advantages provided to either bacteria or tissue cells in our model, is equal on all different materials included, making this type of studies ideally suitable for the comparison of different single factors, such as the materials choice in a given application.

Fig. 4 - Examples of fluorescence images of immuno-cyto-stained HGF cells after 48 h of growth on the different implant materials. Bar marker denotes 100 µm.
The main conclusion of this study, that could never have been reached in mono-culture studies, is that HGF cells are able to withstand low levels of bacterial contamination on smooth Ti implant surfaces. The larger size of HGF cells as compared to bacterial dimensions provides them with a larger foothold that allows cells to successfully compete with bacteria on a smooth surface, whereas on rough surfaces bacteria can anchor themselves more firmly.

However, the true value of co-culture models can only be estimated by comparison of its results with clinical data. Clinical data aimed to reveal the relevant implant properties for the prevention of peri-implantitis are scarce and mostly include only a limited number of different implant types. A recent multi-center study including 281 patients and six different implant types concluded that osseo-integration alone is not a sufficient condition to ensure long-term implant survival and that peri-implantitis develops earlier in implants with rough surfaces [17], which is in line with the conclusions from our co-culture study. Despite the multi-center nature of their study representing the largest clinical and microbiological analysis of patients with peri-implantitis, these authors cautioned for over-interpretation of their results and called for the need of prospective, multi-center studies. Such studies are difficult, if not impossible to carry out because they require huge numbers of patients to be monitored over long time-intervals up to several decades.

This makes the design of in vitro methods of utmost importance, moreover since the oral microflora in animals is so different from the human one [35] that it invalidates the results of animal studies into implant failure due to infection. Our co-culture study indicates that the protective seal constituted by fibroblast tissue around the abutment, has a better chance to form in competition with oral supra-gingival bacteria on smooth than on rough Ti surfaces. This coincides with the conclusions drawn from clinical studies [17]. Therewith our study not only supports clinical data, but clinical data also support our co-culture model although admittedly the number of available clinical studies to support our co-culture model is low. Relevant for future studies is that neither in vitro biofilm studies nor in vitro studies into adhesion, spreading and growth of tissue cells on different implant surfaces in a mono-culture mode yield clinically meaningful conclusions on implant surface properties relevant for early failure of dental implants due to infection in patients, as bacteria and tissue cells interact during their race for the surface.

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