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An extended spectrum bactericidal titanium dioxide (TiO_2) coating for metallic implants: in vitro effectiveness against MRSA and mechanical properties

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Abstract Implant infections remain feared and severe complications after total joint arthroplasty. The incidence of multi-resistant pathogens, causing such infections, is rising continuously, and orthopaedic surgeons are confronted with an ever-changing resistance pattern. Anti-infectious surface coatings aim for a high local effective concentration and a low systemic toxicity at the same time. Antibacterial efficacy and biomechanical stability of a novel broad-spectrum anti-infectious coating is assessed in the present study. Antibacterial efficacy of a sol–gel derived titanium dioxide (TiO_2) coating for metal implants with and without integrated copper ions as antibiotic agent was assessed against methicillin resistant *Staphylococcus aureus* (MRSA 27065). Both bacterial surface adhesion and growth of planktonic bacteria were assessed with bare and various TiO_2-coated Ti6Al4V metal discs. Furthermore, bonding strength of the TiO_2 surface coating, using standard testing procedures, as well as surface roughness were determined. We found a significant reduction of the bacterial growth rate for the coatings with integrated copper ions, with highest reduction rates observed for a four-fold copper TiO_2-coating. Pure TiO_2 without integrated copper ions did not reduce bacterial growth compared to uncoated Ti6Al4V. The coating was not detached from the substrate by standard adhesive failure testing, which indicated an excellent durability of the implant coating. The TiO_2 coating with integrated copper ions could offer a new strategy for preventing implant-associated infections, with antibacterial properties not only against the most common bacteria causing implant infections but also against multi-resistant strains such as MRSA.

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1 Introduction

Ever since the introduction and clinical establishment of total joint replacement, endoprostheses have helped to improve the quality of life of thousands of patients. Nowadays joint replacement is an indispensable component of modern medicine. Especially total hip replacement (THR) is considered to be one of the most successful performable operations. Approximately 1,500,000 primary total hip replacements and additionally about 820,000 primary total knee replacements (TKR) are performed annually worldwide. Despite aseptic operation conditions and perioperative antibiotic prophylaxis, an implant infection remains a feared complication after joint replacement. Infection rates are reported to range between 0.5 and 2% after primary THR [1, 2] and approximately 1% after primary TKR [2]. Implant associated infections occur even more often after revision hip arthroplasty [3]. Furthermore, for the prosthetic reconstruction after the resection of bone tumours, infection rates of up to 35% can be found in literature [4], due to large implants and immunosuppression by adjuvant radiotherapy and chemotherapy.

With the implantation of the biomaterial, a so-called "race for the surface" between bacteria and host tissue

cells begins, which is considered vital for the further fate of the implant [5]. Microbial adhesion triggers a pathogenic sequence and thus results in a resistance against host defence mechanisms [6]. Surface-adherent bacteria may then proliferate and form a bacterial biofilm, which acts as a barrier inhibiting the penetration of bactericidal agents (antibiotics and immune defence). In consequence, a foreign-body triggered infection may, in many cases, not be cured without the removal of the infected device [1]. In general, infections associated with biomaterials have huge clinical and economic implications. The additional average costs of an infected joint prosthesis, for both medical and surgical treatment, are estimated to be US $30,000. In total, roughly US $1.8 billion additional costs are caused by infected joint prosthesis and fracture fixation devices per year in the US [7].

The ability of biofilm formation is considered to be a major factor that influences the pathogenicity of a number of organisms. This especially accounts for staphylococci [8]. Thus *Staphylococcus aureus (S. aureus)* and coagulase-negative staphylococci are the most frequent pathogens isolated from infected joint prostheses [9]. Recently, the implications of a changing pattern of bacterial infections following total joint replacement have been published. A definitive increase of multiple-drug resistant pathogens was hereby observed, with methicillin-resistant *Staphylococcus aureus* (MRSA) being the most common. Up to 46% of the positive cultures in revision hip surgery as well as in revision knee surgery revealed MRSA as the pathogen in single studies. All MRSA associated infections finally showed chronic sepsis [10]. The problems associated with infected medical devices in orthopaedic surgery, especially with resistant bacteria, thus display the necessity of further research and development of alternative treatment and prevention strategies, such as ion based anti-bacterial surfaces [4, 11] as metal ions possess broad biocidal properties [12].

The aim of the present in vitro study was to evaluate the bactericidal effect of a titanium dioxide coating against methicilin-resistant *S. aureus* as well as its mechanical properties. The investigated coating has previously shown both good cytocompatibility and effectiveness against antibiotic susceptible micro-organisms [13].

2 Materials and methods

2.1 In vitro tests with MRSA 27065

The specimens used for the microbiological investigations were small discs (diameter = 14.5 mm, thickness = 0.95 mm) made of titanium alloy (Ti6Al4V), a standard implant material. The discs were coated with a sol–gel derived titanium dioxide surface coating (TiO_2). The following modified TiO_2 coatings were applied:

- a single pure TiO_2-coating,
- a single TiO_2-coating with integrated copper ions ($1 \times$ Cu-TiO_2),
- a twofold TiO_2-coating with integrated copper ions ($2 \times$ Cu-TiO_2),
- a threefold TiO_2-coating with integrated copper ions ($3 \times$ Cu-TiO_2),
- and a fourfold TiO_2-coating with integrated copper ions ($4 \times$ Cu-TiO_2).

The coatings were applied to the titanium discs in a dip-coating procedure (dipping speed: 1.5 mm/s, immersion time: 20 s) as previously described [13, 14]. Copper ions were incorporated into the sol by cold saturation with copper-(II)-acetate monohydrate. After drying of the sol film at room temperature, calcification was performed in a furnace at 500°C in air. The multilayer-coated samples were produced during a repetitive dip-coating procedure. Hence, greater amounts of copper ions can be provided by the multilayer-coated samples, i.e. during repetitive dip-coating procedures.

Prior to the microbiological investigations, the samples were disinfected under ultraviolet (UV) light (590 nm) for 2 h. MRSA 27065 isolated from a patient with an implant-associated infection was used as the pathogen for the microbiological tests. Bacterial colonization of the discs was studied with an assay modified after Christensen et al. [13, 15] with a focus put on microbial proliferation and survival. According to Christensen et al. [15] we therefore used low inocula and long incubation periods under nutritive conditions. The microtiter plate method is hereby described as expedient, inexpensive and reliable [15]. In brief, uncoated and coated Ti6Al4V specimens ($n = 6$) were immersed in 1 ml of growth medium (Gibco™ RPMI 1640 + 10% Gibco™ Fetal Calf Sera (FCS) Invitrogen, New York, USA) containing 1.0×10^5 colony forming units (cfu) of *S. aureus* MRSA 27065 and incubated at 37°C. After 24 h, 200 µl of the incubation fluid were removed and supplemented with 200 µl of a neutralizing solution [16] to stop any further bactericidal action of the copper ions. Serial dilutions of the incubation fluid were hence placed on Mueller–Hinton agar plates and incubated at 37°C for 48 h. After 48 h, the cfu were quantified using a direct counting method. The remaining 800 µl of the incubation fluid were discarded and the metal discs carefully rinsed. Afterwards the colonized metal discs were transferred to vials containing 10 ml of saline solution and sonicated for 7 min (Sonorex RK255H, Bandelin Electronic, Berlin, Germany) to remove the adhering bacteria. This method of sonication has been described as precise, sensitive and with a wide applicability [15]. Furthermore

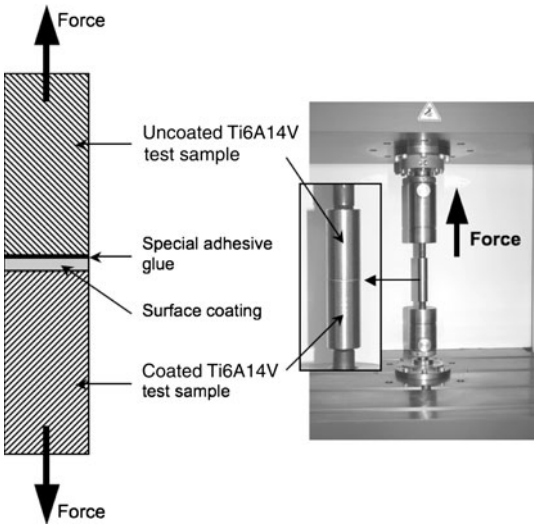

Fig. 1 Test setup for the standard adhesive test

complete and reproducible detachment of bacteria has been described for sonication [17]. Serial dilutions of each sample were placed on Mueller–Hinton agar plates and the cfu were again quantified via the direct counting method after 48 h incubation.

Means and standard deviations were applied for the obtained results and evaluated for statistical significance using non-parametric methods (Kruskal–Wallis and Mann–Whitney test) and the method of closed testing procedures [18], with "$P < 0.05$" being considered significant.

2.2 Mechanical investigations

The adhesive strength of surface coatings can be determined by different methods. According to Fritsche et al. [19] the arbor-bending tests, scratch-tests and standard adhesive tests in conformity with DIN EN 582 (Fig. 1) were carried out using titanium specimens with a 4 × Cu-TiO_2 coating ($n = 3$). Using a surface measuring instrument (Hommel Tester T8000, Hommel-Etamic GmbH, Schwenningen, Germany) the surface roughness of the specimens was recorded. In addition, a hydroxyapatite (HA, Bonit®) coated titanium alloy (Ti6Al4V) was also investigated, in order to be able to correlate the assessed data for the 4 × Cu-TiO_2-coating to a clinically approved bioactive coating.

2.3 Kinetics of copper ion release from the surface

In order to determine the kinetics of the copper ion release from the modified TiO_2-coating, absorption spectroscopy was carried out. Small discs, as used previously in the microbiological investigations, were incubated in 1 ml of growth medium (Gibco™ RPMI 1640 + 10% Gibco™ Fetal Calf Sera (FCS) Invitrogen, New York, USA) for 24 h. As for the microbiological investigations, we used Ti6Al4V discs, as well as TiO_2, 1 × Cu-TiO_2, 2 × Cu-TiO_2, 3 × Cu-TiO_2 and 4 × Cu-TiO_2 coated discs to obtain the copper ion release. Copper ion concentration in mmol/l as well as the standard deviation were then obtained and compared.

3 Results

3.1 Impact of copper ions on adhering bacteria

Pure TiO_2-coating of the discs did not decrease the bacterial growth in comparison to the uncoated Ti6Al4V discs ($P = 0.937$). The integration of copper ions however was followed by a significant decrease of the bacterial growth on the coated discs in comparison to the pure Ti6Al4V discs ($P = 0.002$). The reduction of the adhering bacteria was most distinct for the samples with the 4 × Cu-TiO_2 coating causing a reduction of viable bacteria on the samples by six logarithmic levels compared to the uncoated metal discs ($P = 0.002$). The 4 × TiO_2 discs furthermore exhibited a significantly stronger bactericidal effect than 1 × Cu-TiO_2 and 2 × Cu-TiO_2 discs ($P = 0.002$). The observed reduction of the bacterial growth on the fourfold coated discs (4 × TiO_2) was more obvious in comparison with the threefold coated discs (3 × Cu-TiO_2), however with the lack of statistical significance ($P = 0.485$) (Fig. 2a).

3.2 Impact of released copper ions on planktonic bacteria in the supernatant

As for the adhering bacteria, no reduction of the planktonic bacteria was observed by the pure TiO_2-coating in comparison to the uncoated Ti6Al4V discs ($P = 0.485$). The integration of copper ions caused a highly significant reduction of bacterial growth in the supernatant ($P = 0.002$). Again, strongest reduction of bacterial growth was observed for 4 × $CuTiO_2$. The stronger bactericidal effect of the fourfold-coated discs was highly significant in comparison to the mono- and twofold-coated discs ($P = 0.002$) and more pronounced, but not statistically significant compared to the threefold-coated discs ($P = 0.699$) (Fig. 2b).

3.3 Mechanical properties

The arbor-bending-tests revealed neither cracks nor chipping off from the substrate using the 4 × Cu-TiO_2 coating, whereas the HA-coating showed clear cracks and coating detachment in the sense of adhesive failure. Neither

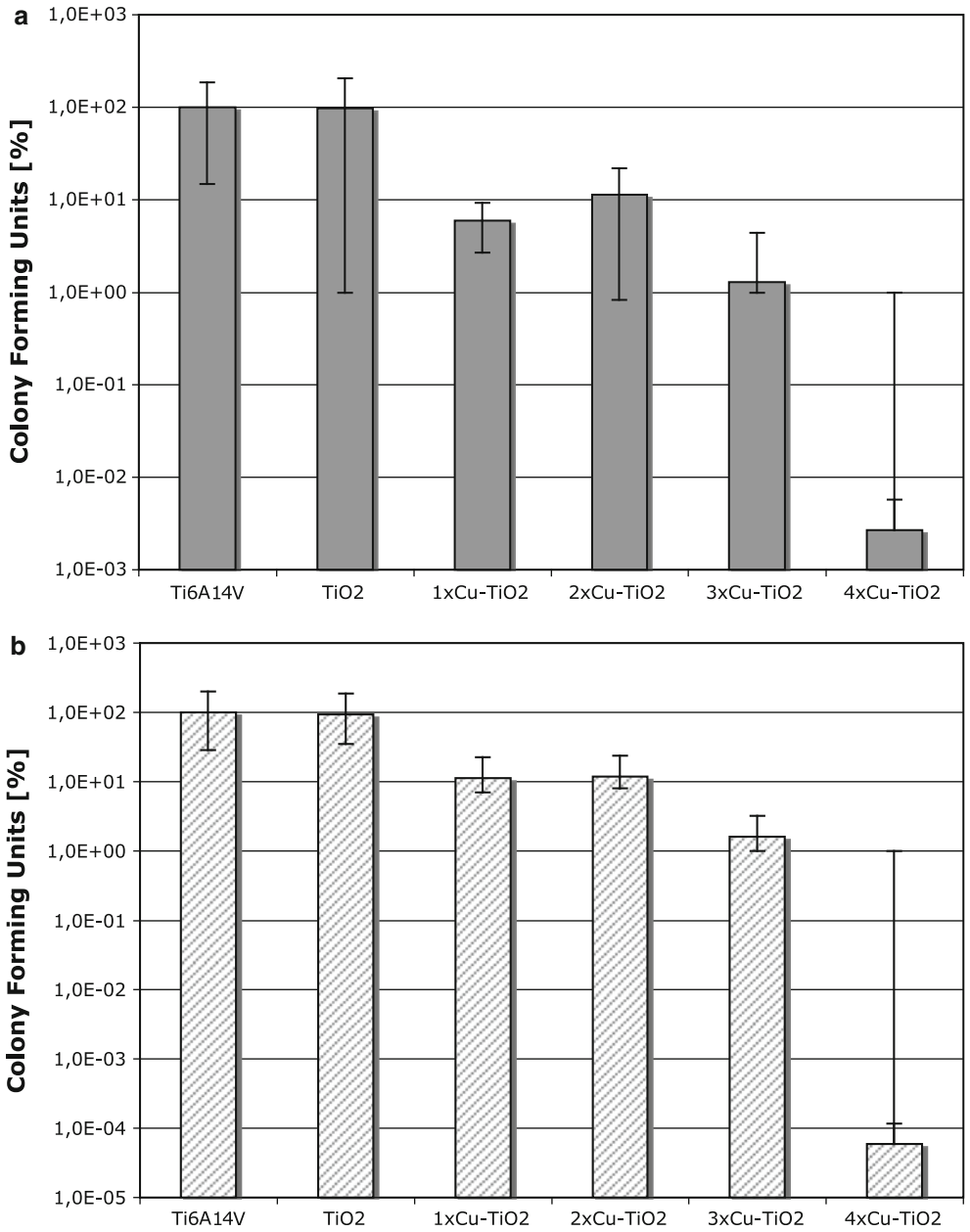

Fig. 2 **a**, **b** Growth of MRSA 27065 (Colony forming units in percent) on Cu-TiO_2-coated and uncoated Ti6Al4V surfaces (**a**) and in the supernatant growth medium (**b**) (RPMI 1640 + 10% FCS)

chipping nor coating penetration could be determined in the scratch-test of the 4 × Cu-TiO_2-coating for axial loads less than 65 N (Fig. 3). The HA-coating also showed no chipping, but the scratching pin penetrated the coating at a load of 5 N (Fig. 3). The standard adhesive strength testing of the 4 × Cu-TiO_2-coating resulted in an average of 89.6 N/mm^2 ($n = 3$), and was ended due to failure of the adhesive glue in all samples. Detachment of the TiO_2-coatings from the Ti6Al4V specimens was not achieved, which indicates that solely the bonding strength of the adhesive glue was overcome. The HA-coating however revealed an average adhesive strength of 32.3 N/mm^2 ($n = 3$), which is less than the bonding strength of the adhesive glue. Complete removal of the HA-coating from the substrate was observed, indicating adhesive failure of the coating.

A roughness (R_z) of 14.3 and 9.6 μm was measured for the uncoated und the 4 × Cu-TiO_2 coated specimens

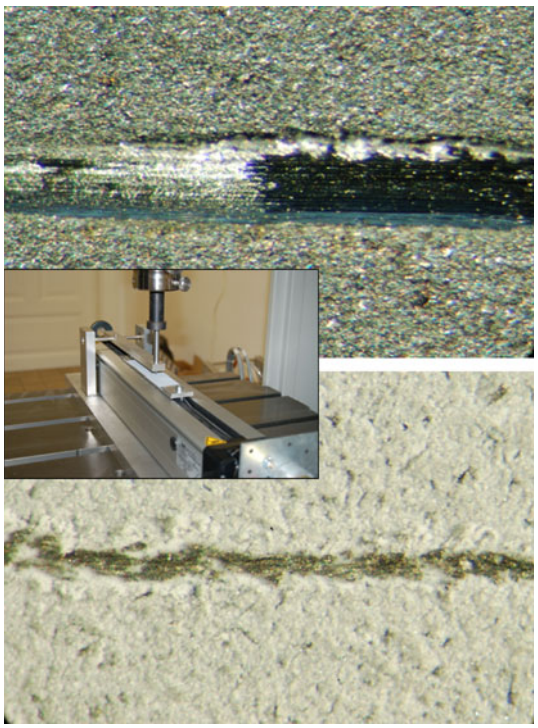

Fig. 3 Results of the scratch test at 100× magnification for the Cu-TiO_2-coating at 65 N (*top*) and HA-coating at 5 N (*bottom*)

respectively. Because of the highly random build up of HA crystals in the HA-coating, its surface roughness varies a great deal and can therefore not be determined properly.

3.4 Kinetics of copper ion release from the surface

Copper ions measured within the growth medium of the pure TiO_2 discs were similarly low as from the uncoated Ti6Al4V discs. The integration of copper ions however was followed by an observable increase of copper ions within the growth medium. The measured copper concentration was hereby following an almost linear increase from the 1 × Cu-TiO_2 via the 2 × and 3 × -Cu-TiO_2 to the 4 × Cu-TiO_2 coating (Fig. 4).

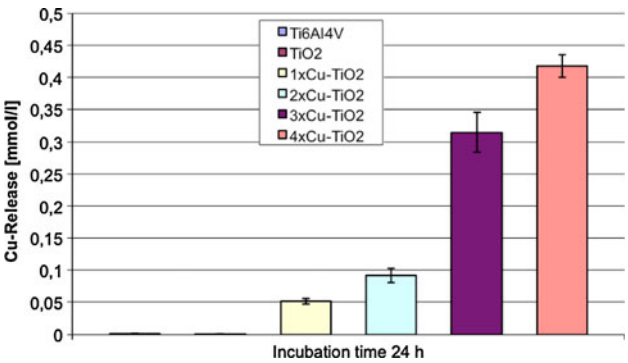

Fig. 4 Copper ion concentration (mmol/l) within the growth medium (RPMI 1640 + 10% FCS) after 24 h of incubation

4 Discussion

Antibiotics and immune cells reach the implant surface only by diffusion, since implants are not vascularized [20]. Many different attempts in order to modify the implant surface, regarding a potential reduction of implant-associated infections have been put forward [21]. Despite many promising approaches of surface alteration, no final breakthrough has been achieved so far.

Generally, anti-infectious surface coatings require a high local effective concentration and a low systemic toxicity at the same time. They are either to be considered anti-adhesive or antimicrobial. Anti-adhesive surface coatings aim to inhibit the initial adherence of bacteria, whereas antimicrobial surface coatings aim to reduce the bacterial growth rate. In both cases however, the bony in-growth of the implant must not be jeopardized [21].

Some implants which are associated with a more frequent rate of infection, like central venous catheters and urinary catheters, have been equipped with modified surfaces meeting the requirements for clinical application [4, 21, 22]. However, the development of antibiotic containing surfaces encounters the problem of changing resistance patterns of bacteria, and thus reflects the necessity to develop alternative antibacterial strategies.

The bactericidal effect of metal ions, especially copper and silver ions, is a well known phenomenon, which has been recognized for centuries [23]. The bactericidal effect of metal ions is due to the so called oligodynamic effect (oligo- little, small; dynamic- force), which is considered to be the noxious effect of tiny amounts of metal ions against living cells [24]. On a molecular scale the exposure of micro-organisms to copper may lead to a decline in membrane integrity and eventually cell death [12]. Furthermore, copper can facilitate hydrolysis or nucleophilic displacement of cell organelles [25]. Copper may also form complexes with proteins or alter the protein structure such that proteins cannot perform their normal function, which results in either cell death or viral inactivation. Due to a specific affinity to the DNA, copper also possesses the ability to break hydrogen bonds within the DNA, thus opening the double helix by cross-linking within the strands [12, 25].

Even though silver ions are particularly used in current clinical applications, Heidenau et al. demonstrated copper to posses the most favourable ratio of antibacterial effectiveness and cell toxicity, and hence proves most adequate to be applied to implant surfaces as an antibacterial agent, without decreasing the biocompatibility in a significant

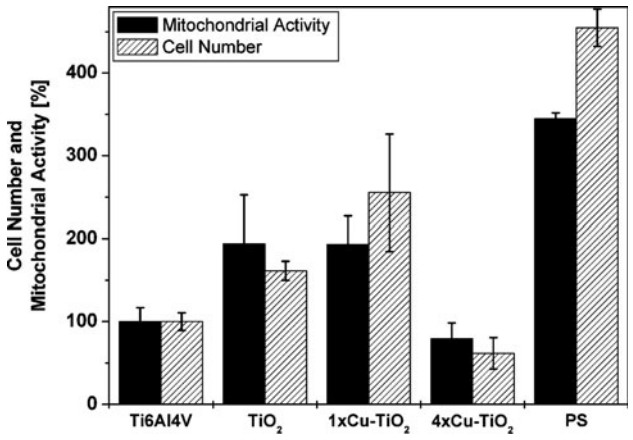

Fig. 5 Growth and mitochondrial activity of osteoblast-like cells MC3T3-E1 on uncoated and coated Ti6Al4V surfaces after 48 h in culture. (Reproduced with kind permission from Heidenau et al. [13])

manner [13]. This was proofed by evaluating growth inhibition tests of bacteria and tissue fibroblasts under corresponding conditions [13]. The sol–gel-derived TiO_2-coating initially aimed for and demonstrated an improvement of the biocompatibility of titanium implants [11]. It also previously proved to possess a good in vitro cytocompatibility [11]. Furthermore, in a recent study, the copper loaded TiO_2-coating revealed significant in vitro bactericidal effectiveness against common sensitive pathogens (*S. aureus* ATCC 25923) with retained excellent cytocompatibility [13]. Hereby, mitochondrial activity as well as cell proliferation on uncoated and coated discs was determined. It was concluded, that TiO_2 and 1 × Cu-TiO_2 discs lead to an increase of both, mitochondrial activity as well as cell number in comparison to uncoated Ti6Al4V coated discs where the results obtained for the 4 × Cu-TiO_2 coated discs where still within the range of the uncoated Ti6Al4V alloy (Fig. 5) [13]. Our current results complement this previous study, as we were able to show the significant dose-dependent bactericidal effect of the copper loaded TiO_2-coating also against a multi-resistant pathogen (MRSA 27065). This extended spectrum bactericidal effect becomes of particular significance, since an increase of MRSA associated orthopaedic implant infections has been reported [10] and a further increase of resistant bacteria isolated from total joint infections is anticipated [26]. Furthermore, copper ions not only possess an anti-microbial effect against bacteria, but also against viruses [12, 25], fungi [12] and other microorganisms [12]. This theoretically leads to a potential antimicrobial effect of this novel TiO_2-coating with integrated copper ions, not only against the most common bacteria and more resistant bacteria associated with orthopaedic implant infections, but also against different pathogens such as fungi which are also reported to cause orthopaedic implant infections [1].

The present study moreover demonstrated that the mechanical properties of this coating fulfil the requirements for a clinical application on orthopaedic implants. The adhesive bonding strength to the Ti6Al4V substrate was by far superior to a HA-coating, which is a non-permanent surface modification and dissolved by the tissue after a certain period of time. Thus, the HA-coating does not need to be as adhesive to the substrate as Cu-TiO_2. According to ASTM standard 1147-F the minimum required adhesive strength for medical implant coatings is 22 N/mm^2 which was accomplished by the tested coatings. Although surface roughness of the TiAl4V discs was reduced after TiO_2 coating, the Cu-TiO_2 coated discs still showed significant surface roughness and similar surface microstructure to corundum-blasted Ti6Al4V surfaces, which indicates a favourable environment for bone ongrowth and cell attachment [27]. All in all, improved adhesion of osteoblast-like cells to TiO_2-coated surfaces has been demonstrated in previous investigations (Fig. 5) [13].

5 Conclusion

The novel antibacterial TiO_2-coating for metal implants was evaluated in vitro regarding a bactericidal effect against antibiotic resistant strains. The integration of copper ions into the coating was followed by a highly significant reduction of bacterial growth of MRSA, both on the implant surface as well as in the supernatant. The observed reduction of bacterial growth was up to $6\log_{10}$ for the fourfold Cu-TiO_2-coating. In addition, the coating demonstrated to possess outstanding mechanical properties.

The investigations of this paper lead to the conclusion that this TiO_2-coating with integrated copper ions, previously proven to be cytocompatible, could offer a broad-spectrum antibacterial prophylaxis against implant-associated infections. This may be particularly interesting for indications such as revision or tumour arthroplasty, which are associated with a higher rate of infection and a compromised immune system.

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