JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE **16** (2005) 883–888

A novel antibacterial titania coating: Metal ion toxicity and *in vitro* surface colonization

F. HEIDENAU[1], W. MITTELMEIER[3], R. DETSCH[2], M. HAENLE[3], F. STENZEL[1], G. ZIEGLER[1,2], H. GOLLWITZER[2,3]
[1]*BioCer EntwicklungsGmbH, Ludwig-Thoma-Straße 36c, 95447 Bayreuth, Germany*
[2]*Friedrich-Baur-Research Institute for Biomaterials, University of Bayreuth, 95440 Bayreuth, Germany*
[3]*Klinik und Poliklinik für Orthopädie und Sportorthopädie der Technischen Universität München, 81675 München, Germany*

Postoperative implant-associated infection is still an unresolved and serious complication in modern surgery. Antibacterial and biocompatible surfaces could both reduce infection rates and promote tissue integration. In this respect, a comparative study of the antibacterial as well as the biocompatible potential of different metal ions *in vitro* is presented. The assays used were growth inhibition tests with different metal salts carried out with tissue cells and bacteria under corresponding culture conditions. Additionally, *in vitro* tests in direct surface contact with tissue cells and bacteria onto a novel copper containing sol-gel derived titanium dioxide coating (Cu-TiO_2) and a fourfold Cu-TiO_2 coating were performed. The values were compared to a non-filled titanium dioxide coating and standard Ti6Al4V alloy. SEM-investigations were performed to approve the results of the *in vitro* tests.

Among Ag^+, Zn^{2+}, Co^{2+}, Al^{3+} and Hg^{2+}, the growth inhibition tests revealed an outstanding position of copper ions as antibacterial but nevertheless bio-tolerant additive. These results were affirmed by the cell tests in direct surface contact and SEM-investigations, where best cell growth was found on the Cu-TiO_2 coatings. Highest antibacterial properties with a tolerable cytocompatibility could be observed on the fourfold Cu-TiO_2 coatings.

Consequently, surfaces with custom-tailored antibacterial properties may be established and could be of particular interest in revision and tumor arthroplasty.

1. Introduction

Biomaterials represent an essential component of modern medical therapy and helped significantly to improve quality of life of thousands of patients. Especially in orthopedic surgery, inauguration of biomaterials in fracture treatment and joint replacement experienced an impressive success story, barely seen for any other medical application. However, biomaterial-associated complications like implant loosening and infection still limit the unrestricted application of biomaterials in daily clinical practice. An effective prophylaxis could be achieved by antibacterial surface modifications preventing implant colonization by microorganisms and formation of bacterial biofilm. In this respect, it is very important not to enforce antibacterial properties of biomaterials at the expense of biocompatibility. Depletion of the oxidative burst of macrophages and local impairment of immune response by medical implants is a well-recognized phenomenon [1, 2]. High biocompatibility of the implant is therefore crucial both for successful implant integration and for prevention of infection by reducing the surrounding "immuno-incompetent fibro-inflammatory zone" [2].

Various investigations have been performed to develop antibacterial surface coatings. Both anti-adhesive and antibacterial surfaces with integrated antibiotics, antiseptics or metal ions have been studied extensively [3–8] and especially antibacterial surfaces with integrated silver have been reported [9, 10]. However, biocompatibility of most of these anti-infective surfaces is still uncertain and needs to be clarified [11].

In the present paper, a comparative study about the antibacterial potential of different metal ions with respect to both cytotoxicity and antibacterial properties is demonstrated. Based on these results, a biocompatible titania surface coating with antibacterial properties was evaluated for its application as implant coating.

2. Material and methods

2.1. Growth inhibition tests with metal ions

Growth inhibition of bacteria and tissue cells by different metal ions was evaluated under corresponding

conditions to compare antibacterial properties and cytotoxicity. Consequently, all test parameters (incubation time, temperature, vials, cell counts, growth medium, etc.) corresponded between bacteria and tissue cell testing.

Growth inhibition tests with tissue cells
Mouse connective tissue fibroblasts (L929, DSMZ GmbH, Braunschweig, Germany) were used for the growth inhibition assays. 100.000 cells were precultured in polystyrene culture plates (Techno Plastic Products, Trasadingen, Switzerland) with 5% CO_2 in 1 ml RPMI 1640 (Gibco Vitrogen Corporation, New York, US) supplemented with 10% (v/v) of fetal calf serum (FCS, Life Technologies, Grand Island, US). After 4 h, culture medium was removed and fresh culture medium with the different concentrations of metal salts was added.

Cell proliferation with serial dilutions of different metal salts ($AgNO_3$, $HgCl_2$, $CuCl_2 \times 2H_2O$, $ZnCl_2$, $AlCl_3 \times 6H_2O$, $Co(C_2H_3O_2)_2 \times 4H_2O$) was determined by quantification of the adherent cells after 24 h. Therefore, attached cells were trypsinized with 300 μl of an aqueous solution containing 0.25% (v/v) trypsin and 0.5 mM EDTA (Sigma, Munich, Germany). The enzymatic reaction was stopped with 700 μl of RPMI 1640 with 10% FCS and the cell number was measured with a cell-counter (Coulter Z2, Beckman, Krefeld, Germany). Cell mitochondrial activity as an indirect marker of cell vitality was investigated with the WST-1 test assay (Roche, Basel, Switzerland), measuring the reduction of a tetrazolium salt to formazan. Briefly, tissue culture plates with the attached fibroblasts were washed with 1 ml of Dulbecco's phosphate buffered saline (PBS, Gibco Vitrogen Corporation, New York, US) by careful rinsing to remove non-adherent cells followed by incubation in a mixture of 1 ml RPMI 1640 with 10% FCS and 10 μl WST-1 '2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt' for 75 min. After complete dissolution of the accumulated formazan in the culture medium, the amount of formazan was quantified with an UV-Vis spectrometer (λ = 430 nm, 690 nm, DU 640, Beckman, Krefeld, Germany).

Growth inhibition tests with bacteria
Similar to the growth inhibition tests with fibroblasts, inhibition of bacterial growth was evaluated with *Staphylococcus epidermidis* ATCC 35984. Test strains were cultured in trypticase soy broth (TSB, Difco Laboratories, Sparks, US) at 37 °C overnight before testing. Bacterial cells were then harvested by centrifugation, washed twice in PBS, resuspended in normal saline and adjusted by densitometry to a MacFarland 0.5 standard. Test strains were then added to RPMI 1640 containing 10% (v/v) FCS and a bacterial concentration of 1.0×10^5 cfu/ml (colony forming units) in the growth medium was reached. Aliquots of this suspension were plated in serial dilutions at Mueller-Hinton agar plates to control inoculum counts.

Serial dilutions of the above mentioned metal salts were also added to the infected growth media and incubated for 24 h. A neutralizing solution, previously described by Tilton and Rosenberg [12] with a concentration of 1.0 g sodium thioglycolate and 1.46 g sodium thiosulfate in 1,000 ml deionized water, was added to all growth media after the incubation period to inhibit reminiscent metal toxicity on bacteria. Serial dilutions of each sample were plated on Mueller-Hinton agar plates and cfu were quantified after incubation for 48 h (37 °C).

2.2. Preparation of the antibacterial modified titania coating

Ti6Al4V round metal plates (diameter 14.5 mm, thickness 1.5 mm, Goodfellow GmbH, Nauheim, Germany) were used for the colonization studies and scanning electron microscopy (SEM) examinations. Prior to the coating procedure, the specimens were washed, sonicated in dry ethanol for 2 min and dried with cyclohexane and acetone. The metal organic sol was a mixture of tetrabutoxytitanate with n-butanol and has been described earlier [13]. Copper ions were incorporated into the sol by cold saturation with copper-(II)-acetate monohydrate (Merck, Darmstadt, Germany) [14, 15]. The samples were coated by a dip coating procedure (dipping speed: 1.5 mm/s, immersion time: 20 s). Hydrolysis of the sol combined with film formation was initiated by moisture in a controlled atmosphere (40% humidity, 25 °C). After drying the sol film at room temperature for 1 h, calcination was performed in a preheated furnace at 500 °C in air.

2.3. Surface colonization studies with tissue cells and bacteria

The specimens used for the surface colonization studies were uncoated Ti6Al4V plates (Ti6Al4V) as reference material, titanium dioxide coated Ti6Al4V plates (TiO_2), Ti6Al4V plates with a single TiO_2-coating with integrated copper ions (Cu-TiO_2) and Ti6Al4V plates with four TiO_2-coatings with integrated copper ions (4 × Cu-TiO_2). Accordingly, greater amounts of copper ions were provided by the multilayer-coated samples. Prior to colonization, the samples were cleaned in an ultrasonic bath with (1) distilled water, (2) sodium lauryl sulphate solution (2.5% v/v SDS, Life Technologies, Karlsruhe, Germany), (3) extran solution (5% v/v; Sigma, Munich, Germany), and dried with isopropanol. Afterwards, the samples were disinfected under UV light (590 nm) for 2 h.

In vitro tests with tissue cells in direct surface contact
Embryo calvaria mouse osteoblast-like cells (MC3T3-E1, DSMZ, Heidelberg, Germany) were used for the cytocompatibility studies. MC3T3-E1 cells were cultured in alpha-minimal essential medium (α-MEM, Life Technologies, UK) in humidified air at 37 °C (5% CO_2). Additionally, 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate, 1 vol% penstrep (Sigma, Germany)

and 5% (v/v) fetal calf serum (FCS, Life Technologies, UK) were added. Metal specimens of each sample group ($n = 4$) were placed in 24-well culture plates and 10×10^4 cells suspended in 1 ml α-MEM were added. Similar to the growth inhibition assays, WST-1 test was used to investigate cell vitality after 48 h of cultivation.

Cell proliferation of MC3T3-E1 cells on uncoated and coated Ti6Al4V specimens was also determined. Attached cells were trypsinized with 300 μl of an aqueous solution containing 0.25 vol% trypsin and 0.5 mM ethylenediaminetetraacetic acid (EDTA, Sigma, Germany). Subsequently, the number of cells was measured with a cell counter (Coulter Z2, Beckman, Germany).

In vitro tests with bacteria in direct surface contact

Parameters of the bacterial colonization studies strictly corresponded to the conditions applied to the tissue cell cultures. Uncoated and coated *Ti6Al4V* specimens were colonized by *Staphylococcus aureus* ATCC 25923 in α-MEM with 20% (v/v) FCS. Adhesion of bacteria to uncoated and coated surfaces was studied with an assay previously described by Christensen *et al*. [16] with minor modifications. Briefly, bacterial suspensions were prepared routinely as described above. Uncoated and coated metal specimens were immersed in 1 ml of the growth medium containing 1.0×10^5 cfu of *S. aureus* ATCC 25923 and incubated at 37 °C. After 24 h, incubation fluid was removed and supplemented with 1 ml of the neutralizing solution [12] to stop antibacterial action. Serial dilutions of the incubation fluid were plated on Mueller-Hinton agar plates and incubated at 37 °C for 48 h. Thereafter, cfu were quantified to evaluate antibacterial effectiveness of released metal ions in the supernatant growth medium.

Colonized metal plates were carefully rinsed, transferred to vials containing 10 ml of normal saline solution and sonicated for 7 min (Sonorex RK255H, Bandelin Electronic, Berlin, Germany) to remove adhering bacteria. Complete detachment of the adhering microorganisms was verified through SEM. Serial dilutions of each sample were plated on Mueller-Hinton agar plates and quantified after incubation for 48 h.

Tissue cell morphology through SEM

After surface colonization for 48 h as described above for the MC3T3-E1 cells, culture medium was withdrawn and the samples were washed with PBS. Subsequently, the MC3T3-E1 cells were fixed with 1.5% (v/v) glutaraldehyde (Sigma, Germany) and 2% (v/v) paraformaldehyde (Sigma, Germany). The solution was buffered with 0.1 M sodium cacodylate solution (Sigma, Germany). Afterwards, the content of glutaraldehyde was increased up to 3% (v/v) to achieve improved fixation. Dehydration was carried out in an ascending acetone series and samples were dried in an autoclave with supercritical carbon dioxide. Cell morphology was investigated using a scanning electron microscope (JEOL 6400, JEOL Germany GmbH, Eching, Germany) with gold-sputtered samples.

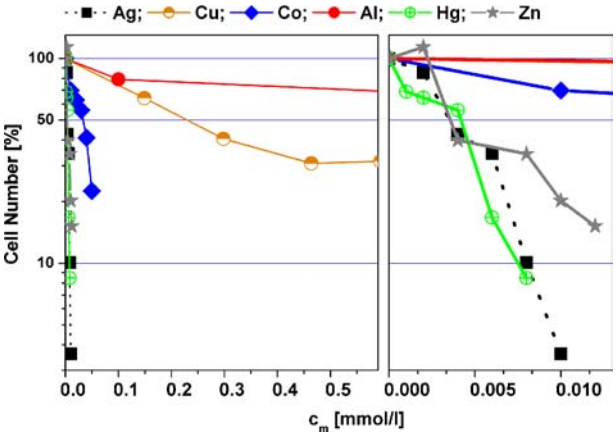

Figure 1 Cell number of fibroblasts L929 with increasing concentrations of metal ions. Insert: Magnification of cell numbers at very low metal ion concentrations.

2.4. Calculations and statistical methods

Means and standard deviations were applied for the presented data and evaluated for statistical significance using non-parametric methods and the method of closed testing procedure [17], with "$P < 0.05$" considered significant (Kruskal Wallis and Mann-Whitney test).

3. Results

3.1. Growth inhibition tests with metal ions

In the performed growth inhibition tests with fibroblasts L929, an increase of the metal ion concentration was generally followed by reduced cell proliferation. Only at trace element concentrations of Zn^{2+} in the range of 0.002 mmol/l, a slight promotion of fibroblast proliferation was observed. Growth inhibition of fibroblasts for all tested metal ions is displayed in Fig. 1. Lethal doses 50 (LD_{50}) are displayed in Table 1 and express the specific metal ion concentrations reducing tissue cell growth by 50%.

Growth inhibition of *S. epidermidis* differed substantially between the various metal ions. With increasing metal ion concentrations, bacterial growth at first showed a plateau of resistance before antibacterial threshold concentrations were reached and an exponential growth inhibition could be observed. Strongest antibacterial properties were exhibited by Hg^{2+}, followed by Ag^+ and Cu^{2+} (Fig. 2).

TABLE I LD_{50} concentrations of different metal ions for fibroblasts L929 in RPMI 1640 supplemented with 10% FCS. Reduction rates of bacterial growth at the LD_{50} concentrations were calculated and displayed in the right column

Metal ions	LD_{50} (L929) (mmol/l)	Reduction rates of bacterial growth at LD_{50}
Ag^+	3.5×10^{-3}	0.93
Zn^{2+}	3.6×10^{-3}	1.11
Hg^{2+}	4.2×10^{-3}	7.58
Cu^{2+}	2.3×10^{-1}	2.5×10^4
Co^{2+}	3.4×10^{-2}	1.42
Al^{3+}	1.8	0.46

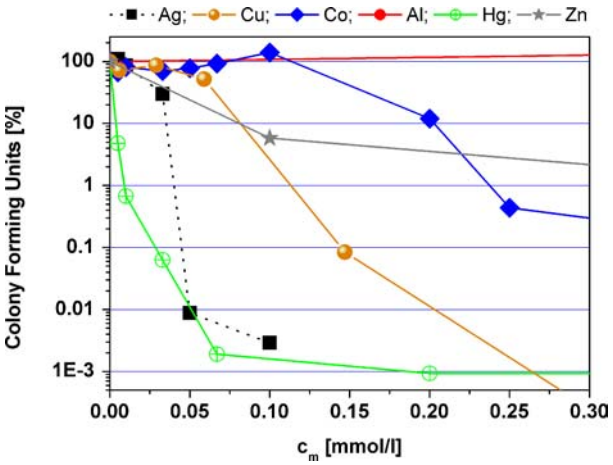

Figure 2 Growth inhibition of *S. epidermidis* (ATCC 35984) with increasing concentrations of metal ions.

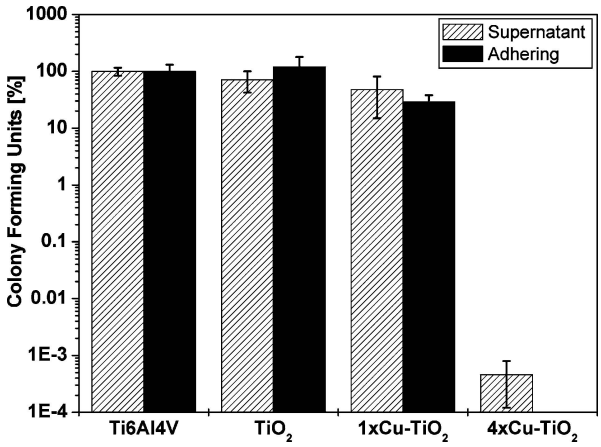

Figure 4 Growth of *S. aureus* ATCC 25923 on coated and uncoated Ti6Al4V surfaces and in the supernatant growth medium.

3.2. *In vitro* tests in direct surface contact

To compare the results of the different surface coatings with the values of a clinically established alloy, all diagrams were standardized to Ti6Al4V, which was set 100%.

In vitro tests with tissue cells in direct surface contact
The number as well as the corresponding mitochondrial activity of MC3T3-E1 cells in contact with the different surfaces is shown in Fig. 3. A significant increase both in cell number ($P = 0.0002$) and mitochondrial activity ($P = 0.0007$) was demonstrated on the TiO_2-coated Ti6Al4V samples compared to uncoated Ti6Al4V. The $1x$Cu-TiO_2-coated specimens showed another slight increase in cell proliferation without proof of significance ($P > 0.05$). At the contrary, a highly significant decrease in cell number ($P = 0.002$) and mitochondrial activity ($P = 0.9 \times 10^{-7}$) was observed on $4x$Cu-TiO_2-coated Ti6Al4V. However, these values are still within the range of the uncoated Ti6Al4V alloy.

In vitro tests with bacteria in direct surface contact
Coating of Ti6Al4V with pure TiO_2 did not influence staphylococcal adhesion *in vitro* after 24 h ($P = 0.699$) (Fig. 4). However, a highly significant reduction of viable adhering staphylococci could be observed on samples coated with copper-filled TiO_2 ($P = 0.002$). This reduction was strongest with samples coated by four layers of Cu-TiO_2, reducing viable bacteria on the surface by more than $6\log_{10}$ compared to uncoated metal plates ($P = 0.002$). Multiple coating layers ($4x$Cu-TiO_2) exhibited significantly stronger antibacterial action than the copper-filled monolayer (Cu-TiO_2) ($P < 0.002$). Fig. 4 also demonstrates, that bacteria seem to tolerate a certain amount of copper ions until a distinct threshold concentration is reached, followed by a significant and disproportionate dropdown in colony forming units of several $\log_{10}$. Similar results were observed in the growth inhibition assays.

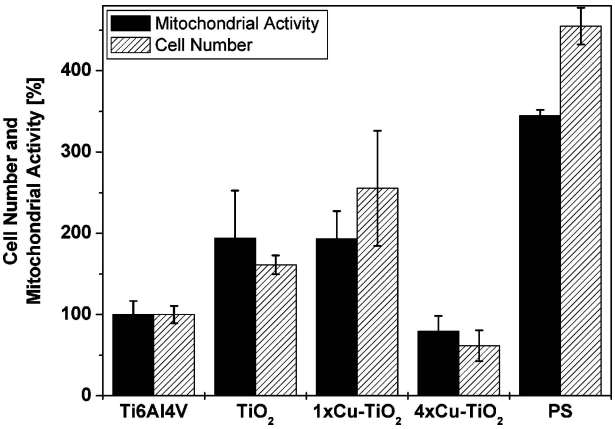

Figure 3 Growth and mitochondrial activity of osteoblast-like cells MC3T3-E1 on uncoated and coated Ti6Al4V surfaces after 48 h in culture.

Impact of released ions on bacteria in the supernatant
Influence of the different surface coatings on the growth of planktonic bacteria in the supernatant growth medium was also studied by quantification of viable organisms. Coating Ti6Al4V with TiO_2 did not influence bacterial growth in the supernatant (Fig. 4). At the contrary, integration of copper ions into the surface coating tendentially reduced staphylococcal growth also in the growth medium ($P > 0.05$). Again, highest reduction of bacterial growth was monitored in the growth medium adjacent to the $4x$Cu-TiO_2-coatings ($P = 0.002$).

Tissue cell morphology through SEM
SEM-images of osteoblast-like cells on the different specimen surfaces are displayed in Fig. 5(a)–(d). The cells on the uncoated Ti6Al4V alloy generally appeared more spindle-shaped compared to the cells on the coated specimens. Ti6Al4V-bound cells showed nearly no spreading on the surface at the time point 48 h (Fig. 5(a)). Tissue cells on the titania coated material revealed similar morphology, but the trend to wider spreading cells is clearly visible on the $1x$Cu-TiO_2-coated and even the $4x$Cu-TiO_2-coated samples. Simultaneously, copper effects producing injured ($1x$Cu-TiO_2) and even dead ($4x$Cu-TiO_2) cells were detectable through SEM. On all images with higher resolution, the cell membrane showed a typical texture and focal contacts were built up to the different surfaces.

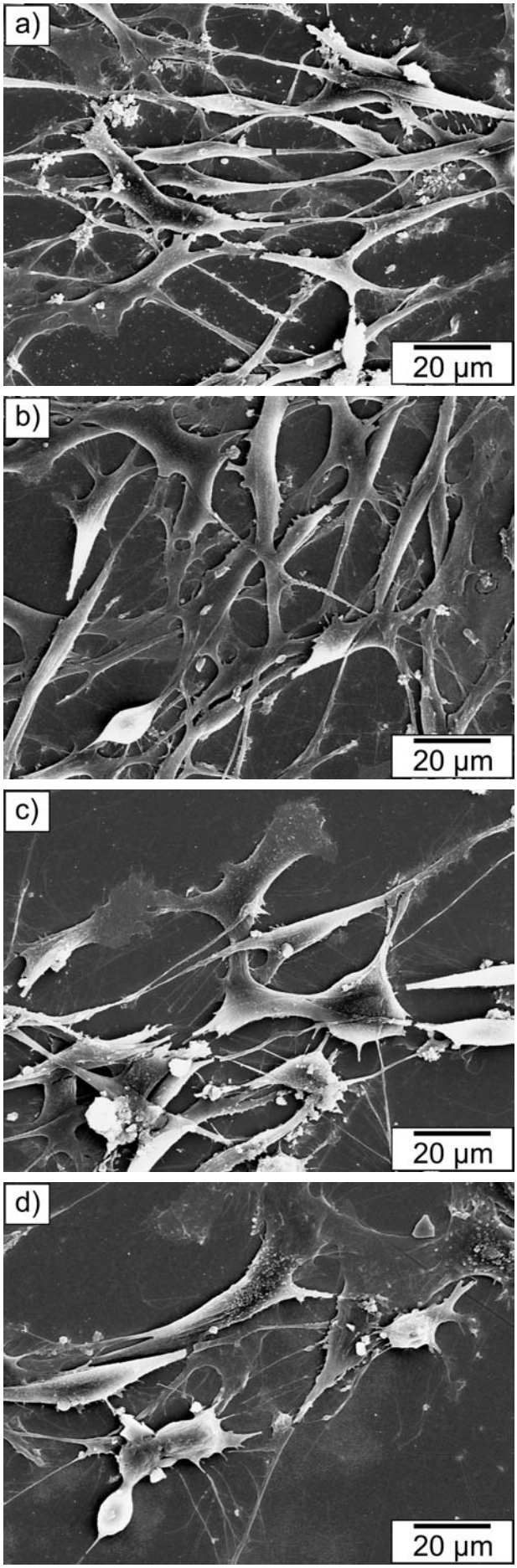

Figure 5 SEM image of MC3T3-E1 cells on uncoated Ti6Al4V (a), TiO_2-coated Ti6Al4V (b), 1*x*Cu-TiO_2-coated Ti6Al4V (c) and 4*x*Cu-TiO_2 coated Ti6Al4V (d) after incubation for 48 h.

4. Discussion

The toxic effect of metal ions on either tissue cells or bacteria is a well-regarded phenomenon and has been reported in a multitude of studies [18–21]. However, those data are inhomogeneous and direct comparison of antibacterial and tissue cell toxicity is impossible due to different test parameters, culture conditions, cell lines and bacteria, respectively. Therefore, in the present study, culture conditions of tissue cells and bacteria were kept identically for all tests to evaluate the relative antibacterial effectiveness of a certain metal ion with respect to the cytotoxicity it induces. Our data are generally in line with those published by other authors [18, 21]. The growth inhibition tests clearly demonstrated that Ag^{+}, as well as Zn^{2+} and Hg^{2+} ions exhibit very strong cytotoxicity already at low concentrations (Fig. 3). Co^{2+} showed intermediate cytotoxicity whereas tissue cells tolerated relatively high concentrations of Cu^{2+} and Al^{3+}. These results are similar for bacteria, except the fact that Co^{2+} and Zn^{2+} exhibited no significant antibacterial effect at the investigated concentrations and that Cu^{2+} ions were significantly more effective against bacteria than toxic for the tissue cells.

To emphasize these differences and to weight up between cytotoxicity and antibacterial effectiveness of the studied metal ions, the values have been put into relation by standardizing the reduction rate of bacterial growth to the LD_{50}-concentration of tissue cells (Table I). This comparison clearly points out that metal ions exhibit markedly different antibacterial properties at corresponding levels of cytotoxicity. The strong antibacterial effect of Hg^{2+} and Ag^{+} is mainly enforced at the expense of cytocompatibility and is clearly reduced, if cytotoxicity is considered (reduction rate: 7.58 and 0.93). In other words, Hg^{2+}, and Ag^{+} are highly antibacterial substances but also extremely cytotoxic. Al^{3+} showed no effective reduction of bacteria at the LD_{50} concentration. Co^{2+} and Zn^{2+} demonstrated only minor effect on bacterial growth but a high cell-toxic potential. In this respect, copper ions clearly proved superior with the highest antibacterial effectiveness (reduction rate: 2.5×10^4) at corresponding LD_{50} concentrations. Our results provide evidence, that significantly higher concentrations of copper ions with corresponding higher antibacterial effectiveness can be safely applied compared to other metal ions. Consequently, copper ions prove most adequate to equip implant surfaces with antibacterial properties without decreasing the biocompatibility in a significant manner.

The tests in direct surface contact additionally affirmed the findings of the growth inhibition tests. Pure TiO_2-coating significantly improved cytocompatibility confirming previous investigations [13], but did not show any antibacterial effect. The morphology of adherent cells is characterized by broader spreading compared to the more spindle-formed cells on uncoated Ti6Al4V alloy. Integrated copper ions in lower concentrations (1*x*Cu-TiO_2) demonstrated excellent cytocompatibility combined with a significant antibacterial effect. These findings also corresponded to the

SEM micrographs, where most of the cells appeared well-spread and well-attached. Nevertheless, due to copper integration, single injured cells with rounded morphology and roundish structure were detected through SEM on $1x$Cu-TiO_2 coated surfaces.

Highest antibacterial effectiveness was reached by the $4x$Cu-TiO_2 surface coating causing a decrease of adhering bacteria by more than $6\log_{10}$ to undetectable levels (Fig. 4). By releasing antibacterial amounts of copper ions to the surrounding medium, bacterial growth was also reduced significantly in the supernatant compared to uncoated specimens. At such elevated copper concentrations in the growth medium, some dead tissue cells were also visible in the SEM images. However, morphology of adherent cells was generally well-spread with a clearly structured cell membrane typical for living cells. Cell number as well as cell proliferation of MC3T3-E1 cells was reduced on $4x$Cu-TiO_2 surfaces, but still within the range of the clinically-established Ti6Al4V alloy. This clearly shows that even high copper ion concentrations causing a tremendous reduction of bacterial growth are tolerable for tissue cells.

The present study clearly demonstrates, that antibacterial properties of the introduced surface coating can be controlled and adjusted to the clinical setting and to specific indications. Custom-tailored surface coatings seem possible and may be of particular interest for high-risk operations like revision and tumor arthroplasty. As infection risk is highest in the perioperative period, a controlled release of metal ions over a period of approximately two weeks may significantly reduce infection rates and release kinetics have to be addressed in further studies. Furthermore, due to leaching of the copper ions, even implants coated with multiple antibacterial layers may exhibit excellent long-term biocompatibility. Biomechanical investigations performed so far showed very promising results in cohesive and adhesive stability of the surface coating. The presented surface coating combining good biocompatibility with distinct antibacterial action may reduce the " immuno-incompetent fibro-inflammatory zone" surrounding any implant and may finally help to improve clinical outcome.

5. Conclusions

Comparing growth inhibition tests with tissue cells and bacteria demonstrated, that among Al^{3+}, Co^{2+}, Zn^{2+}, Hg^{2+}, Ag^{+} and Cu^{2+}-ions, the copper ions delivered by far the best compromise between antibacterial effectiveness and cytotoxicity. These results were confirmed by the tests in direct cell contact, where cell culture results comparable to the uncoated Ti6Al4V were obtained even for titania coating with high copper concentrations ($4x$Cu-TiO_2). With these copper-filled coating, a reduction of bacterial growth by $6\log_{10}$ was achieved.

The investigations of this paper lead to the conclusion, that copper-filled TiO_2 coatings could be a novel approach particularly for revision or tumor arthroplasty, where a compromised immune system has to be supported by an antibacterial but simultaneously biocompatible modified implant.

Acknowledgements

We gratefully acknowledge the contributions of I. Kappstein, Associate Professor of the Abteilung für Infektionshygiene, Technische Universität München, for inspiring discussions and constructive microbiological advice. Furthermore, the authors acknowledge the grateful support from the "Friedrich-Baur-Stiftung", Burgkundstadt, Germany.

References

1. A. G. GRISTINA, *Science* **237** (1987) 1588.
2. *Idem.*, *Clin. Orthop.* (1994) 106.
3. R. O. DAROUICHE, I. I. RAAD, S. O. HEARD, J. I. THORNBY, O. C. WENKER, A. GABRIELLI, J. BERG, N. KHARDORI, H. HANNA, R. HACHEM, R. L. HARRIS and G. MAYHALL, *N. Engl. J. Med.* **340** (1999) 1.
4. H. GOLLWITZER, K. IBRAHIM, H. MEYER, W. MITTELMEIER, R. BUSCH and A. STEMBERGER, *J. Antimicrob. Chemother.* **51** (2003) 585.
5. H. GOLLWITZER, H. MEYER and A. STEMBERGER, *Trauma Berufskrankh.* **5** (Suppl 2) (2003) S347.
6. J. OLSSON, Y. VAN DER HEIJDE and K. HOLMBERG, *Caries Res.* **26** (1992) 428.
7. I. RAAD, R. DAROUICHE, J. DUPUIS, D. ABISAID, A. GABRIELLI, R. HACHEM, M. WALL, R. HARRIS, J. JONES, A. BUZAID, C. ROBERTSON, S. SHENAQ, P. CURLING, T. BURKE, C. ERICSSON, S. GREENBERG, N. HANANIA, D. YOSHER, D. GIBSON, M. REARDON, P. REARDON, T. DARNULE, M. MANSOURI, K. ROLSTON, E. WHIMBEY, C. BIVINS, A. HUARINGA, K. PRICE and H. SAFAR, *Ann. Intern. Med.* **127** (1997) 267.
8. D. L. VEENSTRA, S. SAINT, S. SAHA, T. LUMLEY and S. D. SULLIVAN, *JAMA* **281** (1999) 261.
9. R. T. CARBON, S. LUGAUER, U. GEITNER, A. REGENFUS, M. BOSWALD, J. GREIL, T. BECHERT, S. I. SIMON, H. P. HUMMER and J. P. GUGGENBICHLER, *Infection* **27** (Suppl 1) (1999) S69.
10. M. E. OLSON, B. G. HARMON and M. H. KOLLEF, *Chest* **121** (2002) 863.
11. J. B. HEMMERLEIN, S. O. TREROTOLA, M. A. KRAUS, M. S. MENDONCA and L. A. DESMOND, *Radiology* **204** (1997) 363.
12. R. C. TILTON and B. ROSENBERG, *Appl. Environ. Microbiol.* **35** (1978) 1116.
13. F. HEIDENAU, F. STENZEL, H. SCHMIDT and G. ZIEGLER, *Biomaterialien* **1** (2000) 19.
14. Patent DE 102 43 132.9-45, submitted (2003).
15. H. GOLLWITZER, F. HEIDENAU, F. STENZEL, C. VON EIFF, G. ZIEGLER and W. MITTELMEIER, submitted (2003)
16. G. D. CHRISTENSEN, L. BALDASSARRI and W. A. SIMPSON, *Methods Enzymol.* **253** (1995) 477.
17. R. MARCUS, E. PERITZ and K. R. GABRIEL, *Biometrika* **63** (1976) 655.
18. G. GELLERT, A. STOMMEL and A. B. TRUJILLANO, *Chemosphere* **39** (1999) 467.
19. A. HASSEN, N. SAIDI, M. CHERIF and A. BOUDABOUS, *Bioresour. Technol.* **65** (1998) 73.
20. S. RAMAMOORTHY and D. J. KUSHNER, *Microb. Ecol.* **2** (1975) 162.
21. A. YAMAMOTO, R. HONMA and M. SUMITA, *J. Biomed. Mater. Res.* **39** (1998) 331.

Received 8 March
and accepted 11 March 2005