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(54) Title: DENTAL IMPLANTS FOR HIGH RISK PATIENTS

(57) Abstract: The invention relates to medical and dental prosthetic devices and implants having improved properties for promoting soft tissue healing in high-risk patients. The present invention concerns dental prosthetic devices and implants partially and/or fully coated with covalently bound doxycycline. The invention relates to medical and dental prosthetic devices and implants having improved properties for promoting soft tissue healing. The invention further relates to the use of Doxycycline for promoting soft tissue healing and/or preventing inflammation and/or infection in soft tissue in proximity to a medical prosthetic device or implant deployed in the body of a patient belonging to a group high-risk patient group.

Dental Implants for High Risk Patients

FIELD OF THE INVENTION

The invention relates to medical and dental prosthetic devices and implants having improved properties for promoting soft tissue healing. The present invention concerns dental prosthetic devices and implants partially and/or fully coated with covalently bound Doxycycline and/or Doxycycline bound by electrostatic adhesion. At least a part of the surface of said dental prosthetic devices and implants displays antibacterial properties. The invention further relates to the use of Doxycycline for promoting soft tissue healing and/or preventing inflammation and/or infection in soft tissue adjacent and/or in proximity to a medical prosthetic device or implant, deployed in the body of a patient belonging to a high-risk patient group.

BACKGROUND OF THE INVENTION

Dental implants

Improving techniques of implant production and broadening knowledge about the important factors for implant success, have led to success rates of dental implants well above 90 %. In that respect, diabetic patients, for example, are not seen as contraindicated for dental implants anymore, but rather that a controlled state of the disease is required for successful implantation. Despite these improvements, there are still patient groups that have poor success rates with commercially available implants due to smoking habits, radiation therapy or poor oral hygiene. Furthermore, an increasing number of elderly patients prefer dental implants and implant-retained partial dentures over conventional dentures, creating larger and more complex patient groups. The need for improved dental implants in these patient groups is caused by manifold factors, including general poor bone density, wound healing disruptions or high risk for infections.

For this reason a large proportion of scientific effort has been directed towards treatments that can increase the chances of implant success in poor quality bone.

In the case of diabetes, but also tobacco use, studies suggest, that systemic application of antibiotics could improve the success rates of dental implants for these patients. The

factors that have to be controlled in this context are the stimulation of bone growth around the dental implant for improved osseointegration and the risk of infection at the implant site.

Success of dental implants

The basic criteria for implant success are generally held to depend on the biocompatibility of the implant material, the macroscopic and microscopic nature of the implant surface, the status of the implant bed in both a health (non-infected) and a morphological (bone quality) context, the surgical technique used, the undisturbed healing phase and the subsequent prosthetic design and long-term loading phase.

Nearly all developments that lead to improved implant success rates up to the present were focused on at least one of these factors. The requirements can be further divided in factors that can be controlled by technology, covering treatment method and material used and factors that are given by the patients' condition before, during and after implantation. By far the most effort has been taken to optimize the factors that can be controlled by technology*, such as* the biocompatibility of the implant material, the macroscopic and microscopic nature of the implant surface.

Titanium and its alloys as implant material

Titanium is the material most commonly used today to achieve good biocompatibility and sustainable implant success. The biocompatibility of titanium and its superior properties as an implant material was held to be connected to the 2 - 10 nm thick oxide layer, instantly formed on titanium in the presence of oxygen. Due to this oxide layer, titanium features high polarization resistance, protecting the metal against corrosion and therefore hinders the release of metallic ions into the human body. As a result of titanium oxide's high dielectric constant, the surface oxide film was found to be an attractive site for the establishment of chemical bindings and the attachment of a large spectrum of doxycyclines. This interaction is also the reason why the placement of titanium in bone results in the establishment of a strong bone-implant interface. The establishment of this strong connection between the living bone and tissue and the histological proof thereof is called osseointegration.

Despite the successful application of titanium implants, research constantly aimed on the development of advanced alloying techniques for titanium to optimize biocompatibility and mechanical properties. One of the most recent innovations, the alloying of titanium with zirconium, showed broad success and acceptance. As titanium and zirconium are the only metals that do not show osteoblast growth inhibition, a combination of both is well suited for implants. A significantly higher removal torque and bone area could *in vivo* be shown for a titanium zirconium alloy compared to commercial pure (cp) titanium. Furthermore, it was observed that the oxides on titanium zirconium alloy surfaces are more stable and therefore favorable in terms of corrosion resistance. Also the alloying of titanium with zirconium improves the mechanical strength especially for applications in small diameter implants. While the mechanical strength is high for titanium zirconium alloys, they are well suited for implantation in cortical bone due to a low young's modulus, which prevents stress shielding. In addition, the mechanical properties of titanium zirconium alloys allow the placement of small-diameter implants in critical implantation sites, such as the front of the lower jaw where bone is scarce and the crestal bone is thick.

Surface modification

There are a variety of surface treatments, which are commonly applied for improvement of surface properties of dental implants. Applied techniques can be divided in physiochemical modifications that change the surface topography, morphology and chemistry. Furthermore, additive processes are known to have potential to improve the physiological reaction of implants.

Morphology and topographical surface modifications have the potential to improve the interaction between implant and tissue. In this context, rough implant surfaces were found to promote osseointegration more than smooth surfaces. Mechanical and chemical methods or a combination of both can be used to optimize the surface morphology and topography. Especially blasting with titanium dioxide (TiO2) particles, etching and multistep etching are frequently used techniques.

Yet, hardly any modification of the surface morphology can be done without inducing changes of the chemical surface composition and vice versa. The etching processes used on titanium for surface modifications increases the amount of hydrogen on the Ti surfaces, as liberated hydrogen ions are attached to titanium's outer surface layer in the form of

titanium hydride. This process can be influenced by molar strength of the acid and the etching time. Several studies suggest that the hydrogen content induces faster healing and furthermore better osseointegration. Therefore cathodic polarization (hydridation) has recently been applied to increase layer thickness and concentration of titanium hydride. In addition the hydridation process can be used for attachment of ampholytic biological molecules that bind on the surface during hydridation together with hydrogen.

As the oxide layer on titanium is the most prominent feature of the material, modifications of this layer were tested. Yet, the approaches to increase titanium's biocompatibility by sheer increase of oxide layer thickness by anodic oxidation (hydroxylation) in acidic solutions did not show increased biocompatibility. Nevertheless, if hydroxylation is used with alkaline solutions, an increase of hydroxide (OH) groups on the surface can be achieved.

Biomolecules

A variety of substances would qualify for the creation of bioactive surfaces. The underlying principle of their functionality is always a certain, beneficial stimulation of the host response by the applied substance. Therein the goal is the attachment of bioactive substances like proteins, enzymes or peptides on the surfaces. Cell attachment factors like fibronectin could improve attachment and spreading of cells on the implant surfaces, while the application of growth factors like bone morphogenetic proteins could directly influence the development of osteoblasts on the surfaces. The theoretical possibilities of suitable molecules are extensive, yet limited by the conditions that molecules are exposed during, the coating, solubility and degradation problems or simply destruction due to the attachment process. Furthermore a polarization of the molecule (ampholyte) is required for attachment in an electrochemical process.

As the basic criteria for implant success are generally held to depend on the biocompatibility of the implant material, measured on its effectiveness in promoting and/or stimulating osseointegration, most biomolecules tested today are selected for and evaluated on their effect on hard tissues.

Nearly all developments that lead to improved implant success rates up to the present have thus been focused on employing biomolecules stimulating hard tissue formation.

Doxycycline (Doxy)

The semi-synthetic broad spectrum antibiotic Doxycycline (Doxy/doxycycline) belongs to the group of tetracycline antibiotics and is used for treatment of various infections and works by the inhibition of bacterial protein biosynthesis. In vitro studies suggest the efficiency of systemic Doxycycline application for bone growth promotion and treatment of periodontal disease as well as peri-implantitis. Furthermore, the applicability of Doxycycline for the control of osteogenic differentiation in genetically engineered mesenchymal stem cells has been proven. The administration of Doxycycline for periodontal disease and peri-implantitis treatment has so far been tried using topical drug delivery systems, such as gels.

Both systemic and topical administration of Doxycycline has drawbacks, though; systemic administration of an antibiotic inadvertently leads to systemic interference with the patient's body and necessitates a higher overall dosage regime than topical application. Topical administration to a dental implant in particular, can only be performed during the placing of the implant or with post implant surgery, as the implant is more or less fully incorporated into the patient's body. What is more, topical administration of Doxycycline with a carrier, such as a gel or a matrix, generates a fast release of the active substance, and it also necessitates that said carrier is biodegradable and thus the timespan at which Doxycycline will be available to the surrounding tissue of the implant is limited.

In consequence, it is sought for an administration which will lead to a dosage optimization of the area of drug administration to the tissue directly surrounding the implant. For a combined use of Doxycycline with dental implants, a direct and permanent incorporation of this drug with the implant system would be favorable. Despite the potential benefits, though, successful binding of Doxycycline directly to an implant has not yet been reported in the literature.

DISCLOSURE OF THE INVENTION

Even though modern implants show high success rates, it is still challenging to successfully treat certain groups of patients with the implant systems available. Larger

and more heterogeneous patient groups add to the complexity that implantology faces nowadays.

The present inventors for the first time demonstrate covalent binding of Doxycycline hyclate onto a titanium zirconium (TiZr) alloy surface. The present inventors at the same time demonstrate binding of Doxycycline hyclate onto a titanium zirconium (TiZr) alloy surface by electrostatic adhesion. It was surprisingly found that a hydrogen rich surface, as produced under cathodic reduction in acidic solutions, is suitable and can be efficiently used for the attachment of Doxycycline and that the Doxycycline so bound and/or adhered to the surface is still active as well as remains biologically effective when released from the surface into the adjacent and/or surrounding tissue.

Further, the bioactivity of the Doxycycline on the TiZr surface was assessed under *in vitro* and *in vivo* conditions and it was demonstrated that Doxycycline coated dental implants surprisingly improved not only the biocompatibility of the implant measured on improved osseointegration, but clearly promoted healing of soft tissue adjacent and/or in proximity to the placed implant. At least parts of the surface of the implant displayed antibiotic properties.

The present invention for the first time shows an improved craniofacial and/or dental prosthetic device or implant, containing a metal material, wherein surface parts of the metal material are coated with one or more Doxycycline(s) associated therewith.

In one embodiment, the present invention for the first time shows the efficiency of a dental metal implant surface, such as a titanium zirconium surface, coated with Doxycycline (Doxy), wherein the implant is coated using an electrochemical method in a cathodic reduction setup under acidic conditions.

Doxycycline coated implants were shown to surprisingly improve not only the biocompatibility of the implant measured on improved osseointegration, but especially were able to promote healing of the soft tissue in proximity to the placed implant. It is thus concluded that Doxycycline has the potential to trigger bone growth around the implantation site, and at the same time reduce the risks of poor healing, infection and/or inflammation of the soft tissue adjacent and/or in close proximity to the placed implant, such as in the gum tissue. Thus, for the first time, an improved craniofacial and/or dental

prosthetic device or implant is disclosed that can be implanted even into high-risk patient groups, such as patients with diabetes, smoking habits, poor bone density, wound healing disruptions and/or high risk for infections.

Doxycycline is a semi-synthetic broad spectrum antibiotic, from the group of tetracycline antibiotics, which is used for treatment of various infections and works by inhibition of bacterial protein biosynthesis. Tetracycline is considered to enhance bone formation mostly on the basis of general knowledge about interaction with collagen formation and calcium incorporation. It has been proven to be efficient *in vitro* in application for bone growth promotion and treatment of periodontal diseases. Other studies could furthermore verify the applicability of Doxycycline for the control of osteogenic differentiation in genetically engineered mesenchymal stem cells.

In the present invention, the at least partially doxycycline-coated implant showed a surprisingly strong antibacterial effect.

Administration of Doxycycline for periodontal disease and peri-implantitis treatment is currently mainly done by application of drug delivery systems. Yet, a combined use of Doxycycline with dental implants and its direct incorporation in the implant system has herein for the first time been shown to be favorable, as local administration reduces interference with the patient's body and optimizes the area of drug administration to the bone as well as the soft tissue directly surrounding the implant. The present inventors for the first time demonstrate successful binding of Doxycycline directly to an implant.

What is more, the bound Doxycycline was active both when bound to the implant-surface as well as when released into the adjacent and/or surrounding tissue.

Implant surfaces exposed to biological environments trigger a reaction based on the adsorption of water, ions, proteins and biomolecules. The adsorption of these molecules has an effect on cell attachment to the surface and therefore affects the tissue response. As the chemical properties of the surface directly influence the amount and composition of proteins adsorbed to the surface, coating with antibiotic agents can be used as one form of chemical surface modification. However, binding of biomolecules to titanium`s native TiO_2 layer is difficult due to the low reactivity of this oxide layer.

The dental implant surfaces coated in the present invention are based on a titanium zirconium alloy with grit blasted and acid etched surface, which also served as control. The validation of the coating was done with FTIR, XPS, SIMS and Field emission SEM and its effect in vitro was tested on a MC3T3-E1 murine osteoblastic cell line. A rabbit in vivo study was executed to analyze bone marker gene expression and bone growth in critical size defect.

The Doxycycline molecule was found intact on the surface and quantified using UV-VIS and FTIR, showing a mean concentration of 141 µg/cm² of Doxycycline on the samples. Inclusion of Doxycycline was documented up to a depth of approximately 0.44 µm by tracing the ^{12}C signal in a SIMS analysis. The in vitro study with murine osteoblasts showed significantly increased alkaline phosphatase and osteocalcin gene expression levels after 14 days along with low cytotoxicity. Furthermore the in vivo study showed increased RNA, Coll-I and BMP2 expression at 8 weeks of healing.

In one embodiment, the method of cathodic polarization was shown to be suitable to chemically modify implant and in particular abutment surfaces without significantly altering the surface topography of the original engineered implant or abutment surface.

Bacteria assay was conducted to evaluate the antimicrobial efficacy of Doxycycline-coated surfaces. The bacteria assay was also used as an indication for Doxycycline release. *S. epidermidis* was used for the antibacterial test as it is a common cause of biomaterial associated infections and also highly reproducible in the laboratory.

As can be seen in experiment 2, biofilm formation on the at least partially Doxycycline coated surface was inhibited (**Figure 15**). Steady-state biofilm is difficult to eradicate after formation as it is more resistant to antibiotics than planktonic bacteria and requires up to 250 times greater concentration than inhibiting the same strains grown planktonically.

The present inventors for the first time were able to apply a cathodic reduction process for the binding of Doxycycline to a TiZr surface. As the sample surface was negatively charged under cathodic reduction, the broad spectrum antibiotic Doxycycline was suitable for attachment due to its positive polarization. Doxycycline has several protons that are suitable to bind to the surface like the O-H proton and the A-ring amide. Therefore, it was assumed that Doxycycline would bind instead of the hydrogen formed under cathodic

reduction as documented in the literature. Doxycycline was furthermore chosen with the prospect of creating a novel dental implant surface for treatment of critical implantation sites and contraindicated patients with low bone density.

Even though color change was detected on the coins in the experiments performed, the process did not alter Doxy, as there were no elevated temperatures employed in the process that could trigger degradation. As tetracyclines generally have many potential metal binding sides, a color change due to the binding of liberated metal ions was another possible explanation for the color change. The activity of the molecule was examined by an in vitro study and the integrity was investigated in FTIR.

The FTIR analysis showed that the Doxycycline molecule was present and intact on the surface. The concentration of the coating was analyzed with respect to the peak at 1454 cm^{-1} and 1613 cm^{-1} as those were described in the literature and were most prominent in our observations.

A strong peak at 1459 cm^{-1} was from a CH_2 bending vibration of the aromatic amine compound $N(CH_3)_2$ in Doxy, which was also confirmed in the literature. Furthermore, a secondary amide N-H bending vibration of the amide II band was detected at 1555 cm^{-1}. This structure was rather surprising, as pristine Doxycycline should not contain that compound. The peak at 1575 cm^{-1} was assigned to N-H bending of the aromatic ring A of Doxycycline. Another peak which was visible in both spectra positioned at 1611 cm^{-1} was derived from C=O stretching on ring A. Considering that the peak at 1651 cm^{-1} only appeared in the coated and spiked coated samples, it was likely that this peak was shifted from the peak at around 1660 cm^{-1} of the pure Doxy. This peak was related to the amide I band of the primary amide with its C=O stretching vibration and was observed on the samples in agreement with the literature. Due to the peak shift, the amide was considered a possible binding site as the shifting of carboxyl peaks can occur due to changed hydrogen bonding state.

To analyze the exact amount of Doxycycline on the surfaces, a release study was executed. The total amount of 140.97 µg/cm² released over the seven days period was approximately three times higher than the amount calculated with the FTIR. This was attributed to general inaccuracies of the quantification of compounds in FTIR, as well as the influence of the rough surface and limited penetration depth. Even though no further

measurements were conducted, it was assumed that after seven days all Doxycycline was released from the samples.

Beyond the detection of Doxycycline on the surface, an analysis of the molecules depth integration in the material was performed by SIMS (Figure 4). As Doxycycline contains a significant amount of carbon, tracking of the carbon ^{12}C isotopes depth profile was used to track Doxycycline depth integration in the sample. This method was validated by analysis of a hydrided TiZr sample, to account for the possible carbon contamination, from the buffer solution during the process. As this contamination was negligible in terms of depth integration, ^{12}C must have been derived from Doxycycline (Figure 4 A). Furthermore samples were protected under cover gas to exclude contamination with carbon from other sources and a TiZr SBAE reference sample for assessment of carbon in the source material was used. Based on the experience of previous work, it was believed that the binding of Doxycycline was linked to the buildup and remodeling of the hydrogen layer on the surface (Figure 4 B). Nevertheless, no elevated hydrogen levels were found for the Doxycycline coated samples which suggested that the hydrogen layer was remodeled during the process.

The XPS carbon peaks at 286 eV and at 287.5 eV were found to be corresponding to C-O / C-N and O-C=O / N-C=O respectively. As observed in figure 5, Ti2p intensity decreased strongly over the coating process, which was a clear signal for the coverage of the original surface. The titanium signal in both samples related to TiO_2. The largest peak of nitrogen at 399.5 eV, was attributed to NH_3 according to Moulder et al. Another peak of nitrogen, which was present on both samples at 401.6 eV was probably a C-NH_2^+ compound. Furthermore the Doxycycline coated sample had a nitrogen peak at 402 eV, which was not observed on TiZr SBAE. This peak corresponded to amide and amino groups. Furthermore the literature assigned that region of the N1s peak to NH_4^+. The fact that nitrogen was found on the control samples as well could have been due to the nitrogen cover gas packaging or relics from the processing history of the sample. Nevertheless, the appearance of the peak at 402 eV and the clear increase of nitrogen in general ascertained the presence of Doxycycline on the samples surface.

The XPS and FTIR analysis confirmed the presence of Doxycycline on the sample surface. The clear signal of the amide bands in FTIR and XPS and the appearance of surface masking, as indicated by the decrease of the titanium signal from the XPS,

showed the presence of the molecule on the surface. Furthermore the FTIR measurements demonstrated that the molecule was intact.

The mechanism of Doxycycline attachment to the surface was most likely related to hydrogen bonding in a similar manner as described prior. Potential binding sides could have been the amide group in ring A of Doxycycline. As protonation of Doxycycline occurs in the pH 1 – 6 region, it was likely, that a binding site became available on the amide group of Doxycycline in the form of a zwitterion on the primary amide complex. This could have triggered the reaction of Doxycycline molecules with each other in the powder form, but also the attachment of the biomolecule to the implant surface in the cathodic reduction. Therefore it is possible that Doxycycline was attached to a complex layer of hydrogen and oxygen and the molecule was actively bound to it at one or more binding sites. This mechanism would be similar to the one described in a paper on proline adsorption on titanium due to a zwitterion binding. The theory would sufficiently describe the binding mechanism and explain the appearance of the FTIR peak at 1555 cm^{-1} which is related to a secondary amine. Furthermore it would explain the peak shift observed on Doxycycline powder from 1662 cm^{-1} to 1651 cm^{-1} on Doxycycline coated samples.

Alternatively, Doxycycline (Doxy) is positively charged in the acetic buffer (pH = 3.0) according to the pK_a values (3.02, 7.97 and 9.15) and thus attracted to the negatively charged TiZr cathode during cathodic polarization. Indeed, the protonation of nitrogen in Doxycycline is shown by the increase of $(CH_3)_2$-N+ and O=C-NH_3+. Therefore, in addition to the covalent binding between Doxycycline and TiZr surface as suggested above, Doxycycline is attached to TiZr surface by electrostatic forces. This should theoretically result in a stronger adherence between Doxycycline and the surface. Doxycycline adsorbed by electrostatic force should therefore be more stably adhered onto the surface compared to physically adsorbed Doxycycline by van der Waals force. Electrostatic adhesion also has the advantage over covalent immobilization in that it allows the release of Doxycycline from the surface, and thus, its antibacterial effect in the adjacent and/or close proximity. Unlike other surface coating approaches by using polymers and cements as drug carriers, this electrochemical method introduces no foreign substances other than Doxycycline onto the surface.

As seen in figure 6, the micro topography of both surfaces was similar, yet, the Doxycycline coated sample seemed to be smoothened in comparison to TiZr SBAE. Both

surfaces of the present study were well suited for mechanical bone anchoring. Yet, this parameter is of greater importance for bone interlocking than for cell attachment. The smooth edges on the sub-micron scale and the white spherical structures observed on the Doxycycline coated sample were important in terms of their effect for cell attachment. It was assumed that the rough surface on the TiZr SBAE base material was covered with a film containing Doxy, gradually built during the coating. This coating smoothed the surface in the Doxycycline coated group and concealed the rough structures created by the etching process. Nevertheless, the optimal surface nano topography for cell attachment is unknown and always depends on both, surface topography and chemistry.

As the two analyzed surfaces exhibited lower cytotoxicity than the reference tissue cultured plastic and differences between the groups were marginal, a cytotoxic effect of Doxycycline or any side products could be excluded. After 14 days of cell culture ALP and OC were found significantly increased on MC3T3-E1 cells seeded on Doxycycline coated samples, while Coll-I was lower at the same time. The high expression of ALP and OC on the Doxycycline coated samples was an indication that the osteoblastic cells on these surfaces were in the mineralization phase, which points to fast differentiation. These results were in accordance with the literature and represented a typical effect of Doxycycline increasing osteoblast differentiation.

The in vivo model used in this study was chosen due to its capacity to project the response in humans.

Inflamed bone tissue is known to release lactate dehydrogenase after cell death. The increase in cytotoxicity by assessment of LDH activity in the Doxycycline coated group after 8 weeks was interpreted as a late tissue response of bone remodeling. This assumption was furthermore supported by the low LDH activity observed for the Doxycycline coated group after 14 days in vitro (Figure 7).

Alkaline phosphatase is an early marker of osteoblast growth and its expression declines before the mineralization phase. As the relative ALP activity was significantly decreased after 8 weeks for both, hydrided and Doxycycline coated groups, the wound fluid analysis showed a late differentiation stage. A significant increase of total RNA content after 8 weeks, as well as the significantly higher Coll-I mRNA levels for the Doxycycline coated group showed the bioactivity of the surface coating with Doxycycline and indicate a

proliferative stage. Coll-I is the main organic contributor to the mechanical strength of bone and induces high density bone formation. This was in accordance with the high BMP-2 gene expression levels in the Doxycycline coated group after 8 weeks, which is relevant for ossification and directly influences bone formation. The increased activity of osteocalcin in the Doxycycline coated group furthermore indicated the maturation of bone tissue organization after 8 weeks.

Bone resorption due to remodeling of bone by osteoclasts is connected to the mRNA levels from H^+-ATPase and TRAP. While H^+-ATPase had highest expression after 8 weeks in the Doxycycline coated group, TRAP was lowered in the Doxycycline coated group compared to TiZr SBAE at 8 weeks. This behavior could indicate the presence of a resorption / deposition state due to bone remodeling. The significant increase of pro-inflammatory IL-6 was countered in the present study by the presence of increased, anti-inflammatory IL-10 mRNA levels in the Doxycycline coated group. A conclusive remark on the inflammation status of the peri-tissue is therefore difficult.

The devolution of the 2 dimensional BMD showed the most significant results for the Doxycycline coated group. Its high depth integration suggested the release of the molecule from the implant and its transport through the defect. The Doxycycline coated group was besides the high BMD, promising due to the high BMD at the end of the volume of interest.

After 8 weeks of healing time increased healing of the Doxycycline coated group compared to all other reference groups was observed. The Doxycycline coated group showed higher values of bone density, bone volume was furthermore increased. The high bone surface / tissue ratio furthermore suggested the presence of a highly porous bone structure with large quantities low diameter trabecular bone. This theoretical analysis was also confirmed by the 3D images.

DETAILED DISCLOSURE

The present invention relates a craniofacial and/or dental prosthetic device or implant, containing a metal material selected from the group consisting of titanium or an alloy thereof, zirconium or an alloy thereof, tantalum or an alloy thereof, hafnium or an alloy

thereof, niobium or an alloy thereof and a chromium-vanadium alloy, wherein surface parts of the metal material are coated with one or more Doxycycline(s) associated therewith, for use in high-risk patient groups.

In one embodiment, the present invention relates to a hydrided metal dental implant which is partially and/or fully coated with covalently bound Doxycycline(s) and which displays an improved in-healing in soft tissue. It was surprisingly found that a hydrogen rich surface, e.g. as produced under cathodic reduction in acidic solutions, is suitable for the covalent attachment of Doxycycline and that covalently bound Doxycycline is biologically effective.

In an alternative and/or supplemental embodiment, the present invention relates to a hydrided metal dental implant which is partially and/or fully coated with Doxycycline(s) bound to the surface by electrostatic adhesion, and which displays an improved in-healing in soft tissue. The electrostatically adhered Doxycycline is over time controllably released into the soft tissue in proximity and/or adjacent to the implant.

The bioactivity of the biomolecule on the hydrided metal dental implant surface was assessed under *in vitro* and *in vivo* conditions and it was demonstrated that Doxycycline coated dental implants surprisingly improved not only the biocompatibility of the implant measured on improved osseointegration, but clearly were able to promote healing of the soft tissue in proximity to the placed implant such as of gum tissue. The Doxycycline coated dental implants of the present invention were found to have antibacterial properties.

Consequently, the present invention relates to a craniofacial and/or dental prosthetic device or implant, containing a metal material selected from the group consisting of titanium or an alloy thereof, zirconium or an alloy thereof, tantalum or an alloy thereof, hafnium or an alloy thereof, niobium or an alloy thereof and a chromium-vanadium alloy, wherein surface parts of the metal material are coated with a layer of a corresponding hydride material selected from titanium hydride, zirconium hydride, tantalum hydride, hafnium hydride, niobium hydride and chromium and/or vanadium hydride, respectively, characterized in that the layer of hydride material comprises one or more Doxycycline(s) associated therewith.

In one embodiment, a device or implant is related to, wherein the metal material is titanium or an alloy thereof, preferably titanium.

Typically, the one or more Doxycycline(s) is present on the surface of the hydride material or trapped in the hydride material of the device or implant, and in a presently preferred embodiment, the one or more Doxycycline (C) is associated with surfaces that are in contact with soft tissue when the device is deployed in the body of a mammal. Nonetheless, also related to herein is a device or implant, wherein essentially all surface parts of the craniofacial and/or dental prosthetic device or implant are coated with a layer of a corresponding hydride material and one or more Doxycycline(s) associated therewith.

In one embodiment, a craniofacial and/or dental prosthetic device or implant, such as a dental implant is described, wherein selectively the surface parts that are in contact with soft tissue, such as in gum tissue, when the craniofacial and/or dental prosthetic device or implant is deployed in the body of a mammal, are coated with a layer of a corresponding hydride material and one or more Doxycycline(s) associated therewith.

The craniofacial and/or dental prosthetic device or implant, such as a dental implant as described in the present invention is particularly intended for use when the craniofacial and/or dental prosthetic device or implant is deployed in the body of patient with smoking habits, radiation therapy and/or poor oral hygiene.

In another, equally preferred embodiment, the craniofacial and/or dental prosthetic device or implant, such as a dental implant as described in the present invention, is particularly intended for use when the craniofacial and/or dental prosthetic device or implant is deployed in the body of patient with general poor bone density, poor quality bone, wound healing disruptions and/or high risk for infections.

Consequently, the present invention in one aspect relates to a craniofacial and/or dental prosthetic containing a metal material, wherein surface parts of the metal material are optionally coated with a layer of a corresponding hydride material, characterized in that the layer of hydride material or at least parts of metal material comprises one or more Doxycycline(s) associated therewith, for use in promoting soft tissue healing and/or preventing inflammation and/or infection in soft tissue adjacent and/or in proximity to the craniofacial and/or dental prosthetic device or implant, when deployed in the body of a

high-risk patient, such as a patient with smoking habits, radiation therapy and/or poor oral hygiene, general poor bone density, poor quality bone, wound healing disruptions and/or high risk for infections.

In one embodiment, the Doxycycline (C) is associated with the hydride material in an amount from 1 picogram per mm^2 to 1 mg per mm^2, preferably in an amount from 0.1 nanogram to 100 microgram per mm^2.

A device or implant as presently disclosed is typically a device or implant which replaces anatomy or restores a function of the body such as a craniofacial and/or dental prosthetic device or implant, such as a dental implant.

In the present context; craniofacial prostheses are prostheses made by individuals trained in anaplastology or maxillofacial prosthodontics who medically help rehabilitate those suffering from facial defects caused by disease (mostly progressed forms of skin cancer, and head and neck cancer), trauma (outer ear trauma, eye trauma) or birth defects (microtia, anophthalmia). They have the ability to replace almost any part of the face, but most commonly is the ear, nose or eye/eyelids. An ocular prosthesis and hair prosthesis can also be classified as craniofacial prostheses. Prostheses are held in place either by biocompatible drying adhesives, osseointegrated implants, magnets, or another mechanical form (although rare) such as glasses or straps. Prostheses are designed to be as similar as possible to the natural anatomy of each individual. Their purpose is to cover, protect, and disguise facial disfigurements or underdevelopments.

In the present context; a dental implant is a "root" device, usually made of titanium or an alloy thereof, used in dentistry to support restorations that resemble a tooth or group of teeth to replace missing teeth.

Virtually all dental implants placed today are root-form endosseous implants, i.e., they appear similar to an actual tooth root (and thus possess a "root-form") and are placed within the bone. The bone of the jaw accepts and osseointegrates with the titanium post. Osseointegration refers to the fusion of the implant surface with the surrounding bone. Dental implants will fuse with bone.

Dental implants can be used to support a number of dental prosthetic devices, including abutments, crowns, implant-supported bridges or dentures, such prostetic devices are typically in direct contact with soft tissue, such as gum tissue and/or oral mucosa. They can also be used as anchorage for orthodontic tooth movement. The use of dental implants permits undirectional tooth movement without reciprocal action.

A dental implant can comprise one or several parts. Single-piece dental implants can comprise and/or include a variety of prosthetic devise, including abutments, crowns, implant-supported bridges or dentures, such prostetic devices are typically in direct contact with soft tissue, such as gum tissue and/or oral mucosa.

In one embodiment, only the surface of the abutment is coated with one or more Doxycycline(s).

One embodiment of the present invention consequently relates to dental implants either supporting, including, containing and/or comprising a dental prosthetic device, selected from the group consisting of abutments, crowns, implant-supported bridges or dentures, being in direct contact with soft tissue, such as gum tissue and/or oral mucosa, wherein essentially all surface parts of the craniofacial and/or dental prosthetic device or implant are coated with one or more Doxycycline(s).

Another embodiment of the present invention relates to dental implants either supporting, including, containing and/or comprising a dental prosthetic device, selected from the group consisting of abutments, crowns, implant-supported bridges or dentures, being in direct contact with soft tissue, such as gum tissue and/or oral mucosa, wherein, wherein selectively the surface parts that are in contact with soft tissue, such as gum tissue, when the craniofacial and/or dental prosthetic device or implant is deployed in the body of a mammal, are coated with one or more Doxycycline(s).

Also disclosed herein is a method for preparing a craniofacial and/or dental prosthetic device or implant as defined herein, said method comprising subjecting surface parts of the metal material to an electrolysis treatment to form the layer of hydride material, said electrolysis treatment being carried out in the presence of one or more Doxycycline(s).

In addition, a craniofacial and/or dental prosthetic device or implant as defined herein can alternatively be prepared by other methods, such as dipping, spraying, nano-coating, plasma spraying, sputtering, deposition, chemical treatment with acids or alkali, hydrogen peroxide treatment, sol-gel, chemical vapor deposition, anodization, drying-on, and/or vaporizing of the one or more Doxycycline(s) to at least parts of the metal surface (A).

A use is further related to herein of a craniofacial and/or dental prosthetic device or implant as defined herein, for promoting soft tissue healing and/or preventing inflammation and/or infection in soft tissue in proximity to the craniofacial and/or dental prosthetic device or implant as defined herein, when deployed in the body of a mammal, in particular in a patient belonging to a high-risk patient group.

Further, Doxycycline is related to for use in promoting soft tissue healing and/or preventing inflammation and/or infection in soft tissue in proximity to a craniofacial and/or dental prosthetic device or implant as defined herein, when deployed in the body of a mammal, wherein said Doxycycline is covalently bound and/or electrostatically adhered selectively to the surface parts of the craniofacial and/or dental prosthetic device or implant as defined herein that are in contact with soft tissue, when the craniofacial and/or dental prosthetic device or implant, such as an abutment, as defined herein is deployed in the body of a mammal.

In the present context, the craniofacial and/or dental prosthetic device or implant as defined herein can be employed as a drug delivery device for a pharmaceutical composition comprising Doxycycline.

One aspect of the invention therefore relates to the use of Doxycycline for the preparation of a pharmaceutical composition for use in promoting soft tissue healing and/or preventing inflammation and/or infection in soft tissue in proximity to a craniofacial and/or dental prosthetic device or implant as defined herein, when deployed in the body of a mammal, wherein said Doxycycline covalently bound and/or electrostatically adhered selectively to the surface parts of the craniofacial and/or dental prosthetic device or implant as defined herein that are in contact with soft tissue, when the craniofacial and/or dental prosthetic device or implant, such as the abutment, as defined herein is deployed in the body of a mammal.

In particular, a use of Doxycycline is herein related to, wherein the craniofacial and/or dental prosthetic device or implant as defined herein is deployed in a patient belonging to a high-risk patient group, such as patients with general poor bone density, wound healing disruptions or high risk for infections, such as a patient group that has poor success rates with commercially available implants, e.g. due to diabetes, smoking habits, radiation therapy and/or poor oral hygiene.

One aspect of the invention consequently relates to the use of Doxycycline for the preparation of a pharmaceutical composition for use in promoting soft tissue healing and/or preventing inflammation and/or infection in soft tissue in proximity to a craniofacial and/or dental prosthetic device or implant as defined herein, when deployed in the body of a mammal, wherein the craniofacial and/or dental prosthetic device or implant as defined herein is deployed in a patient belonging to a high-risk patient group, such as patients with general poor bone density, wound healing disruptions or high risk for infections, such as a patient group that has poor success rates with commercially available implants, e.g. due to diabetes, smoking habits, radiation therapy and/or poor oral hygiene.

Doxycycline is herein also related to for use in promoting soft tissue healing and/or preventing inflammation and/or infection in soft tissue in a patient carrying a craniofacial and/or dental prosthetic device or implant containing a metal material selected from the group consisting of titanium or an alloy thereof, zirconium or an alloy thereof, tantalum or an alloy thereof, hafnium or an alloy thereof, niobium or an alloy thereof and a chromium-vanadium alloy, and wherein surface parts of the metal material are coated with a layer of a corresponding hydride material selected from titanium hydride, zirconium hydride, tantalum hydride, hafnium hydride, niobium hydride and chromium and/or vanadium hydride, respectively.

In one embodiment, Doxycycline is disclosed for use in promoting soft tissue healing and/or preventing inflammation and/or infection in soft tissue in a patient carrying a craniofacial and/or dental prosthetic device or implant containing a metal material selected from the group consisting of titanium or an alloy thereof, zirconium or an alloy thereof, tantalum or an alloy thereof, hafnium or an alloy thereof, niobium or an alloy thereof and a chromium-vanadium alloy, and wherein surface parts of the metal material are coated with a layer of a corresponding hydride material selected from titanium hydride, zirconium hydride, tantalum hydride, hafnium hydride, niobium hydride and chromium and/or

vanadium hydride, respectively, wherein said Doxycycline covalently bound and/or electrostatically adhered to surface parts of the a craniofacial and/or dental prosthetic device or implant, such as an abutment.

"Soft tissues", (i.e. non-mineralized tissues), can in the present context be used interchangeably with gingival tissue and/or gum tissue and may be defined as collagen or epithelium containing tissues, including skin and mucosa, such as oral mucosa, muscle, blood and lymph vessels, nerve tissues, glands, tendons, eyes and cartilage. In general the fraction and/or polypeptide fragments of the present invention can be used to promote healing or for manufacturing a pharmaceutical composition for promoting healing of a wound not only in skin and mucosa, but in any gingival tissue of the patient in need thereof.

The term "hard-tissue formation" in "mineralised tissue" may be summarised as the production by cells of an organic matrix capable of accepting mineral, with the activity of the enzyme alkaline phosphatase and a good blood supply prerequisites.

In the present context, the phrase a craniofacial and/or dental prosthetic device or implant includes within its scope a device intended to be implanted into the body of a vertebrate animal, in particular a mammal such as a human.

The device or implant according to the invention can be used for a number of purposes.

When the metal material is an alloy of titanium, zirconium, tantalum, hafnium or niobium, it may be an alloy between one or more of these metal elements; or it may be an alloy containing one or more other metals such as aluminum, vanadium, chrome, cobalt, magnesium, iron, gold, silver, copper, mercury, tin or zinc; or both.

It is preferred that the metal material is titanium or an alloy thereof, e. g. an alloy with zirconium, tantalum, hafnium, niobium, aluminum, vanadium, chrome, cobalt, magnesium, iron, gold, silver, copper, mercury, tin or zinc. In a particularly preferred embodiment, the metal material is titanium.

The corresponding hydride material is preferably titanium hydride.

The amount of Doxycycline (C) present on or in the hydride layer (B) of the parts of the prosthesis, device or implant coated with the hydride may vary within wide limits. Thus, the Doxycycline (C) associated with the hydride material may be present in amounts ranging from as low from 1 picogram per mm^2 to as high as 1 mg per mm^2 of hydride-coated device or implant surface.

However, it is contemplated that most useful Doxycycline coatings will range from 0.1 nanogram to 100 microgram per mm^2.

As indicated above, the method of the invention involves subjecting surface parts of the metal material to a electrolysis treatment to form the hydride layer (B), said treatment being carried out in the presence of one or more Doxycycline(s) as discussed above. It has been found that it is important that the conditions in the electrolyte (pH, ionic strength etc.) are such that the Doxycycline (C) has a net positive charge.

Consequently, the main concern for incorporation thereof in a hydride layer is stability under the conditions needed for bio-hydride preparation; I. e. an environment that supplies enough H+ ions for hydride preparation and at the same time keeps the net charge of the Doxycycline (C) positive. This mostly means that the electrolyte should have a high salt concentration and hence ionic strength; a comparatively high temperature, although preferably below any denaturing temperature of the Doxycycline (C); and a low pH.

Thus, the electrolyte may be any salt solution, preferably aqueous, e. g. a solution of sodium chloride, sodium sulphate, calcium phosphate, calcium chloride, phosphate buffered saline (PBS), saline, a salt solution mimicking physiological conditions, bi-carbonates, carbonates etc., in which the Doxycycline (C) is dissolved. The ionic strength of the salt is typically 1M, but concentrations can be adjusted to as low as 0.01 M and as high as 10 M according to the chemical properties and concentration of the Doxycycline(s).

The temperature of the electrolyte containing the Doxycycline (C) may range from ambient (20°C) to as high as the boiling point of the electrolyte, typically around 100°C, although the use of temperatures in the upper part of this range clearly depends on the ability of the Doxycycline (C) to withstand such temperatures without damage. If the Doxycycline (C) can withstand it, an optimum temperature for the formation of hydride is around 80°C.

The pH of the electrolyte is typically adjusted to the desired pH by means of a strong acid, e. g. HCl, HF, H2504 etc. although it should be taken into account that a pH below 2 will produce an irregular, corroded implant surface on titanium while a pH above 2 conserves the original surface. The pH is adjusted according to the desired Hydride/ Doxycycline (C) ratio; Low pH produces an implant surface with a high hydride/ Doxycycline (C) ratio (=more metal hydride), whereas a high pH close to the pI of the Doxycycline (C) in question will produce a surface with a low hydride/ Doxycycline (C) ratio (=more biomolecules).

Accordingly, while any pH between 0 and 10 can be used, the preferred pH for hydride preparation is between 5 and 2, depending on the chemical characteristics and concentration of the Doxycycline(s), the electrolyte used and the preferred hydride/ Doxycycline (C) ratio. For higher hydride/ Doxycycline (s) ratios (=more hydride), adjust pH more acidic, for lower hydride/ Doxycycline (s) ratios (=more Doxycycline (s)) adjust pH closer to, but not above, PI Doxycycline (C). The only requirement is that there are hydrogen ions (H+) and positively charged Doxycycline (C) (Doxycycline (C)+, net charge) present in the electrolyte.

The concentration of the Doxycycline(s) (one or any combinations of two or more) in the electrolyte may vary over a very wide range depending on, if it is to be released or not from the hydride layer, stability in vivo, stability in the electrolyte, availability, optimal pH, etc., Thus, the concentration of the Doxycycline(s) in the electrolyte may be within the range of 1 pg to 50mg per milliliter. A preferred range is between 10 pg and 1 mg per milliliter, but the optimal Doxycycline(s) concentration should always be by finally determined in pilot experiments with each Doxycycline(s) or Doxycycline(s)-mix.

Also, the time span over which the electrolysis is performed may vary but chiefly influences the thickness of the hydride layer and hence the concentration of Doxycycline(s) in the hydride layer.

An electrolysis cell for use in the method of the invention may be of any conventional design but is typically a two-chamber cell without any conducting connections between the chambers except for the electrolyte. The metal implant to be hydride-modified is placed in the cathode (i.e. the negatively charged electrode) chamber whereas the anode (the

positively charged electrode), typically made of carbon, is placed in a separate chamber. The electrolytes of each chamber are connected through a porous glass or porcelain filter allowing the current to pass unhindered but without any exchange of electrolytes between the two chambers. This is important because the products from the anode reaction, e. g. chloride or hypo-chlorites etc., could potentially interfere with the formation of the Doxycycline -hydride layer or destroy or modify the Doxycycline in the cathode electrolyte.

The separation of the two cells also allows the use of a smaller cathode electrolyte volume and thus a more effective use of Doxycycline(s) as well as the possibility to use a two-electrolyte system that allows optimization of the electrolytic process, e. g. one electrolyte optimal for Doxycycline(s) on the cathode side and an electrolyte on the anode side which is optimized for the efficacy of the electrolysis per se (conductivity, avoiding toxic products, or even producing useful byproducts/coatings).

As indicated above, the temperature in the cathode cell (Teat) should be as high as possible with an optimum for hydride preparation at 80°C.

The electrolytic process itself also produces heat which can pose two problems ; constituents of the electrolyte will evaporate so that the volume decreases and the ionic strength and the concentration of Doxycycline(s) increase above the preferred range, and the increase in temperature might cause precipitation, coagulation, denaturation, degradation or destruction of the Doxycycline (s) present. Therefore, the cathode compartment of the electrolysis cell is preferably equipped with a cooled lid for condensation of vaporized electrolyte and a temperature regulated radiator shell for stabilizing temperatures and volumes during electrolysis.

By adjusting current, charge and electrolyte composition it may also be possible to provide a favorable milieu for positive charge for most doxycycline(s). If not, a pulse field electrolysis set-up where the polarity of the electrodes is switching in controlled cycles during preparation of the biohydride layer could be one way to omit a negative net charge problem.

The power supply is typically a so-called current pump, i.e. a device delivering a constant current even if the resistance within the circuit varies. Although voltages between 0.1 and 1000 volts can be used, the voltage is typically below 10 volts. The current density during

electrolysis is typically in the range of 0.1 mA to 1 A per square centimeter (cm^2) of implant specimen. A preferred charge density is 1 mA/cm^2 although adjustments in the electrolyte, pH and temperature to increase Doxycycline compatibility may command minor or major deviations from this value.

The duration of the process depends on several parameters such as the desired thickness of the bio-hydride layer, the composition and characteristics of the electrolyte, the characteristics of the doxycycline, the temperature and pH, the desired hydride/bio-molecule ratio, the size of the implant specimen, the volume of the cathode electrolyte, the concentration of the doxycycline, etc. Thus, the duration of the process may be between 0.5 hours and several days. However, an optimal time-span is generally between 8 and 24 hours.

In one embodiment, TiZr implants are machined and/or machined and acid etched in HCl/H_2SO_4, cleaned in a succession of ultrasonic baths with NaOH (40 wt.%), HNO_3 (50 wt.%) and Milli-Q water and then coated with Doxycycline. The implants are treated by cathodic polarization in 2 M acetic acid/sodium acetate buffer containing 1 mg/ml Doxycycline hyclate. Three densities (1, 5 and 15 mA/cm^2) and three coating durations (1, 3 and 5 h) are preferred.
Doxycycline content on the surface can be increased with polarization time. Higher current density results in more Doxycycline on the surface.

A particularly preferred method of producing the at least partially Doxycycline coated implants according to the present invention comprises cathodic polarization with Doxycycline at 5 mA/cm^2 for 3 h.

The present invention consequently relates to a craniofacial and/or dental prosthetic device or implant comprising a metal material selected from the group consisting of titanium or an alloy thereof, zirconium or an alloy thereof, tantalum or an alloy thereof, hafnium or an alloy thereof, niobium or an alloy thereof and a chromium-vanadium alloy, such as TiZr, wherein surface parts of the metal material are coated with a layer of a corresponding hydride material selected from titanium hydride, zirconium hydride, tantalum hydride, hafnium hydride, niobium hydride and chromium and/or vanadium hydride, respectively, characterized in that the layer of hydride material comprises one or more Doxycycline(s) associated therewith and which is produced by cathodic polarization

with Doxycycline, wherein the implants and or medical devices are optionally machined and/or machined and acid etched, e.g. in HCl/H_2SO_4, and then treated by cathodic polarization in a Doxycycline comprising buffert, such as in 2 M acetic acid/sodium acetate buffer, comprising 1 mg/ml Doxycycline hyclate at a density of between 1-15 mA/cm^2, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 mA/cm^2, during 1-5 hours, such as during 1, 2, 3, 4 or 5 h.

To monitor the bio-hydride process, a calomel electrode may typically be placed in the cathode chamber. When the hydride layer formation process at the cathode is optimal, a difference of-1 Volt is observed between the calomel electrode and the cathode. If the current differs much from this value, the process will be running under sub-optimal conditions and a change in the set-up should be considered. Furthermore, a temperature probe and a pH probe may typically be placed in the cathode chamber to monitor that the process is running within the desired pH and temperature limits. A stirring device such as a magnetic stirrer may also be applied in the cathode cell to continuously mix the electrolyte and keep the temperature homogenous and avoid variations in local ionic strength, pH and Doxycycline concentrations.

After the electrolysis step, the now doxycycline/hydride-coated metal device or implant is immediately removed from the electrolyte and treated according to the requirement of the Doxycycline (s) in question. Typically, the device or implant specimen is allowed to air-dry and is then packaged in a sterile, airtight plastic bag in which it is stored until use for implantation. However, some Doxycycline(s) might be sensitive to drying, and consequently a wet storage system might be desired, e. g. like canning or storage in a fluid like saline or simply the electrolyte from the manufacturing process.
Although the electrolysis can be run under aseptic or even sterile conditions, the need for doing this may be avoided by including a sterilization step prior to use, using conventional methods such as ionizing radiation, heating, autoclaving, or ethylene oxide gas etc. The choice of method will depend on the specific characteristics and properties of the Doxycycline (s) present in the metal hydride layer.

Prior to the electrolysis treatment, the device or implant should be thoroughly cleaned.

This may typically consist in the implant being mechanically pre-treated by electropolishing or sandblasting to modify surface structure if desired, and subsequently

thoroughly cleaned using hot caustic soda followed by a de-greasing step, e. g. in concentrated trichloro-ethylene, ethanol or methanol, before being treated in a pickling solution, e. g. hydrofluoric acid, to remove oxides and impurities on the surface. After pickling the implant specimen is washed thoroughly in hot, double distilled, ion-exchanged water.

The invention is further illustrated by the following, non-limiting examples.

LEGENDS TO FIGURES

Figure 1. Surface analysis Doxycycline coated and untreated sample (SBAE) in comparison. The low resolution images show distinct differences in coloration of the coated sample in comparison to the untreated sample.

Figure 2. FTIR of Doxycycline coated sample, one curve of pure Doxycycline powder (Doxycycline pure, purple line) and one spiked sample (blue line) are displayed in the figure.

Figure 3. UV-VIS release in a 40% ACN, 3% TFA solution over a total time of 7 days. Absorbance was measured at 263 nm.

Figure 4. SIMS curves. Carbon depth profile over one Doxycycline coated and one TiZr SBAE coin (A). 1H Depth profile over one Doxycycline coated and one control coin (B).

Figure 5. XPS detail spectra of Doxycycline coated (green) and TiZr SBAE (red) samples for C 1s, N 1s (A) and Ti 2p (B).

Figure 6. FE-SEM TiZr SBAE (A, C), Doxycycline coated (B, D) samples in two different magnifications. Pictures A and B show 10.000x magnification of the surface, pictures C and D show 30.000x magnification.

Figure 7. In vitro after 14 days: ALP: Paired t-test; significant difference between Doxycycline electrocoated and untreated (P=0.038), data normally distributed and therefore displayed. (A)Toxicity, (B) Coll-I, (C) ALP, (D) OC: ANOVA on Ranks with Kruskal-Wallis, Multiple comparison with Dunn's Method; Data displayed as mean ± SEM.

Significance levels; highly significant for P ≤ 0.01 displayed as **, significant differences for P ≤ 0.05 displayed as *.

Figure 8. Analysis of wound fluid of implantation sites after retraction of TiZr SBAE, Hydrided and Doxycycline coated samples after time points of 4 and 8 weeks. Cytotoxicity was expressed by LDH activity (A) as a percentage of TiZr samples at 4 weeks, which were set to 100%. ALP activity was corrected for total protein (B). Values represent the mean ± SEM. Student t-test: (*) ($p < 0.05$) indicates significant differences compared to TiZr at each time point; (#) ($p < 0.05$) indicates significant differences compared to TiZr-H at each time point.

Figure 9. 4 and 8 weeks total RNA content (A) gene expression of bone formation markers Coll-I, BMP2 and OC (B, C, D), bone resorption markers H+ATPase and TRAP (E, F), pro-inflammatory IL-6 (G) and anti-inflammatory IL-10 (H) markers at the bone implant interface. Data presents changes of target genes normalized with reference genes (GAPDH and 18S), expressed as percentage of TiZr SBAE samples at 4 weeks, which represent 100%. Values are displayed as mean ± SEM. Student t-test: (*) indicates $p < 0.05$ compared toTiZr SBAE at each time point, (#) indicates $p < 0.05$ compared to Hydrided at each time point.

Figure 10. 2D BMD average of defects after retraction of TiZr SBAE, Hydrided and Doxycycline coated samples after healing times of 4 (A) and 8 weeks (B).

Figure 11. Atomic ratio of nitrogen on the surface from XPS analysis (n = 1). M: machined; MA: machined and acid etched; control 1: no cathodic polarization; control 2: cathodic polarization without Doxycycline at 5 mA/cm^2 for 3 h.

Figure 12. Detailed XPS spectra of N 1s, C 1s (A), O 1s and Ti 2p (B) on machined surface. Control 1: no cathodic polarization; control 2: cathodic polarization without Doxycycline at 5 mA/cm^2 for 3 h; 1mA-1h: cathodic polarization with Doxycycline at 1 mA/cm^2 for 1 h; 5mA-3h: cathodic polarization with Doxycycline at 5 mA/cm^2 for 3 h.

Figure 13. Topographical parameters on the surface. M: machined; MA: machined and acid etched; control 1: no cathodic polarization; control 2: cathodic polarization without Doxycycline at 5 mA/cm^2 for 3 h; 1mA-1h: cathodic polarization with Doxycycline at 1

mA/cm^2 for 1 h; 5mA-3h: cathodic polarization with Doxycycline at 5 mA/cm^2 for 3 h. Values represent the mean ± SD (n = 8). * $p < 0.05$ vs. respective control 1.

Figure 14. FE-SEM image of surfaces before and after *S. epidermidis* incubation. M: machined; MA: machined and acid etched; control 1: no cathodic polarization; control 2: cathodic polarization without Doxycycline at 5 mA/cm^2 for 3 h; 1mA-1h: cathodic polarization with Doxycycline at 1 mA/cm^2 for 1 h; 5mA-3h: cathodic polarization with Doxycycline at 5 mA/cm^2 for 3 h.

Figure 15. Optical density of biofilm (n = 8) and planktonic biomass (n = 5) of samples. M: machined; MA: machined and acid etched; control 1: no cathodic polarization; control 2: cathodic polarization without Doxycycline at 5 mA/cm^2 for 3 h; 1mA-1h: cathodic polarization with Doxycycline at 1 mA/cm^2 for 1 h; 5mA-3h: cathodic polarization with Doxycycline at 5 mA/cm^2 for 3 h. a: $p < 0.05$, aa: $p < 0.001$ vs. control 1; b: $p < 0.05$, bb: $p < 0.001$ vs. M with the same treatment; c: $p < 0.05$, cc: $p < 0.001$ vs. respective 1mA-1h.

EXPERIMENTAL SECTION

Experiment 1:

Material and Methods

Sample preparation

This study employed coin shaped samples with a diameter of 6.25 mm and a height of 2 mm. The samples were made of titanium zirconium (TiZr) with a zirconium content of 13 % - 17 % zirconium, they were grit blasted and acid etched in hydrochloric and sulfuric acid (SBAE). After production, handling and packaging was done under nitrogen cover gas and the samples were stored in 0.9 % NaCl, making the surfaces comparable to the commercially available SLActive® surface (Institute Straumann AG, Basel, Switzerland).

Before coating, the test coins were unpacked under laminar flow and washed for 5 minutes in an ultrasound bath with deionized reverse osmosis water.

The hydridation buffer for Doxycycline was a 2 M acetate buffer solution with a pH of 3 mixed at a volume of 200 ml of acetic acid (Fluka, Oslo, Norway) and sodium acetate (Sigma Aldrich, Oslo, Norway). This buffer was used directly for production of the hydrided coins from TiZr SBAE base material. For the Doxycycline coated samples, 200 mg of Doxycycline were dissolved in the buffer to achieve a final concentration of 1 mg/ml. Doxycycline Hyclate was delivered by Yangzhou Pharmaceutical (Yangzhou Pharmaceutical Co Ltd, Jiangsu, China).

The coating was performed on a custom made setup, controlled by the software LabView, providing a constant direct current of 0.65 mA on individual channels for each sample. The current was controlled by a feedback loop with a PID controller. Thereby the sample coins were connected to the cathode of the system, whereas a platinum anode served as the counter electrode.

The TiZr coins were mounted on individual titanium holders, Teflon caps protected the interface between the holder and the coin from the electrolyte. After the process, the Doxycycline coated coins were dried under nitrogen gas flow and packed in containers filled with nitrogen prior to further analysis. Control samples were washed with ultrasound

and dried under nitrogen in the same fashion as the Doxycycline coated samples. All samples were kept under cover gas before analysis as the exposure to air results in the attraction of environmental carbon, which subsequently lowers the signal intensity. Storage conditions were cold (< 10 °C), dry and dark.

Optical Microscopy

A Leica DMRB Fluorescence Microscope (Wetzlar, Germany) was used for the optical assessment of the coin surface. Main focus was homogeneity and color shade differences in comparison to untreated TiZr SBAE coins. The microscope was used with external lightning and a 1.25 x objective, which in combination with the camera system added up to a total of 12.5 x magnifications. The software for image capturing was used with exposure time of 59 ms and ISO 100 sensitivity. Images were saved in bitmap format.

Fourier transformed infrared spectroscopy (FTIR)

A PerkinElmer Spectrum 400 FT-IR/FT-NIR spectrometer (PerkinElmer, Waltham, MA, USA) was used to analyze the chemical composition of the surface. For analysis of the Doxycycline powder the ATR accessory was used, while the Doxycycline coated coins were measured with a diffuse reflectance (DR) accessory in order to compensate for the rough surfaces. The DR accessory is designed with an adjustable mirror to collect the scattered beams by the rough surface. After recording of a background spectrum from an untreated TiZr SBAE coin, the samples were scanned on three different spots each. The scans were executed on a wavelength between 4000 cm^{-1} and 1000 cm^{-1} in the mid infrared region. Every measurement was constituted of 8 individual measurements with a resolution of 8 cm^{-1}. After scanning all samples were background corrected, normalized and the Kubelka-Munk algorithm was applied to the datasets in order to quantify certain peaks with Beer's Law. Peak area quantification was carried out for two characteristic peaks of Doxycycline at 1454 cm^{-1} and at 1613 cm^{-1}. Four reference coins were spiked with 10 µl of water dissolved Doxycycline to obtain a defined Doxycycline coverage on the reference sample. The amount of applied Doxycycline was 11.5 µg per coin. The solution covered the whole top coin surface and samples were dried under nitrogen flow. The spiked reference coins were measured in the same fashion as Doxycycline coated and control samples and their average signal intensity was calculated over all measurements for the characteristic peaks.

UV- VIS Spectroscopy (UV-VIS)

A release study was executed to assess the total amount of Doxycycline released from the coins when submerged in liquid. The release was performed in a solution of 60 % acetonitrile and 3 % trifluoroacetic acid (ACN-TFA). Two time points with three Doxycycline coated samples per group were analysed after release in ACN-TFA. The release fluids were analysed after 6 hours and after 7 days in release with a Nano-Drop ND 1000 spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts) in UV-VIS mode. A calibration curve was recorded at Doxycycline concentrations between 0.005 mg/ml and 0.25 mg/ml.

Secondary Ion Mass Spectroscopy (SIMS)

Depth profiles of carbon and hydrogen were measured by secondary ion mass spectroscopy (Cameca IMS 7f, Paris, France) on the test coins. The analysis was carried out for ^{1}H and ^{12}C ions. One control sample was included as background and a hydrided sample was used as control for carbon contamination. A 50 nA primary beam of 15 keV Cs^{+} was used for rastering over a surface area of 200 μm x 200 μm whereas negative secondary ions were collected from central part (67 μm x 67 μm) of the crater at room temperature. Doxycycline inclusion in the surface was detected by analysis of the depth profile of the ^{12}C isotope.

The depth of each sputtering crater was analyzed by a blue light profilometer (SensofarPLμ 2300, Sensofar-Tech S.L., Terrassa, Spain), and the aspect heights calculated using an advanced software package (Sensomap, Sensofar-Tech S.L., Terrassa, Spain). The crater depth was measured with a 50 x objective (50 x PI, Nikon, Tokyo, Japan) over an extended topography of 2 x 2 images, each with a viewing area of 253 μm x 190 μm at 20 % overlapping. The result data from the SIMS measurement was calibrated according to the obtained crater depth.

X-ray photoelectron Spectroscopy (XPS)

The XPS analysis was carried out on an Axis Ultra^{DLD} XP spectrometer (Kratos Analytical, Manchester, UK) using monochromatic Al Kα radiation (hv = 1486.6 eV). Survey spectra were recorded in a range between 1100 eV and 0 eV binding energy (BE). Detail spectra were acquired in the energy regions of O 1s, Ti 2p, C 1s, N 1s and Zr 3d. The instrument resolution was 1.1 eV for the survey scans and 0.55 eV for the detail scans. The analysis area was 300 μm x 700 μm.

The samples used for the XPS analysis were not stored under nitrogen cover gas after coating and washing. Samples were mounted on a sample bar with conductive carbon tape. The energy shift due to surface charging was below 1 eV based on the C 1s peak position relative to established BEs, therefore the experiment was performed without charge compensation. All Bes were referenced to the C 1s peak 284.5 eV.

Field Emission Scanning Electron Microscope (Fe-SEM)

The micro- and sub-micro structural analysis of the surface topography and morphology was performed with a FEI Quanta 200 FE-SEM (FEI Hillsboro, Oregon, USA). The samples were platinum sputtered prior to analysis and mounted on a 45° aluminum rack with conductive carbon tape. Images were acquired with a working distance between 5 mm and 7 mm. Acceleration voltages of 3 kV– 5 kV were applied to achieve magnifications of 10.000x and 30.000x. High vacuum (HV) operation mode was used for the examination of all samples.

In vitro study

Cell culture

The mouse osteoblastic cell line MC3T3-E1 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). MC3T3-E1 cells were routinely cultured at 37 °C in a humidified atmosphere of 5% CO_2, and maintained in α-MEM supplemented with 10% fetal calf serum (FCS) and antibiotics (50 IU penicillin/ml and 50 μg streptomycin/ml). Cells were subcultured 1:50 before reaching confluency using PBS and trypsin/EDTA. All experiments were performed in the same passage of the MC3T3-E1 cells (passage 16).

The coins were placed in a well plate and$7x10^3$cells were seeded on each well to study cell differentiation and cell toxicity. The same numbers of cells were cultured in parallel in plastic tissue culture dishes in all experiments. Trypan blue stain was used to determine total and viable cell number. For the experiments, MC3T3-E1 cells were maintained for 14 days on the implants in α-MEM supplemented with 10% FCS and antibiotics. Culture media was changed every other day. Culture media was collected after 24 hours to test the toxicity of the treatments and of the different implant surfaces (LDH activity). To study cell differentiation, cells were harvested at 14 days and collagen 1 (Coll-1), alkaline phosphatase (ALP) and osteocalcin (OC) gene expression were analyzed using real-time RT-PCR.

Lactate dehydrogenase (LDH) activity in the culture media

The presence of LDH activity in the culture media was used as an index of cell death. LDH activity is determined spectrophotometrically after 30 min incubation at 25°C using 50 µl of culture media and adding 50 µl of the reaction mixture, by measuring the oxidation of NADH at 490 nm in the presence of pyruvate, according to the manufacturer's kit instructions (Cytotoxicity Detection kit, Roche Diagnostics).Positive control (100%) was cell culture media from cells seeded on plastic culture dishes and incubated with Triton X-100 at 1%. Negative control (0 %) was cell culture media from cells seeded on plastic culture dishes without any treatment.

RNA isolation

Total RNA was isolated with Tripure (Roche Diagnostics), following the instructions of the manufacturer. RNA was quantified using a spectrophotometer set at 260 nm (Nanodrop).

Real-time RT-PCR

Real-time PCR was performed for two *housekeeping genes*, 18S ribosomal RNA (18S rRNA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and 3*target genes,* ALP, collagen-I and osteocalcin. The primer sequences are detailed in Table 1.

Gene	Primer sequence
ALP	S 5´- AACCCAGACACAAGCATTCC -3´ (SEQ ID NO:1)
	A 5´- GAGAGCGAAGGGTCAGTCAG -3´ (SEQ ID NO:2)
Collagen-I	S 5´- AGAGCATGACCGATGGATTC -3´ (SEQ ID NO:3)
	A 5´- CCTTCTTGAGGTTGCCAGTC -3´ (SEQ ID NO:4)
Osteocalcin	S 5´- CCGGGAGCAGTGTGAGCTTA -3´ (SEQ ID NO:5)
	A 5´- TAGATGCGTTTGTAGGCGGTC -3´ (SEQ ID NO:6)
18S rRNA	S 5'- GTAACCCGTTGAACCCCATT -3'

		(SEQ NO:7)
		A 5´- CCATCCAATCGGTAGTAGCG -3´
		(SEQ ID NO:8)
GAPDH		S 5´- ACCCAGAAGACTGTGGATGG -3´
		(SEQ ID NO:9)
		A 5´- CACATTGGGGGTAGGAACAC -3´
		(SEQ ID NO:10)

Table 1 Primers used in the real-time PCR of housekeeping and target genes.

The same amount of total RNA (0.25µg) from each sample was reverse transcribed to cDNA at 37 °C for 60 min in a final volume of 20µl, using High Capacity RNA to cDNA kit (Applied Biosystems). Each cDNA was diluted 1/4.

Real-time PCR was performed in the Lightcycler 480® (Roche Diagnostics, Mannheim, Germany) using SYBR green detection. Each reaction contained 7 µl Lightcycler-FastStart DNA Master^{PLUS} SYBR Green I (*containing Fast Start Taq polymerase, reaction buffer, dNTPs mix, SYBRGreen I dye and* $MgCl_2$), 0.5 µM of each, the sense and the antisense specific primers and 3µl of the cDNA dilution in a final volume of 10 µl. The amplification program consisted of a preincubation step for denaturation of the template cDNA (5 min 95 °C), followed by 45 cycles consisting of a denaturation step (10 s 95 °C), an annealing step (10 s 60 °C, except for ALP that was 5s 65ºC) and an extension step (10 s 72 °C, except for ALP that was 12s 65ºC). After each cycle, fluorescence was measured at 72 °C. A negative control without cDNA template was run in each assay.

Real-time efficiencies were calculated from the given slopes in the LightCycler 480 software using serial dilutions, showing all the investigated transcripts high real-time PCR efficiency rates, and high linearity when different concentrations are used. PCR products were subjected to a melting curve analysis on the LightCycler and subsequently 2% agarose/TAE gel electrophoresis to confirm amplification specificity, Tm and amplicon size, respectively.

Relative quantification after PCR was calculated by dividing the concentration of the target gene in each sample by the mean of the concentration of the two reference genes (housekeeping genes) in the same sample using the Advanced relative quantification

method provided by the LightCycler 480 analysis software version 1.5 (Roche Diagnostics, Mannheim, Germany).

In vivo rabbit study

Animals

An animal study for assessment of the in vivo performance of SVS and Doxycycline was executed with 14 Grey Bastard Chinchillas. The animals were 6 months old and had a weight of 2.86 kg in average (Crl:CHB, Charles River Laboratories International, Inc., Research Models and Services, Sulzfeld, Germany). The animals were kept in cages during the experimental period the two days post-surgery, and one day prior to euthanization. Room temperature, humidity and diet were standardized. Sedation and anesthesia were done according to the Norwegian School of Veterinary Science, Laboratory Animal Unit; SOP on anesthesia of rabbits. The experiments had been approved by the Norwegian Animal Research Authority (NARA) and registered by this authority. The procedures have thus been conducted in accordance with the Animal Welfare Act of June 01st 2010, No 94 and Regulation on Animal Experimentation of January 15th 1996.

Surgical procedures, sedation and euthanasia

Sedation of the rabbits as well as euthanasia and all surgical procedures were done as described by Rønold et al. (Rønold HJ, Ellingsen JE. The use of a coin shaped implant for direct in situ measurement of attachment strength for osseointegrating biomaterial surfaces. Biomaterials. 2002;23(10):2201-9. doi:10.1016/s0142-9612(01)00353-2.). The only difference to this process was the integration of a central defect, drilled into the bone marrow region, with a diameter of 3.5 mm as described by Haugen et al. (Haugen HJ, Monjo M, Rubert M, Verket A, Lyngstadaas SP, Ellingsen JE et al. Porous ceramic titanium dioxide scaffolds promote bone formation in rabbit peri-implant cortical defect model. Acta Biomater. (0). doi:10.1016/j.actbio.2012.09.009.).

Wound fluid analysis

After implant detachment, wound fluid sampling and LDH analysis were carried out analogous to Haugen et al.. Furthermore an aliquot of 25 µl of wound fluid in duplicate was assayed for alkaline phosphatase (ALP) activity. ALP activity as well as total protein were assessed according to Monjo et al. (Monjo M, Rubert M, Wohlfahrt JC, Rønold HJ,

Ellingsen JE, Lyngstadaas SP. In Vivo Performance Of Absorbable Collagen Sponges With Rosuvastatin In Critical-Size Cortical Bone Defects. Acta Biomater. 2009.).

Gene expression

Total RNA was isolated from peri-implant bone tissue as described already elsewhere (Monjo M, Lamolle SF, Lyngstadaas SP, Rønold HJ, Ellingsen JE. In vivo expression of osteogenic markers and bone mineral density at the surface of fluoride-modified titanium implants. Biomaterials. 2008;29(28):3771-80.).

An amount of 280 ng of extracted RNA was reverse transcribed to cDNA at 42 °C for 60 min using High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA), containing both oligo(dT) and random hexamers. Each cDNA was diluted 1/4 and aliquots were frozen (-20 °C) until the PCR reactions were carried out.

Real-time PCR was performed with a Lightcycler-480® (Roche Diagnostics, Mannheim, Germany) using SYBR green detection. Real-time PCR was done for three housekeeping genes: 18S ribosomal RNA (18S rRNA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-actin, and nine target genes: bone morphogenetic protein 2 (BMP2), collagen type I (Coll-I), osteocalcin (OC), vacuolar type proton ATPase (H+-ATPase), tartrate-resistant acid phosphatase (TRAP), interleukin-6 (IL-6) and interleukin-10 (IL-10). Each reaction contained 7 µl Lightcycler-FastStart DNA MasterPLUS SYBR Green I (containing Fast Start Taq polymerase, reaction buffer, dNTPs mix, SYBRGreen I dye and MgCl2), 0.5 µM of each, the sense and the antisense specific primers and 3µl of the cDNA dilution in a final volume of 10 µl. The amplification program consisted of a preincubation step for denaturation of the template cDNA (10 min 95 °C), followed by 45 cycles consisting of a denaturation step (10 s 95 °C), an annealing step (8-10 s 60 °C) and an extension step (10 s 72 °C). After each cycle, fluorescence was measured at 72 °C (λex 470 nm, λem 530 nm). A negative control without cDNA template was run in each assay.

Real-time efficiencies were calculated from the given slopes in the Lightcycler-480® software using serial dilutions, showing all the investigated transcripts high real-time PCR efficiency rates, and high linearity when different concentrations are used. PCR products were subjected to a melting curve analysis on the LightCycler and subsequently 2%

agarose/TAE gel electrophoresis to confirm amplification specificity, Tm and amplicon size, respectively.

All samples were normalized by the geometric mean of the expression levels of β-actin and GADPH and fold changes were related to 4 weeks of implant placement using the mathematical model described by Pfaffl (Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 2002;30(9):e36-e.), as follows: ratio = $E_{target}^{\Delta Cp\ target\ (mean\ control\ 4\ weeks - sample)} / E_{reference}^{\Delta Cp\ target\ (mean\ control\ 4\ weeks - sample)}$, where Cp is the is the crossing point of the reaction amplification curve as determined by the Lightcycler-480® software. Stability of reference genes was calculated using the BestKeeper tool (Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations. Biotechnol Lett. 2004;26(6):509-15.). Thus, values were expressed as a percentage of TiZr (reference implant) at 4 weeks, which were set to 100 %.

µ-CT analysis

After extraction of the coins and wound fluid sampling, the tibia samples were prepared for analysis and scanned with µ-CT as described elsewhere. Furthermore reconstruction and bone mineral density (BMD) calibration was done according to the same description (Monjo M, Lamolle SF, Lyngstadaas SP, Rønold HJ, Ellingsen JE. In vivo expression of osteogenic markers and bone mineral density at the surface of fluoride-modified titanium implants. Biomaterials. 2008;29(28):3771-80.).

VOI for each sample was chosen: a cylinder with a diameter of 3.5 mm and a height of 2.5 mm starting from the outer cortical bone defect (which was in contact with the implant surface) and towards the marrow space. This VOI is identical to the defect created in the cortical bone prior to placement of the samples. Furthermore analysis of the BMD devolution dependent on the distance to the implant surface (2D BMD) was executed with a diameter of 6.2 mm and a height of 2.5 mm. The threshold level was set from 65 to 255.

Statistics

The statistical significance of the results from the in vitro study was assessed as follows.

Analysis of the raw data was carried out with Excel 2007 (Microsoft, Redmond, USA) and SigmaPlot 11.0 (Systat Software Inc, Chicago, USA). All datasets were examined for parametric or non-parametric distributions (Shapiro-Wilk test). For the in vitro study, ANOVA on Ranks with Kruskal-Wallis multiple comparison was used for the assessment of OC. One way ANOVA was used for cytotoxicity Coll-I and osteocalcin. ALP was analyzed with paired t-test. The statistical analysis of the in vivo results was done with student t-test and Doxycycline coated samples were compared to TiZr SBAE and hydrided at each time point. Differences were considered significant (*) for $p \leq 0.05$ and highly significant (**) for $p \leq 0.01$, and labeled respectively. For differences compared to the hydrided group (#) for $p \leq 0.05$ and highly significant (##) for $p \leq 0.01$ were used, and labeled respectively All parametric data are presented as mean values ± standard deviation and non-parametric data as median ± interquartile range.

Results

Optical Microscope

As the Doxycycline coated samples showed a different color after coating compared to the grey color of the control TiZr SBAE samples, a low resolution microscopy analysis was used to document the coin color. Figure 1 displays pictures of the surfaces recorded with a microscope. Doxycycline coated coins adopted a light brownish color after coating, which was equally distributed over the sample surface.

FTIR

The FTIR was used to assess the integrity of Doxycycline on the surface after coating and to quantify its amount. All samples analyzed in FTIR showed stable expressions of the significant peaks at 1613 cm^{-1} and 1454 cm^{-1} as displayed in Figure 2. Furthermore peaks at 1558 cm^{-1}, 1576 cm^{-1}, 1651 cm^{-1}, 1660 cm^{-1} and 1672 cm^{-1} were detected. In comparison to the spiked sample, the coated samples showed higher absorbance values for both of the significant peaks (Figure 2). Spiked and coated samples showed a slight shift of the spectrum to higher wavelengths in comparison to the spectrum of pure Doxy. Pure Doxycycline showed mainly the absorption peaks at 1611 cm^{-1}, 1575 cm^{-1}, 1555 cm^{-1} and 1459 cm^{-1}. In addition a weaker peak at 1662 cm^{-1} appeared in this spectrum. The peaks at 1611 cm^{-1}, 1575 cm^{-1} and 1555 cm^{-1} were pronounced stronger in the spectrum of pure Doxycycline in comparison to the spiked and coated samples. Therein the peaks at 1575 cm^{-1} and 1555 cm^{-1} were expressed stronger than the ones at 1611 cm^{-1} and 1662 cm^{-1}, relative to their respective spiked and coated counterparts. The uniformity of

the four coated samples analyzed was visible in the spectra as well as the compliance of the coated coins with the Doxycycline powder.

The amount of Doxycycline on the coins was obtained by comparison of the samples absorbance values with the values of a spiked sample with a known concentration of Doxycycline (Table 2). The peak area underneath the significant peaks was calculated and set into relation to the known concentration on the spiked coin. The distribution was homogenous for the individual peaks, yet comparing the loading calculation for the peak at 1454 cm^{-1} to the peak at 1613 cm^{-1}, a six times higher Doxycycline concentration was found for the peak at 1613 cm^{-1} (Table 2).

	Doxy coated mean	**spiked**
Peak 1454 cm^{-1} Doxy (µg/cm2)	6.9	11.5
Peak 1613 cm^{-1} Doxy (µg/cm2)	45.1	11.5

Table 2 Amount of Doxycycline over four coins - 3 measurements per coin. Spiked consists of 4 samples with 3 measurements each.

UV-VIS

As the quantification of loading with the FTIR provided a rather rough estimation, a release experiment was performed over a total period of 7 days. A first approach with water as release medium was not successful. Therefore, a solution containing 40% acetonitrile with 3% trifluoroacetic acid (ACN-TFA) was used as a release medium. With this method it was possible to detect a release of 10.27 µg/cm² after 6 hours and 140.97 µg/cm² after 7 days at a wavelength of 263 nm. The absorbance was measured at 263 nm as some peak shifting was detected in comparison to the literature.

SIMS

SIMS measurements were obtained to confirm the integration of the Doxycycline in the depth of the material. Figure 4 A shows the depth profile of a Doxycycline coated sample in comparison to a TiZr SBAE control sample and a hydrided sample. Doxycycline inclusion was detected up to a depth of 0.44 µm into the surface. At this sputtering depth, the concentration of the coated sample was approximately equal to the baseline carbon concentration of the bulk material. While the Doxycycline coated sample showed very high

surface carbon levels, the control sample showed only slightly increased concentration in comparison to the baseline carbon level of the bulk material. The carbon concentration of the hydrided sample was elevated on the outer surface, but decreased rapidly.

Due to the proposed importance of hydrogen for the binding of Doxy, Figure 4 B displays the hydrogen profiles of a control and a Doxycycline coated sample. It could be seen that the control TiZr SBAE and the hydrided TiZr samples showed similar maximum concentration of hydrogen compared to the Doxycycline coated sample. Furthermore, the depth of the hydrogen layer was approximately 1 µm for all samples.

XPS

The XPS analysis was performed to evaluate the chemical bonding of Doxycycline on the surface. Figure 5 shows the detail spectra of C 1s, N 1s and Ti 2p. It could be observed that the intensity of carbon and nitrogen peaks generally increased, whereas the peaks for titanium were strongly reduced on the Doxycycline coated sample in comparison to the control TiZr SBAE. The Ti 2p 3/2 peak was found at 459 eV and the Ti 2p 1/2 peak 5.5 eV higher. Whereas for control both Ti 2p peaks were well visible, the Ti 2p1/2 peak could not be observed on the Doxycycline coated sample. In addition, the carbon signal exhibited another peak at 286 eV and one at 287.5 eV. Nitrogen was present on both samples, exhibiting the major peak at 399.5 eV, and a smaller one at 401.6 eV. Furthermore the Doxycycline coated sample had another peak at 402 eV, which could not be observed on TiZr SBAE.

SEM

A SEM study was conducted to optically assess the samples' surfaces. SEM images with 10.000x resolution revealed a smoother surface morphology for the Doxycycline coated samples compared to TiZr SBAE (Figure 7 A, B). Peaks on the Doxycycline coated surface appeared smoothened and sharp edges were not visible. Furthermore the surface showed no deep pits. In contrast, the control samples seemed rougher with sharp peaks on the edges. Despite those observations surface micro topography was similar for the two groups. The higher magnification (30.000x) images emphasized the differences between the samples (Figure 6 C, D). At this magnification, structures on Doxycycline coated sample expressed few topographical features and distinct structures were not visible. Edges were rounded and concave structures on the samples were smooth. In contrast, structures on the control sample were better visible, pits were identifiable and peaks showed sharp edges. In addition to that the Doxycycline coated samples showed

white spherical structures on the sample surface that could not be observed on the control sample.

In Vitro

The in vitro study was executed to evaluate the effect of the Doxycycline coated TiZr surfaces on the biological response of murine osteoblastic cells. Doxycycline coated and TiZr SBAE samples expressed generally low cytotoxicity values and differences between the samples were minimal and not significant. Cytotoxicity based on LDH activity, was generally lower in all TiZr surfaces compared to the tissue culture plastic reference group. ALP and OC were significantly increased on Doxycycline coated samples after 14 days, whereas Coll-I was slightly lower, without significance (Figure 7).

In vivo

Wound fluid analysis

Wound fluid sampled from the implantation site after extraction of the implants was analyzed with regard to LDH activity as an indicator of cytotoxicity, ALP activity and protein content (Figure 8). Cytotoxicity according to LDH activity of TiZr at 4 weeks served as control and was set to 100 %. At the 4 week's time point the hydrided group expressed lower LDH activity than TiZr SBAE, while Doxycycline coated samples had lower activity compared to hydrided samples. Nevertheless, differences were not significant. At 8 weeks TiZr SBAE samples expressed slightly lower LDH activity compared to 4 weeks TiZr SBAE. In contrast, LDH activity for hydride and Doxycycline coated groups was higher than TiZr SBAE. This increase was found significant for the Doxycycline coated group (Figure 8 A).

The ALP activity was corrected for total protein content in the wound fluid to calculate specific ALP activity. The resulting factor was increased at 4 weeks for hydrided samples and significantly increase was found for Doxycycline coated samples compared to TiZr SBAE. As LDH activity is gradually decreasing during bone healing, hydrided and Doxycycline coated groups were found significantly lower than TiZr SBAE at 8 weeks (Figure 8 B).

Peri implant bone tissue analysis

Total RNA in the 4 weeks samples was relatively similar for all groups. In contrast, at 8 weeks of healing time, the amount of RNA was significantly increased on hydrided and Doxycycline coated samples compared to TiZr SBAE (Figure 9 A).

The levels of Coll-I were decreased significantly for hydrided samples at 4 weeks in comparison to TiZr SBAE, while no significant difference was observed for the Doxycycline coated group. Coll-I an early marker of osteoblast differentiation, was significantly increased in Doxycycline coated samples, compared to TiZr SBAE and hydrided samples, after 8 weeks of healing time (Figure 9 B).

Levels of BMP-2 gene expression showed no significant differences at any of the time points, yet a trend of increasing BMP-2 expression with healing time was observed. Furthermore, at both time points, hydrided samples showed higher expressions than TiZr SBAE but lower expression than Doxycycline coated samples (Figure 9 C).

Although OC gene expression did not change significantly, its expression was elevated after 8 weeks of healing time for hydrided and Doxycycline coated samples, compared to TiZr SBAE. Therein hydrided samples had higher OC gene expression levels than Doxycycline coated samples (Figure 9 D).

Both, H+-ATPase and TRAP, indicators of bone resorption, did not exhibit significant differences in mRNA levels for all examined groups at the 4 weeks. Nevertheless, H+-ATPase levels were significantly increased after 8 weeks in Doxycycline coated samples compared to the hydrided group and TRAP was found significantly lower than TiZr SBAE (Figure 9 E, F).

The inflammatory response to the tested surfaces was analyzed by gene expression of the pro-inflammation IL-6 cytokines and the anti-inflammatory IL-10 cytokines (Figure 9 G, H). IL-6 expression was increased significantly for Doxycycline coated samples after 8 weeks compared to TiZr SBAE, while no significant differences were found for IL-10. Nevertheless, IL-10 showed a trend of increasing gene expression after 8 weeks in Doxycycline coated samples compared to TiZr SBAE and hydrided.

2D BMD

The devolution of BMD was analyzed with increasing distance from the sample surface in the defect regions of the tibia pieces (Figure 10). Highest depth integration and density of

bone could be seen for the hydrided group after 4 weeks of healing time. The group with Doxycycline coated samples had similar maximum BMD values, but those were found at a lower distance from the implant compared to hydrided. Yet, both of the treated groups had higher BMD values compared to the TiZr SBAE control (Figure 10 A).

After 8 weeks, the hydrided group expressed lower maximum BMD values than hydrided and Doxycycline coated groups. Furthermore, the depth integration of bone was higher for Doxycycline coated samples than in TiZr SBAE and hydrided (Figure 10 B).

3D bone properties

The analysis of the bone in the 3.5 mm diameter volume of interest showed significant increase of bone surface in the Doxycycline coated group and the factor relating bone surface to tissue volume compared to the TiZr SBAE group. The latter parameter was also significant for the hydrided group. While the BMD showed only slight increase for hydrided and Doxycycline coated compared to TiZr SBAE, total bone volume increased for both treated groups approximately 16 % (Table 3).

	BMD (g/cm^3)		Bone surface/ tissue volume (1/µm)		Bone surface/ Volume ratio (1/µm)		Bone surface ($µm^2$)		Bone volume fraction (%)		Bone volume ($µm^3$)	
weeks	**4**	**8**	**4**	**8**	**4**	**8**	**4**	**8**	**4**	**8**	**4**	**8**
TiZr SBAE	5.34 ±0.371	5.26 ±0.371	7.30 ±0.919	8.16 ±0.919	19.00 ±2.076	21.68 ±2.076	176.97 ±22.21	197.21 ±22.21	39.11 ±4.643	40.01 ±4.643	9.49 ±1.125	9.67 ±1.125
Hydrided	5.42 ±0.415	5.09 ±0.415	11.47 *, # ±1.027	6.59 # ±1.027	25.34 ±2.321	19.22 ±2.321	277.55 # ±24.832	159.26 # ±24.83	45.56 ±5.191	39.90 ±5.191	11.02 ±1.258	9.65 ±1.258
Doxy coated	5.46 ±0.415	5.72 ±0.415	11.69 * ±1.027	10.20 ±1.027	26.87 ±2.321	22.93 ±2.321	283.30 * ±24.832	247.57 ±24.832	45.34 ±5.191	49.63 ±5.191	10.99 ±1.258	12.04 ±1.258

Table 3 3D parameter analysis of defect after 4 weeks. Statistical analysis with two way ANOVA. (*) indicates $p < 0.05$ compared toTiZr SBAE at each time point, (#) indicates $p < 0.05$ compared to same treatment at the other time point. Values are displayed as mean ± SEM.

After 8 weeks of healing time the increase in BMD amounted to 8.7 % and in bone volume to 24 % in the Doxycycline coated group, compared to TiZr SBAE . Bone surface, bone surface / volume ratio and bone surface / tissue volume were increased furthermore. In contrast to that, the hydrided group had lower values compared to TiZr SBAE for all parameters (Figure 10 B).

Conclusion

The study demonstrated the efficiency of the process of coating a TiZr implant surface with Doxy. A hypothesis on the mechanism of binding based on a zwitterionic mechanism was postulated. It was possible to verify the integrity of the molecule on the surface and show its integration into the outer layer of the sample surface. In addition, the in vitro results demonstrated enhanced bioactivity of the Doxycycline coated samples after 14 days in comparison to TiZr SBAE, without negative effects on cell viability. Furthermore Doxycycline coated samples had positive effects on RNA content as well as Coll-I, BMP2 and OC mRNA levels in vivo after 8 weeks of healing time. Higher BMD values and total bone volume were detected in µ-CT after 8 weeks. Therefore it was concluded that Doxycycline coated surfaces positively influenced bone remodeling.

Experiment 2:

In this study, a method is introduced to coat Doxycycline onto dental implant abutment surface for the purpose of preventing biofilm formation on the surface. In addition to evaluating the antibacterial effect of Doxy-coated abutment surface, the abutment surface after Doxycycline coating was analyzed, and the effect of current density and reaction time on the amount of Doxycycline on coated surface studied.

Materials and methods

Doxycycline coating on the surface

Titanium-zirconium coin-shaped samples (TiZr, 13~17% zirconium, thickness 2 mm, diameter 6.2 mm/4.4 mm for agar plate test) were kindly provided by Institut Straumann (Basel, Switzerland): machined (M) and machined, acid etched in HCl/H_2SO_4 (MA). Upon receiving, M coins were cleaned in a succession of ultrasonic baths with NaOH (40 wt.%), HNO_3 (50 wt.%) and Milli-Q water (18.2 MΩ·cm, Millipore, Billerica, MA, US) (Lamolle et al., 2009; Xing et al., 2013). MA were washed in ultrasonic bath with Milli-Q water. M and MA were further coated with Doxycycline as described by Walter et al (Walter et al., 2013). Briefly, the samples were treated by cathodic polarization in 2 M acetic acid/sodium acetate buffer containing 1 mg/ml Doxycycline hyclate (98%, AK Scientific, Inc. Union City, CA, US). The samples were then rinsed with Milli-Q water for 10 seconds and dried under nitrogen gas for 30 minutes. Three current densities (1, 5 and 15 mA/cm^2) and three coating durations (1, 3 and 5 h) were used to evaluate the effect of current density and reaction time on Doxycycline coating. Samples without cathodic polarization were used as

control 1. Samples treated by cathodic polarization at 5 mA/cm^2 for 3 h but without Doxycycline were used as control 2.

Surface chemistry

X-ray photoelectron spectroscopy (XPS, Axis Ultra^{DLD} XP spectrometer, Kratos Analytical, Manchester, UK) using monochromatic aluminum Kα X-rays (1486.69 eV/15kV) was employed to quantify elemental compositions on the topmost surface (n = 1). The analysis area was 300 × 700 μm^2 and the detection limit was 0.1~0.5 at.%. Survey spectra were recorded between 1100 eV and 0 eV binding energy. Detailed spectra were acquired in the energy regions of N 1s, C 1s, O 1s and Ti 2p. XPS spectra were analyzed by CasaXPS (Casa Software Ltd, Teignmouth, UK).

Surface topography

Surface topography was examined on 255 × 191 μm^2 area (n = 8) by a blue light laser profilometer (Sensofar Plμ 2300, Terrassa, Spain) with 50× Nikon objective. Three surface amplitude parameters (S_a, S_{sk} and S_{ku}) and one hybrid parameter (S_{dr}) were calculated with SensoMap software (SensoMap Plus 4.1, Sensofar, Terrassa, Spain).
Field emission scanning electron microscope (FE-SEM, Quanta 200F, FEI, Eindhoven, The Netherlands) was used to image surface morphology.

Biofilm and planktonic assay

S. epidermidis (ATCC-35984) was cultured in Brain Heart Infusion (BHI) broth (Oxoid Ltd, Basingstoke, UK) at 37°C in an aerobic atmosphere. Before optical density (OD_{600}) reached 0.4~0.5, the cells were diluted 1:100 in BHI and incubated overnight before inoculation to the samples.

Eight groups were selected for bacterial test (four from both M and MA): samples without cathodic polarization (control 1), with cathodic polarization but no Doxycycline at 5 mA/cm^2 for 3 h (control 2), coated with Doxycycline at 1 mA/cm^2 for 1 h (1mA-1h) and 5 mA/cm^2 for 3 h (5mA-3h). The overnight culture was diluted 1:100 in BHI (OD_{600} = ~0.02). 0.5 ml of the cell suspension was inoculated to the samples in 48 multi-well plate (Nunc Hydrocell, Thermo Fisher Scientific-Nunc AS, Roskilde, Denmark) and incubated for 6 hours.
The biofilm on the samples (n = 8) were stained with 0.1% safranin (Merck KGaA, Darmstadt, Germany) for 10 min and washed with PBS. After drying, the safranin stain

was dissolved in 30 % acetic acid and measured at OD_{530}. The samples without bacteria were used as blank for each corresponding group. The planktonic cells were measured at OD_{600} (n = 5). BHI without bacteria were used as blank.

The biofilms were fixed with 2.5 % glutaraldehyde and examined by FE-SEM (n = 2).

Agar diffusion test

S. epidermidis was cultured in BHI broth until OD_{600} reached 0.1. 100 µL of the bacterial culture was spread out on BHI agar plate (Becton Dickinson, Sparks, MD, US) using an L-shaped spreader. The samples were placed on the plates (n = 9) with the modified surface facing down and incubated with bacteria for 16~18 hours. The diameter of the inhibition zone was then measured.

Statistical analyses

Topographical parameters (S_a, S_{sk}, S_{ku} and S_{dr}) and biomass results passed normality test (Shapiro-Wilk method) and were presented as mean values ± SD (standard deviation). Differences of topographical parameters compared to control 1 were assessed by One-Way ANOVA followed by multiple comparisons versus control group (Holm-Sidak). Significance levels were notified by * if $p < 0.05$ and ** if $p < 0.001$. All pairwise multiple comparison procedures (Holm-Sidak) were used to compare biomass differences. The agar diffusion result did not pass normality test (Shapiro-Wilk) and was presented as descriptive statistics with median and range. Friedman test was used to assess differences among M groups and among MA groups. Multiple comparisons of every two groups were assessed with post hoc analysis (Wilcoxon Signed Rank Test). A Bonferroni adjustment was used for corrected significance level (significantly different if $p < 0.017$). Differences between M and MA surfaces with the same treatment, and between 5mA-3h and 1mA-1h were analyzed by Wilcoxon Signed Rank Test (significantly different if $p < 0.05$). SigmaPlot 12.0 (Systat Software, San Jose, CA) and IBM SPSS Statistics 20 were used for statistical analyses.

Results

Surface chemistry

Elements of N, C, O, Ti and Zr were detected on all the samples by XPS analysis (**Table 4**). The Doxy-coated abutments showed increase of nitrogen and carbon ratios compared to control samples, indicating the effective coating of Doxycycline onto the surfaces. Doxycycline content on the surface was increasing with polarization time as indicated by

the increased nitrogen ratio on surfaces treated with longer time (**Figure 11**). Higher current density resulted in more Doxycycline on the surface for both M and MA abutments when coating time was 1 h. However, current density did not seem to affect Doxycycline amount when samples were coated for 3 h and 5 h.

Table 4. Atomic ratios of N, C, O, Ti and Zr on the surface from XPS analysis (n = 1). M: machined; MA: machined and acid etched; control 1: no cathodic polarization; control 2: cathodic polarization without Doxycycline at 5 mA/cm^2 for 3 h.

		M (at. %)					**MA** (at. %)				
Current density	**Time**	**N**	**C**	**O**	**Ti**	**Zr**	**N**	**C**	**O**	**Ti**	**Zr**
1 mA/cm^2	1 h	2.8	58.31	32.02	6.07	0.79	2.67	59.83	31.52	5.15	0.83
	3 h	3.68	69.85	25.85	0.52	0.1	3.66	72.2	23.98	0.16	0.01
	5 h	3.96	72.13	23.86	0.04	0.02	4.13	71.94	23.87	0.05	0
5 mA/cm^2	1 h	3.52	69.4	25.65	1.22	0.21	3.06	69.5	25.74	1.47	0.24
	3 h	3.77	73.53	22.68	0.02	0	3.32	72.38	24.09	0.16	0.05
	5 h	3.84	71.15	24.95	0.05	0.02	4.1	72.09	23.78	0.04	0
15 mA/cm^2	1 h	3.8	71.4	24.41	0.32	0.06	3.36	72.13	23.95	0.43	0.13
	3 h	3.82	70.98	25.16	0.02	0.03	3.97	73.08	22.83	0.12	0
	5 h	4.17	71.73	24.09	0	0	4.19	71.96	23.8	0.06	0
control 1		0.74	24.82	53.49	18.88	2.07	0.15	33.07	47.61	16.71	2.47
control 2		0.61	35.29	52.65	10.09	1.35	0.44	30.9	51.47	14.94	2.24

The detailed spectra of N 1s, C 1s, O 1s and Ti 2p for machined surfaces containing high (5mA-3h) and low (1mA-1h) Doxycycline amount are shown in **Figure 12**. The two N 1s peaks on Doxy-coated samples suggested two binding sites of Doxycycline onto the surface: $(CH_3)_2$-N+ and O=C-NH_3+ (**Figure 12A**). The intensity of the two characteristic bonds of Doxycycline was higher on 5mA-3h sample than on the 1mA-1h sample. A low intensity peak at similar binding energy could also be detected on the control groups, which was assigned to adsorbed N_2 on the surface as the prepared samples were stored in nitrogen cover gas. C-N/N-C=O bonds and more C-H/C-C, OH/CO bonds were introduced onto Doxy-coated samples (**Figure 12B**, **Figure 12C**). Titanium oxide decreased from control 1 to control 2, followed by 1mA-1h and 5mA-3h (**Figure 12C**, **Figure 12D**). The spectra of MA samples showed similar trend as M (data not shown).

Surface topography

Topographical parameters were measured for the abutment surface. Surface area roughness (S_a, absolute average height deviation from a mean plane) of MA groups were higher than that of M groups (**Figure 13A**). Three modified M surfaces showed significantly higher roughness than control 1 ($p < 0.05$). No significant difference was observed among MA groups. Skewness of the height distribution (S_{sk}) represents the shape of surfaces to be dominated by valleys ($S_{sk} < 0$) or peaks ($S_{sk} > 0$). M groups showed nearly equivalent existence of peaks and valleys, while MA groups mainly consisted of peaks on the plane (**Figure 13B**). S_{ku} indicates the kurtosis of the height distribution. Three modified M groups had significantly less sharp peaks and valleys than control 1 (**Figure 13C**). No significant difference was found among MA groups. Complexity of surfaces is indicated by a hybrid parameter S_{dr} (developed interfacial area ratio). All MA groups revealed higher complexity than M groups (**Figure 13D**).

The eight surfaces were also imaged by FE-SEM (**Figure 14**). Similar machined structures were observed on all M samples. MA samples presented irregular ridges and valleys. No significant difference could be noticed on control 1, control 2 and 1mA-1h surfaces for both M and MA. However, the M sample coated with Doxycycline at 5 mA/cm^2 for 3 h showed white particles, suggesting the successful coating of Doxycycline on the surface. The sample coated with Doxycycline at 5 mA/cm^2 for 3 h revealed less sharp contour of the ridges compared to the other three groups, indicating a layer of Doxycycline covering the surface.

FE-SEM examination of biofilm

The eight surfaces were incubated with *S. epidermidis* and analyzed with FE-SEM after 6 hours (**Figure 14**). For both M and MA samples, control 1 and control 2 which had no Doxycycline on the surface were covered by a similar layer of bacteria. Less bacteria was observed on the 1 mA-1 h surfaces. In comparison, the samples which were coated with Doxycycline at 5 mA/cm^2 for 3 h seemed to significantly inhibit bacteria attachment.

Biomass quantification

For M and MA samples, there were significantly less bacteria in 1mA-1h and 5mA-3h groups both on the surface and in the media compared to control 1, indicating the antibacterial effect of Doxycycline on these surfaces (**Figure 15**). Bacteria were significantly more reduced on 5mA-3h surfaces than on 1mA-1h. Control 1 and control 2 of M samples presented similar biofilm and planktonic biomass, while MA control 2

showed significantly more biofilm than MA control 1. MA control 2 also exhibited significantly higher biomass than M control 2. On the contrary, MA 1mA-1h showed significantly lower bacteria presence compared to M 1mA-1h. For the other groups, there was no significant difference of biofilm and planktonic biomass between M and MA samples after the same treatment.

Zone of inhibition

The diameters of the clear zone around the coins were determined for each group (**Table 5**). There were no clear zones for control 1 but for 1mA-1h and 5mA-3h groups. Doxy-coated groups showed significantly different clear zones compared to non-coated group except 1mA-1h of M. 5mA-3h significantly reduced bacteria around them than 1mA-1h. The clear zones around MA Doxy-coated surfaces were significantly larger than respective M surfaces.

Table 5. The size of inhibition zones in agar diffusion test. M: machined; MA: machined and acid etched; control 1: no cathodic polarization; 1mA-1h: cathodic polarization with Doxy at 1 mA/cm^2 for 1 h; 5mA-3h: cathodic polarization with Doxy at 5 mA/cm^2 for 3 h.

		Number	Range (Ø, mm)	Median (Ø, mm)	Significant difference
M	control 1	9	0.0 - 0.0	0.0	-
	1mA-1h	9	0.0 - 8.0	5.0	-
	5mA-3h	9	6.0 - 12.5	9.0	**a**, **c**
MA	control 1	9	0.0 - 0.0	0.0	-
	1mA-1h	9	6.0 - 10.0	6.0	**a**, **b**
	5mA-3h	9	7.0 - 15.0	10.0	**a**, **b**, **c**

a: **vs. control 1**; b: **vs. M with the same treatment**; c: **vs. respective 1mA-1h.**

Discussion

Binding of biomolecules onto TiZr surface is difficult due to the low reactivity of the inert oxide layer on the surface. In this study, we used the same method to coat Doxycycline on TiZr dental implant abutment as described in experiment 1. The presence of Doxycycline on the surface was confirmed by the increase in nitrogen and carbon concentrations (**Table 4**). Furthermore, the peak intensity of chemical bonds in Doxycycline containing carbon, oxygen, nitrogen and hydrogen also increased, whereas titanium oxide bonds on the surface decreased in intensity after the coating process (**Figure 12**).

Doxycycline is positively charged in the acetic buffer (pH = 3.0) according to the pK_a values (3.02, 7.97 and 9.15) (Shariati et al., 2009), and thus attracted to the negatively charged TiZr cathode during cathodic polarization. Indeed, the protonation of nitrogen in Doxycycline is shown by the increase of $(CH_3)_2$-N+ and O=C-NH_3+. Therefore, in addition to the covalent binding between Doxycycline and TiZr surface which has been previously suggested, we believe that Doxycycline is attached to TiZr surface by electrostatic forces. This should be a stronger adherence between Doxycycline and the surface. Doxycycline adsorbed by electrostatic force should be more stably adhered onto the surface compared to physically adsorbed Doxycycline by van der Waals force. Electrostatic adhesion also has the advantage over covalent immobilization in that it allows the release of Doxycycline from the surface, and thus, its antibacterial effect (Khoo and Grinstaff, 2011). Unlike other surface coating approaches by using polymers and cements as drug carriers, this electrochemical method introduces no foreign substances other than Doxycycline onto the surface.

Nitrogen was used as the indicator for Doxycycline when analyzing the influence of reaction parameters such as current density and reaction time. It was considered more precise to use nitrogen than carbon due to the possible carbon contamination from air and the acetic buffer. As previously mentioned, the small amount of nitrogen on uncoated samples may have resulted from the nitrogen cover gas packaging. As shown in the XPS results, reaction time is one of the decisive factors for Doxycycline amount. The Doxycycline amount on the surface increased with longer reaction time at the same current density. A similar correlation has also been found between surface hydrogen and reaction time when hydrogen instead of Doxycycline was coated onto Ti abutment surface by cathodic polarization. However, current density seems to affect the amount of Doxycycline ony at short reaction time (1 h). It could be possible that Doxycycline reacts more rapidly with the surface under higher current density due to the higher charge density on the surface. After the negatively charged surface is fully covered by a layer of Doxy, the reaction speed between Doxycycline and the surface may be more dependent on the time instead of current density.

Only nano scale alterations of topographical parameters were observed on the Doxy-coated surface. Nano particles and less sharp contours could also be seen on 5mA-3h abutment surfaces in FE-SEM images. Therefore, the method of cathodic polarization can

be used to chemically modify abutment surfaces without significantly altering the surface topography of the original engineered implant surface.

Bacteria assay was conducted to evaluate the antimicrobial efficacy of Doxy-coated surfaces. Because the direct measurement of Doxycycline release was found challenging in the present study, the bacteria assay was also used as an indication for Doxycycline release. *S. epidermidis* was used for the antibacterial test as it is a common cause of biomaterial associated infections and also highly reproducible in the laboratory (Barth et al., 1989).

Biofilm formation on the Doxy-coated surface (5mA-3h) was inhibited (Figure 15). This is important because steady-state biofilm is difficult to eradicate after formation as it is more resistant to antibiotics than planktonic bacteria (Gilbert et al., 1997) and requires up to 250 times greater concentration than inhibiting the same strains grown planktonically. As a tetracycline analogue, Doxycycline inhibits ribosomal protein synthesis after being translocated into the cytoplasm of bacteria. Thus, the inhibited biofilm formation could be attributed to the released Doxycycline at the bacteria-abutment interface. The release of Doxycycline into the media is confirmed by the reduced planktonic biomass. Bacterial inhibition zones were observed around Doxy-coated samples, indicating the diffusion of released Doxycycline into the agar media. In comparison to fixed antibiotics, released Doxycycline has the advantage of killing bacteria which are not in contact with the surface. It should be noted that despite the antibacterial effect of released Doxy, direct contact between fixed Doxycycline and bacteria might occur, which might interrupt electrostatic balance of the bacterial membrane and eventually cause membrane destruction. The sample with lower amount of Doxycycline (1mA-1h) also reduced both biofilm and planktonic bacteria but not as much as the samples with higher amount of Doxy. This shows that the antibacterial effect is dependent on the Doxycycline amount on the surface.

Two different types of surfaces (M and MA) with roughness difference of more than 100 nm were used in the study. Unlike a previous human biofilm study in which more bacteria harboured on rougher surfaces, surface roughness seemed not to influence the antibacterial performance in the present study. This is in accordance with the assumption that gravitational forces overcome the influence of surface roughness on bacterial attachment under the static plate culture condition. As a preliminary bacteria study, the

incubation time was limited to 6 hours. Longer incubation time should be tested for long term efficacy of the surface coating.

Conclusion

Doxycycline (Doxy) was coated onto TiZr dental implant abutment surface by the electrochemical method of cathodic polarization. Higher amount of Doxycycline on the surface can be obtained by increasing reaction time. Current density affects Doxycycline amount only at short time (1 h). Doxycycline covering on the surface could be achieved without significantly altering the surface topography. The growth of both planktonic bacteria and biofilm was significantly inhibited by Doxy-coated abutment surfaces. The antibacterial efficacy is more significant for the abutments with higher amount of Doxy. Therefore, Doxycycline coating on implant abutment shows potential for preventing biofilm formation in particular on the abutment surface.

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CLAIMS

1. A craniofacial and/or dental prosthetic device or implant,
   a) containing a metal material selected from the group consisting of titanium or an alloy thereof, zirconium or an alloy thereof, tantalum or an alloy thereof, hafnium or an alloy thereof, niobium or an alloy thereof and a chromium-vanadium alloy,
   b) characterized in that surface parts of the metal material comprise one or more Doxycycline(s).

2. A craniofacial and/or dental prosthetic device or implant,
   a) containing a metal material selected from the group consisting of titanium or an alloy thereof, zirconium or an alloy thereof, tantalum or an alloy thereof, hafnium or an alloy thereof, niobium or an alloy thereof and a chromium-vanadium alloy,
   b) wherein surface parts of the metal material are coated with a layer of a corresponding hydride material selected from titanium hydride, zirconium hydride, tantalum hydride, hafnium hydride, niobium hydride and chromium and/or vanadium hydride, respectively,
   c) characterized in that the layer of hydride material comprises one or more Doxycycline(s) associated therewith.

3. A device or implant as claimed in claim 2, wherein the one or more Doxycycline(s) is (are) present on the surface of the hydride material or trapped in the hydride material.

4. A device or implant as claimed in claim 2, wherein the one or more Doxycycline(s) is(are) covalently attached to the layer of hydride material.

5. A device or implant as claimed in claim 2, wherein the one or more Doxycycline(s) is (are) attached to the layer of hydride material by electrostatic adhesion.

6. A device or implant as claimed in claims 2-5, which is produced by cathodic polarization with doxycycline.

7. A device or implant as claimed in claims 2-5, which is produced by cathodic polarization with doxycycline, wherein the device or implant is optionally machined and/or machined and acid etched, and then subjected to cathodic polarization in a Doxycycline comprising buffert, comprising 1 mg/ml Doxycycline hyclate at a density of between 1-15 mA/cm^2, during 1-5 hours.

8. A dental implant as claimed in any of the preceding claims, which supports a dental prosthetic device, selected from the group consisting of abutment, crown, implant-supported bridge or denture, being in direct contact with soft tissue, such as gum tissue and/or oral mucosa.

9. A dental implant as claimed in any of the preceding claims, which comprises a dental prosthetic device, selected from the group consisting of abutment, crown, implant-supported bridge or denture, being in direct contact with soft tissue, such as gum tissue and/or oral mucosa.

10. A dental prosthetic device as claimed in any of the preceding claims, which is selected from the group consisting of abutment, crown, implant-supported bridge or denture.

11. A device or implant as claimed in any of the preceding claims wherein the one or more Doxycycline(s) is(are) selectively associated with one or more surfaces that are in contact with soft tissue when the device or implant is deployed in the body of a mammal.

12. A device or implant as claimed in any of the preceding claims, wherein essentially all surface parts of the metal material of the craniofacial and/or dental prosthetic device or implant are at least partially coated with a layer of a corresponding hydride material and one or more Doxycycline(s) associated therewith.

13. A device or implant as claimed in in any of the preceding claims, wherein the metal material is titanium or an alloy thereof, preferably titanium.

14. A method for preparing a craniofacial and/or dental prosthetic device or implant as defined in any of claims 1-14, said method comprising subjecting surface parts of the metal material to an electrolysis treatment to form the layer of hydride material,

said electrolysis treatment being carried out in the presence of one or more Doxycycline(s).

15. A craniofacial and/or dental prosthetic device or implant as defined in any of claims 1-14 for use in promoting soft tissue healing and/or preventing inflammation and/or infection in soft tissue in proximity to the craniofacial and/or dental prosthetic device or implant, when deployed in the body of a mammal.

16. A craniofacial and/or dental prosthetic device or implant as defined in any of claims 1-14 for use in promoting soft tissue healing and/or preventing inflammation and/or infection in soft tissue in proximity to the medical prosthetic device or implant, when deployed in the body of a patient with smoking habits, radiation therapy, poor oral hygiene, general poor bone density, poor quality bone, wound healing disruptions and/or high risk for infections.

17. Use of a craniofacial and/or dental prosthetic device or implant as defined in any of claims 1-13 for promoting soft tissue healing and/or preventing inflammation and/or infection in soft tissue in proximity to the craniofacial and/or dental prosthetic device or implant, when deployed in the body of a mammal.

18. Doxycycline for use in promoting soft tissue healing and/or preventing inflammation and/or infection in soft tissue in proximity to a craniofacial and/or dental prosthetic device or implant as claimed in any of claims 1-13.

19. Doxycycline for use in promoting soft tissue healing and/or preventing inflammation and/or infection in soft tissue in proximity to a craniofacial and/or dental prosthetic device or implant, when deployed in the body of a mammalian patient, wherein said Doxycycline is covalently bound selectively to the surface parts of the craniofacial and/or dental prosthetic device or implant that are in contact with soft tissue in said patient.

20. Doxycycline for use according to claim 16, wherein said Doxycycline is covalently bound selectively to the surface parts of the craniofacial and/or dental prosthetic device or implant that are in contact with gum tissue and/or oral mucosa in said patient.

21. Doxycycline for use according to claims 16 or 17, wherein the craniofacial and/or dental prosthetic device or implant is deployed in the body of patient with smoking habits, radiation therapy, poor oral hygiene, general poor bone density, poor quality bone, wound healing disruptions and/or high risk for infections.

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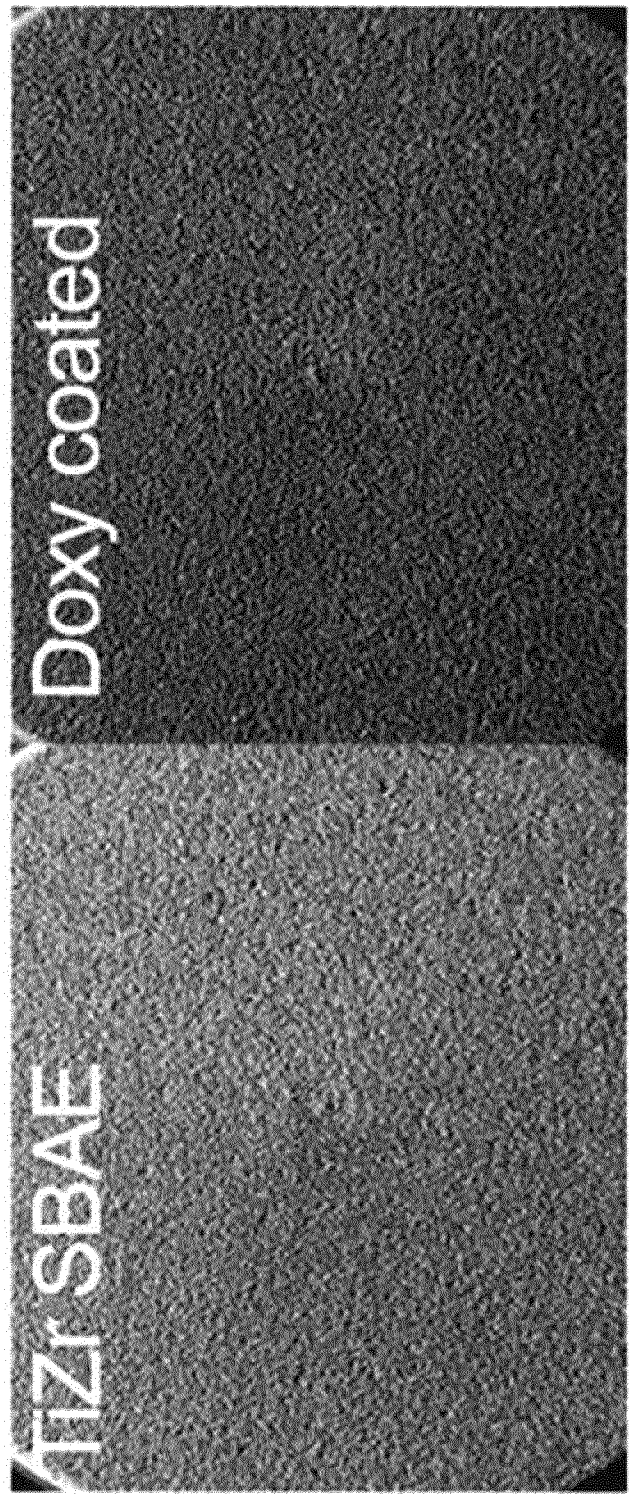


Fig. 1

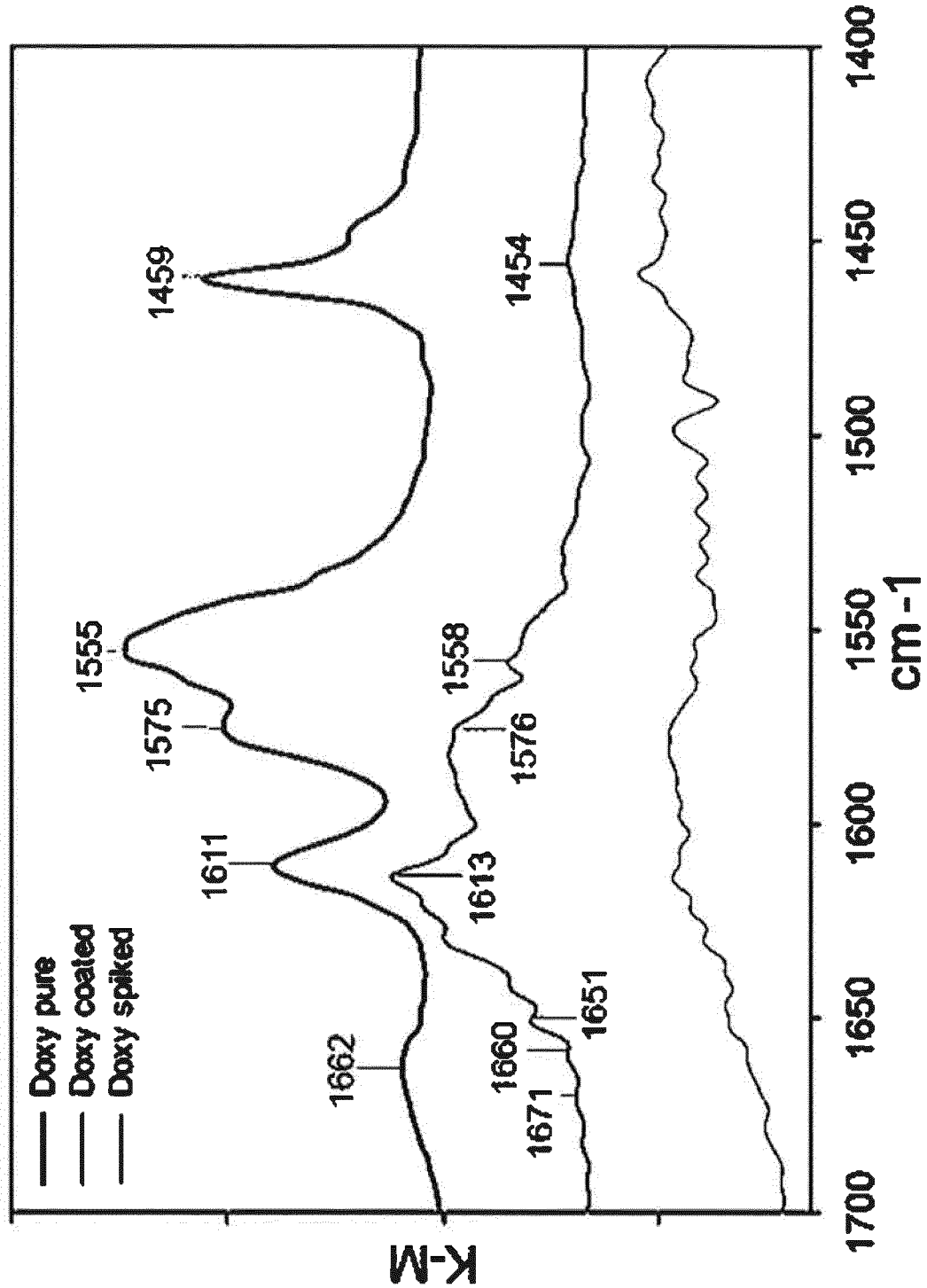

Doxy pure
Doxy coated
Doxy spiked
1459
1555
1575
1611
1662
1454
1558
1576
1613
1660
1671
1651
K-M
1700
1650
1600
1550
1500
1450
1400
cm-1

Fig. 2

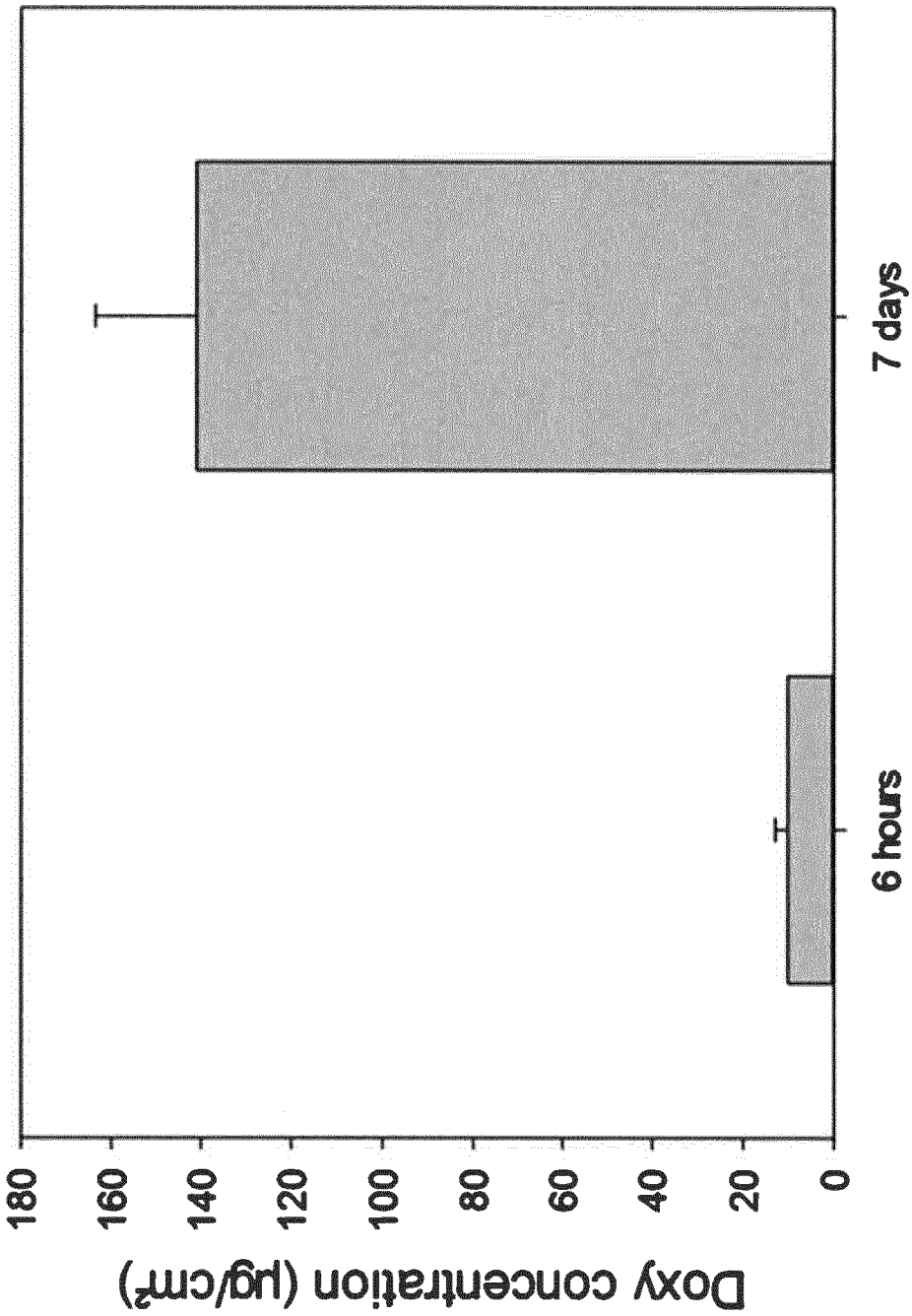

Doxy concentration (µg/cm²)
180
160
140
120
100
80
60
40
20
0
7 days
6 hours

Fig. 3

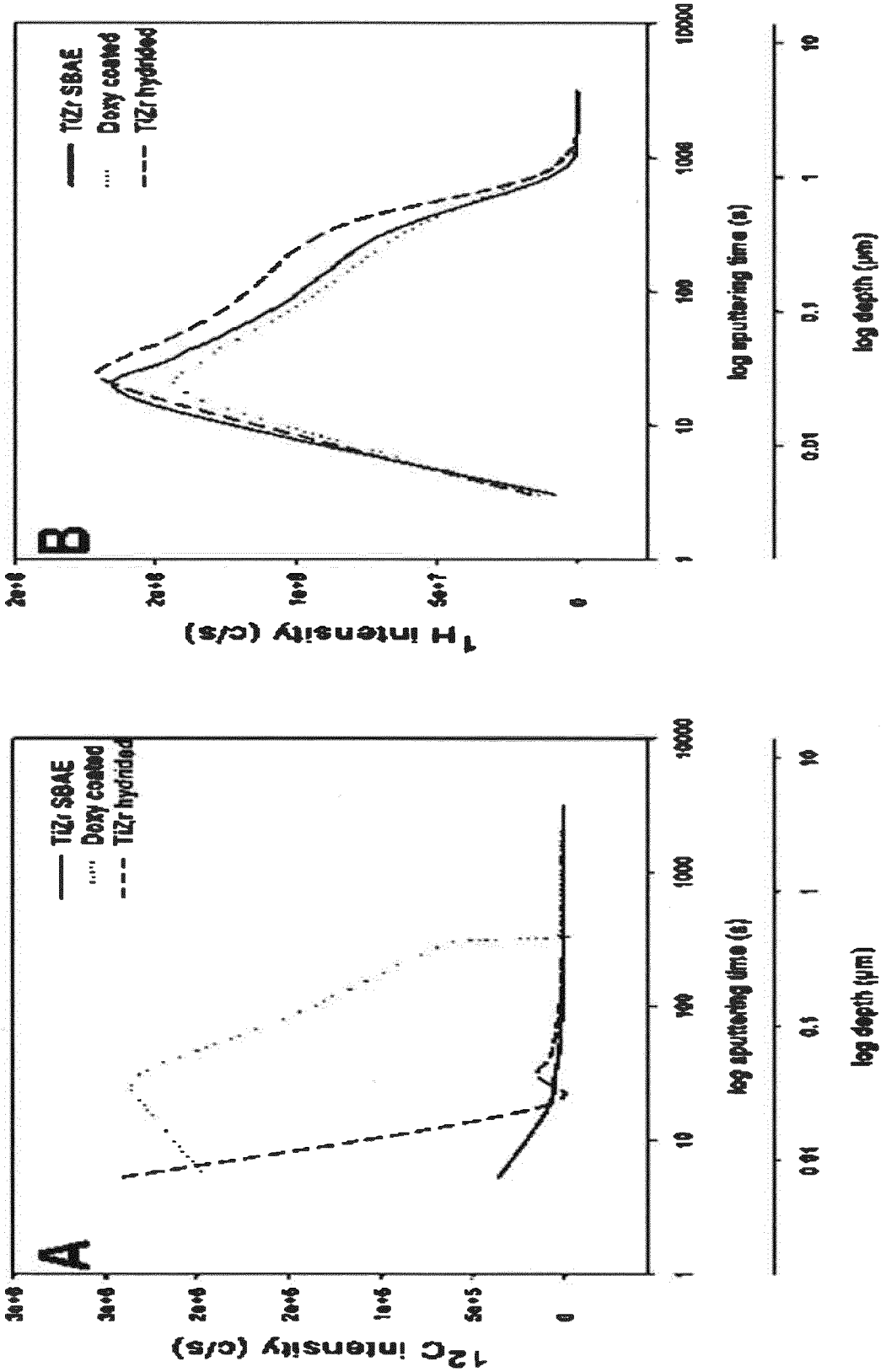

B
TiZr SBAE
Doxy coated
TiZr hydrided
^{1}H intensity (c/s)
log sputtering time (s)
log depth (µm)
A
TiZr SBAE
Doxy coated
TiZr hydrided
^{12}C intensity (c/s)
log sputtering time (s)
log depth (µm)

Fig. 4

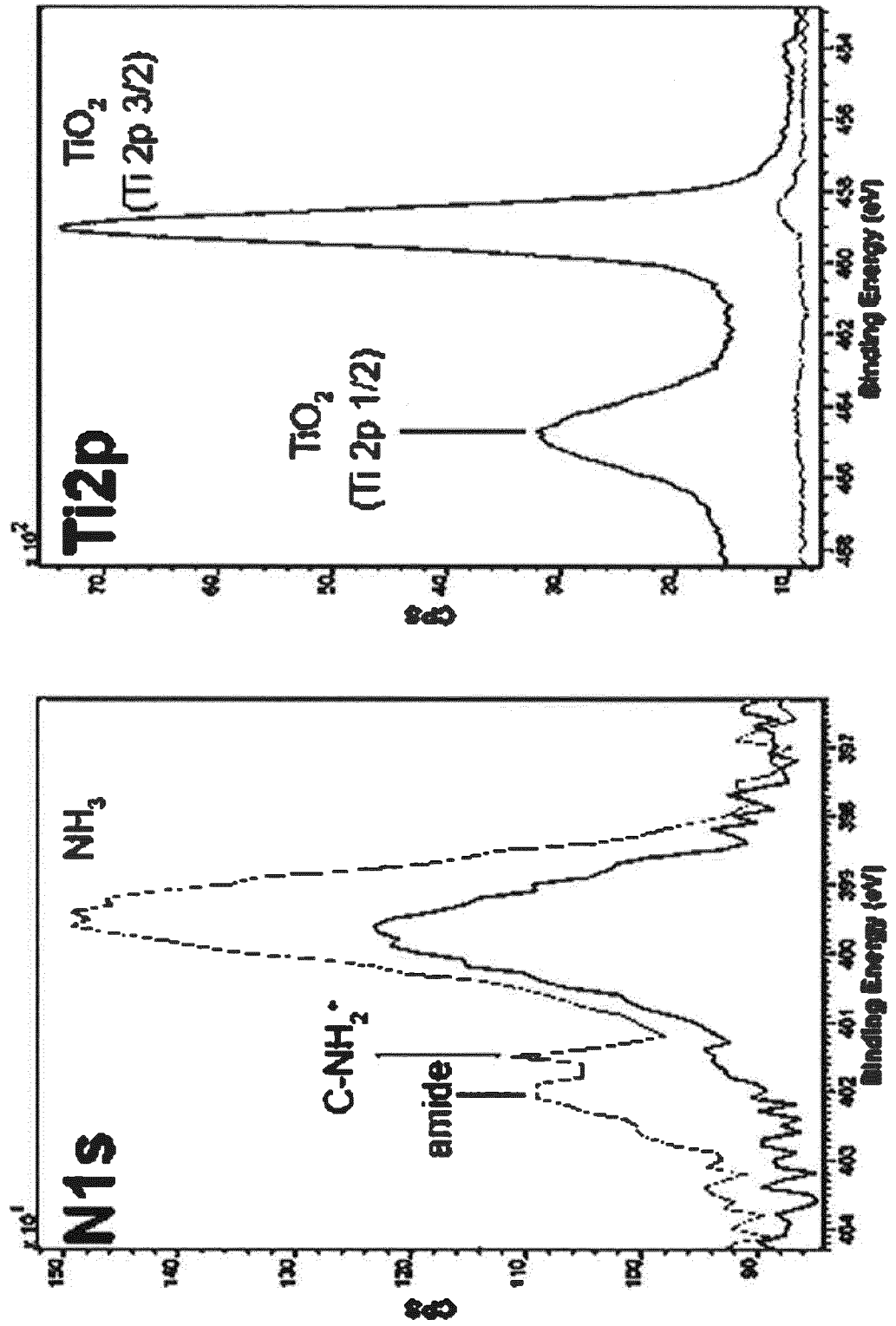

Ti2p
TiO2
(Ti 2p 3/2)
TiO2
(Ti 2p 1/2)
Binding Energy (eV)
N1s
NH3
C-NH2
amide
Binding Energy (eV)

Fig. 5A

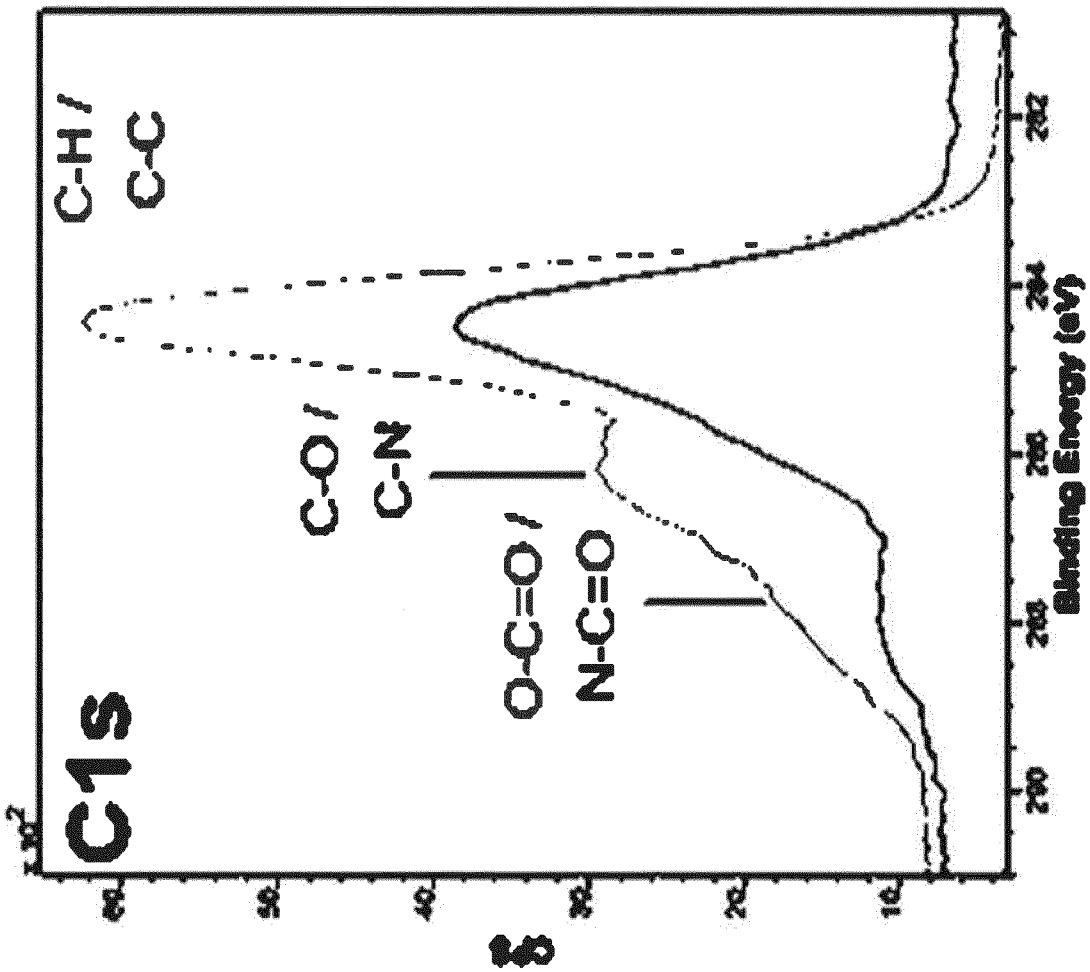

C1s
C-H /
C-C
C-O /
C-N
O-C=O /
N-C=O
Binding Energy (eV)
292
288
284
280
276
272
268
264
260
x10²
CPS

Fig. 5B

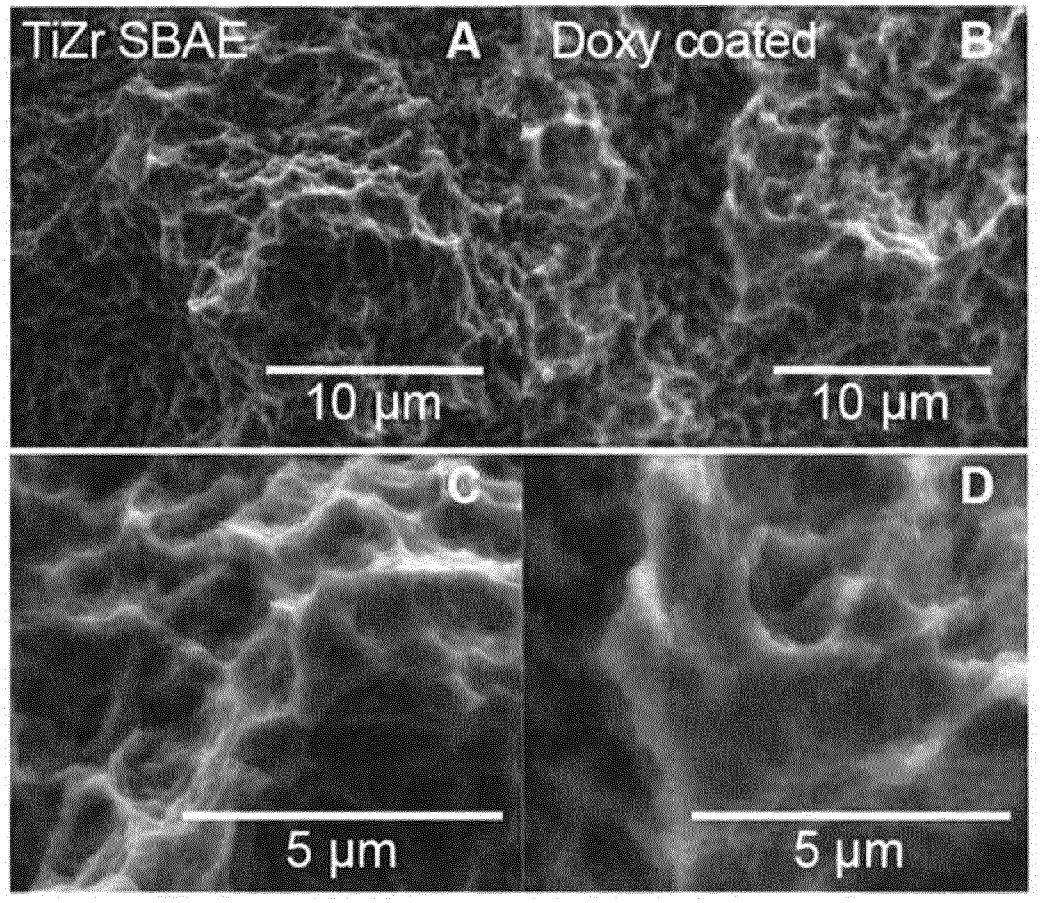


Fig. 6

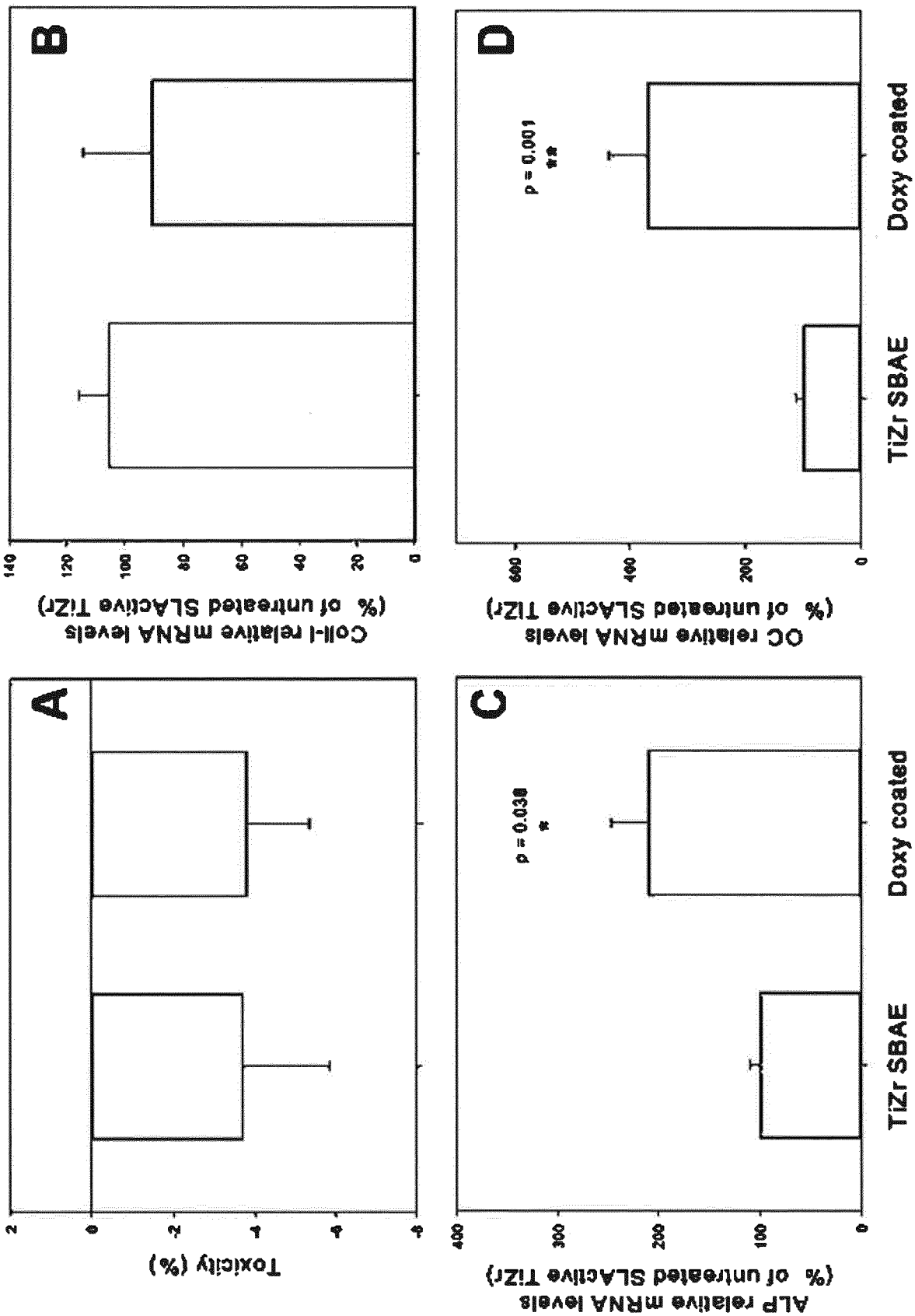

A
Toxicity (%)
2
0
-2
-4
-6
B
Coll-I relative mRNA levels
(% of untreated SLActive TiZr)
140
120
100
80
60
40
20
0
C
p = 0.038
*
ALP relative mRNA levels
(% of untreated SLActive TiZr)
400
300
200
100
0
TiZr SBAE
Doxy coated
D
p = 0.001
**
OC relative mRNA levels
(% of untreated SLActive TiZr)
600
400
200
0
TiZr SBAE
Doxy coated

Fig. 7

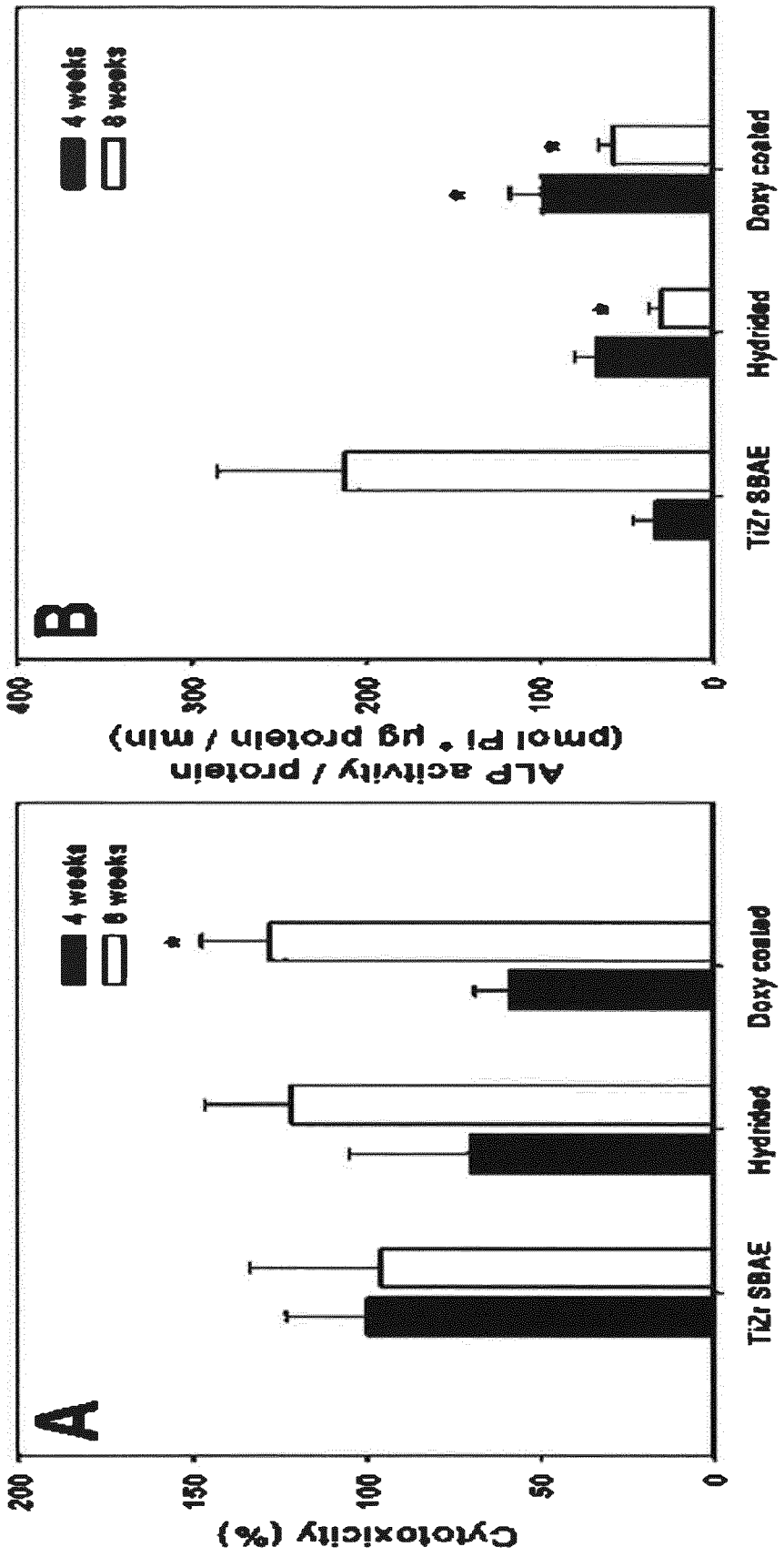

B
4 weeks
8 weeks
ALP activity / protein
(pmol Pi * µg protein / min)
400
300
200
100
0
TiZr SBAE
Hydrided
Doxy coated
A
4 weeks
8 weeks
Cytotoxicity (%)
200
150
100
50
0
TiZr SBAE
Hydrided
Doxy coated

Fig. 8

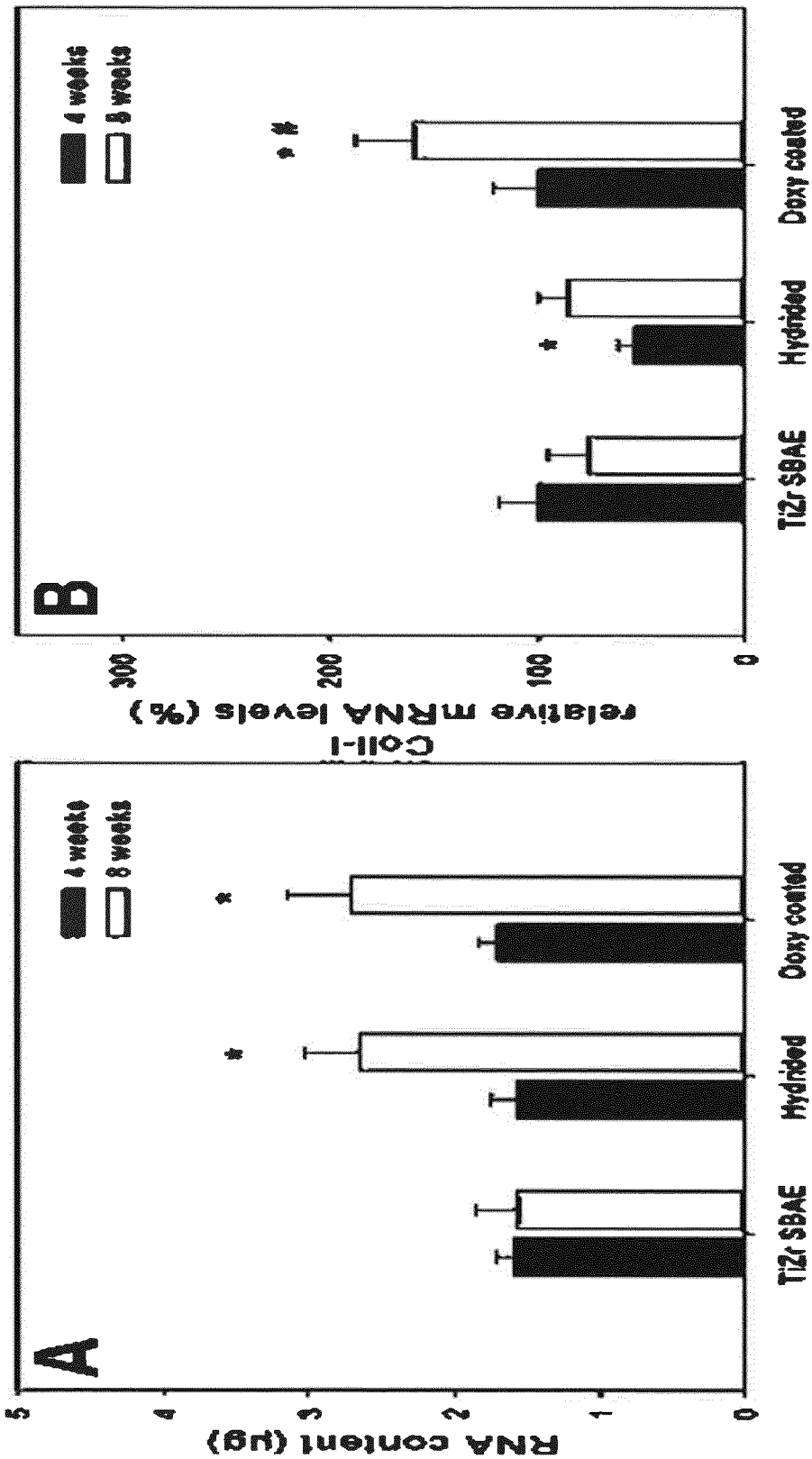

A
4 weeks
8 weeks
RNA content (µg)
0
1
2
3
4
5
Ti2r SBAE
Hydrided
Oxy coated
B
4 weeks
8 weeks
Coll-I
relative mRNA levels (%)
0
100
200
300
Ti2r SBAE
Hydrided
Doxy coated

Fig. 9 A-B

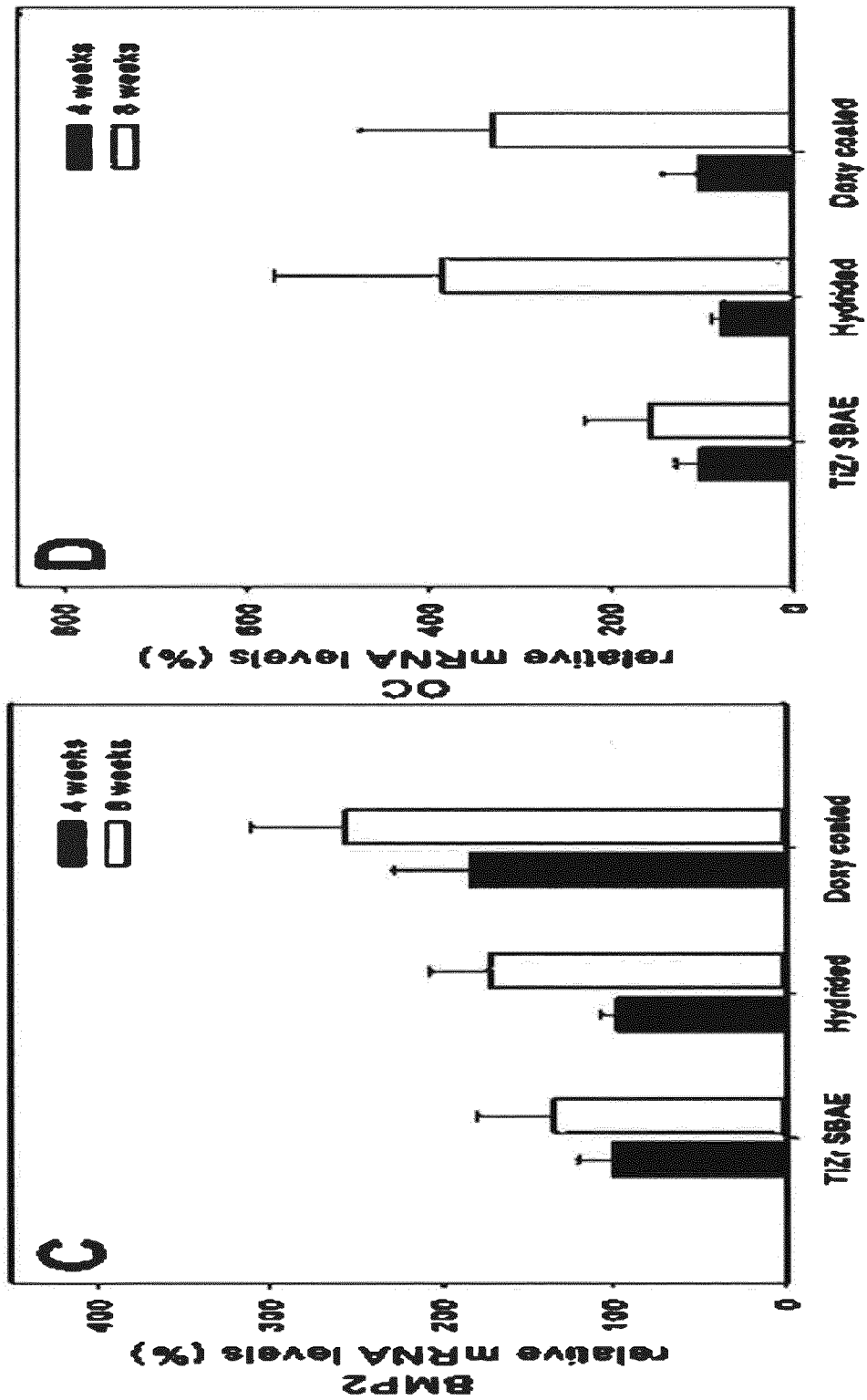

C
4 weeks
8 weeks
BMP2
relative mRNA levels (%)
0
100
200
300
400
TIZ/ SBAE
Hydrided
Oxy coated
D
4 weeks
8 weeks
relative mRNA levels (%)
0
200
400
600
800
TIZ/ SBAE
Hydrided
Oxy coated

Fig. 9 C-D

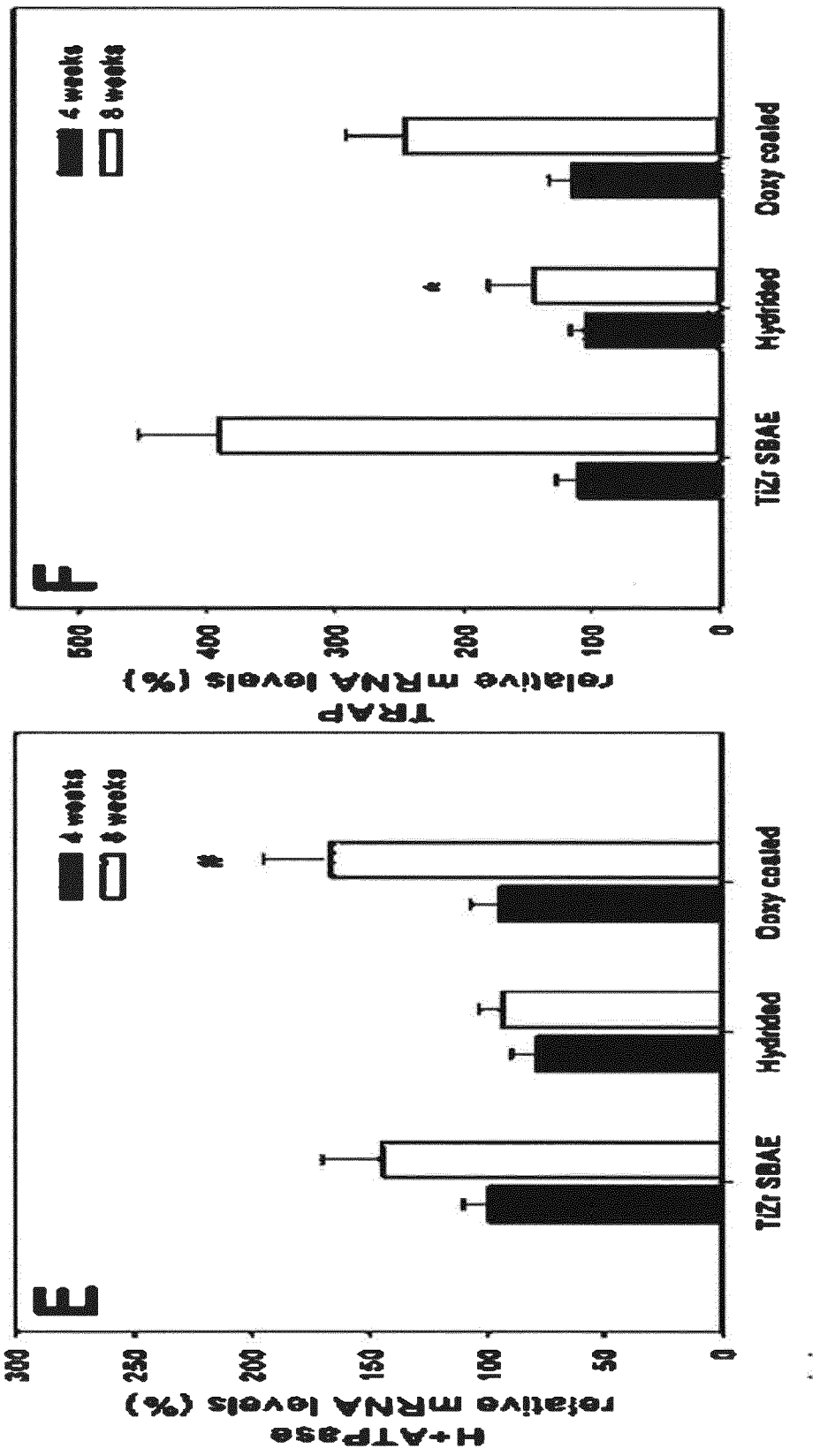

F
4 weeks
8 weeks
TRAP
relative mRNA levels (%)
500
400
300
200
100
0
TiZr SBAE
Hydrided
Oxy coated
E
4 weeks
8 weeks
H+-ATPase
relative mRNA levels (%)
300
250
200
150
100
50
0
TiZr SBAE
Hydrided
Oxy coated

Fig. 9 E-F

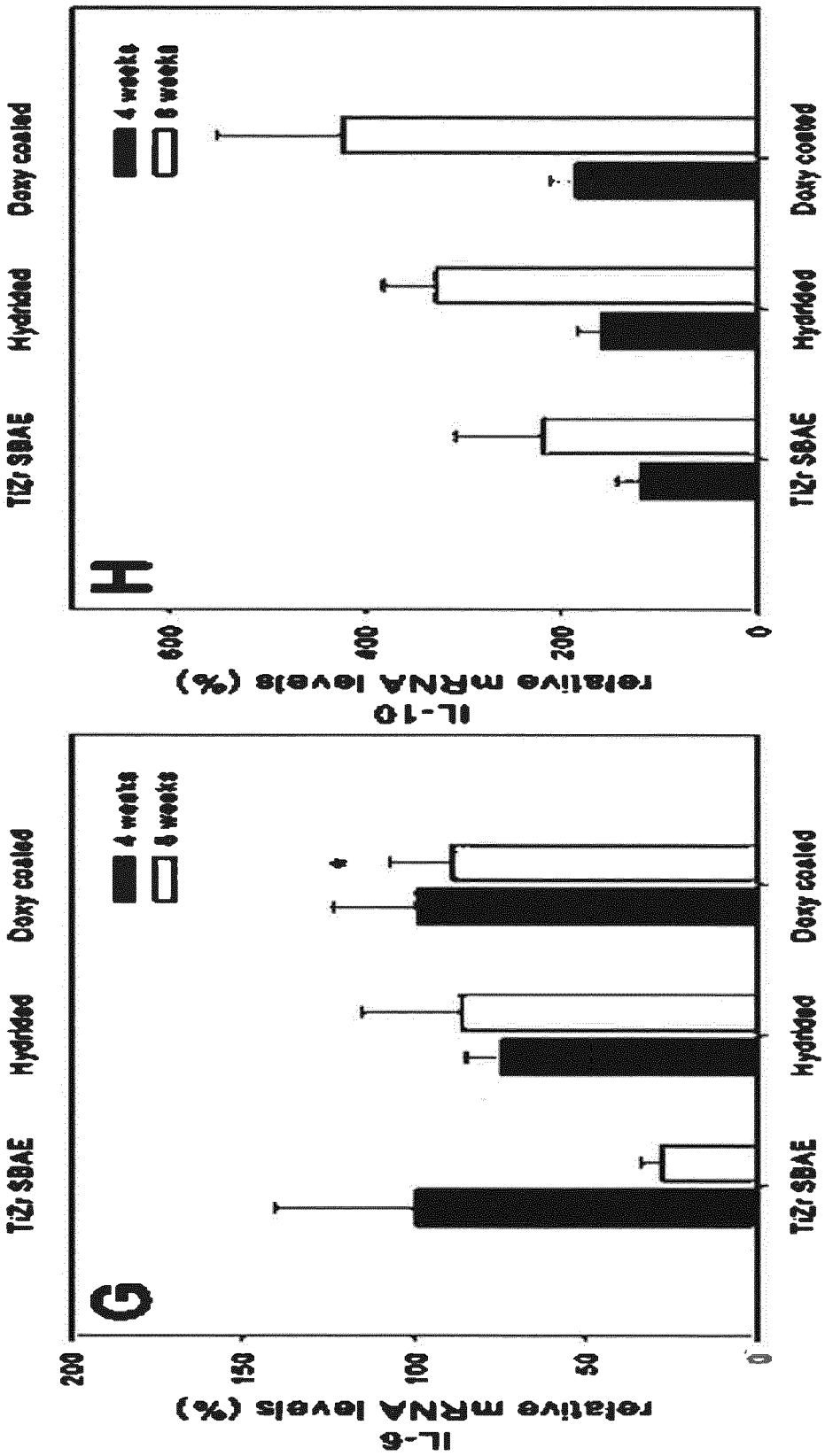

H
IL-10
relative mRNA levels (%)
0
200
400
600
4 weeks
8 weeks
TiZr SBAE
Hydrided
Oxy coated
G
IL-6
relative mRNA levels (%)
0
50
100
150
200
4 weeks
8 weeks
TiZr SBAE
Hydrided
Oxy coated

Fig. 9 G-H

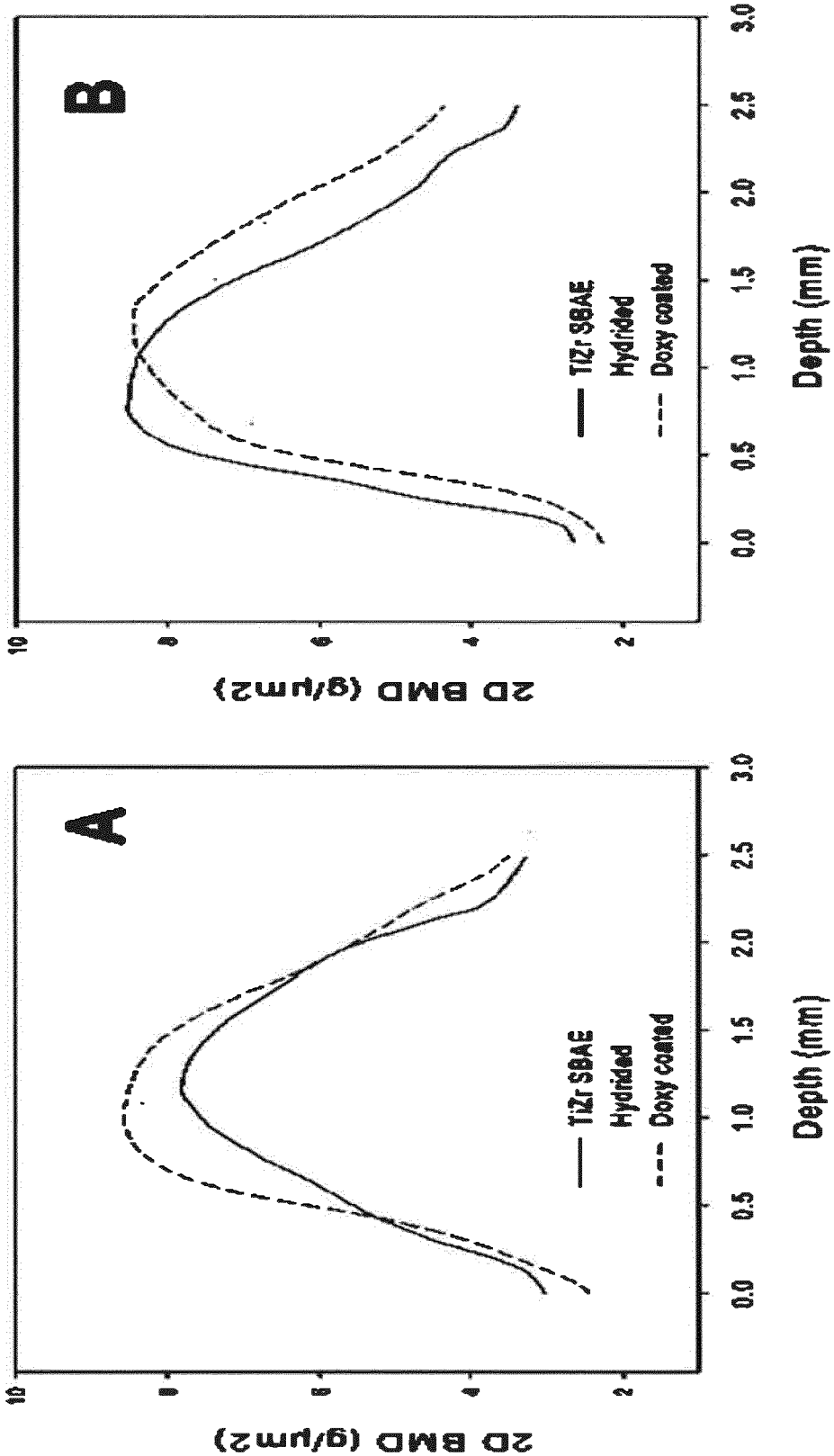

B
A
2D BMD (g/µm2)
Depth (mm)
Ti2r 98AE
Hydrided
Doxy coated
0.0
0.5
1.0
1.5
2.0
2.5
3.0
2
4
6
8
10

Fig.10

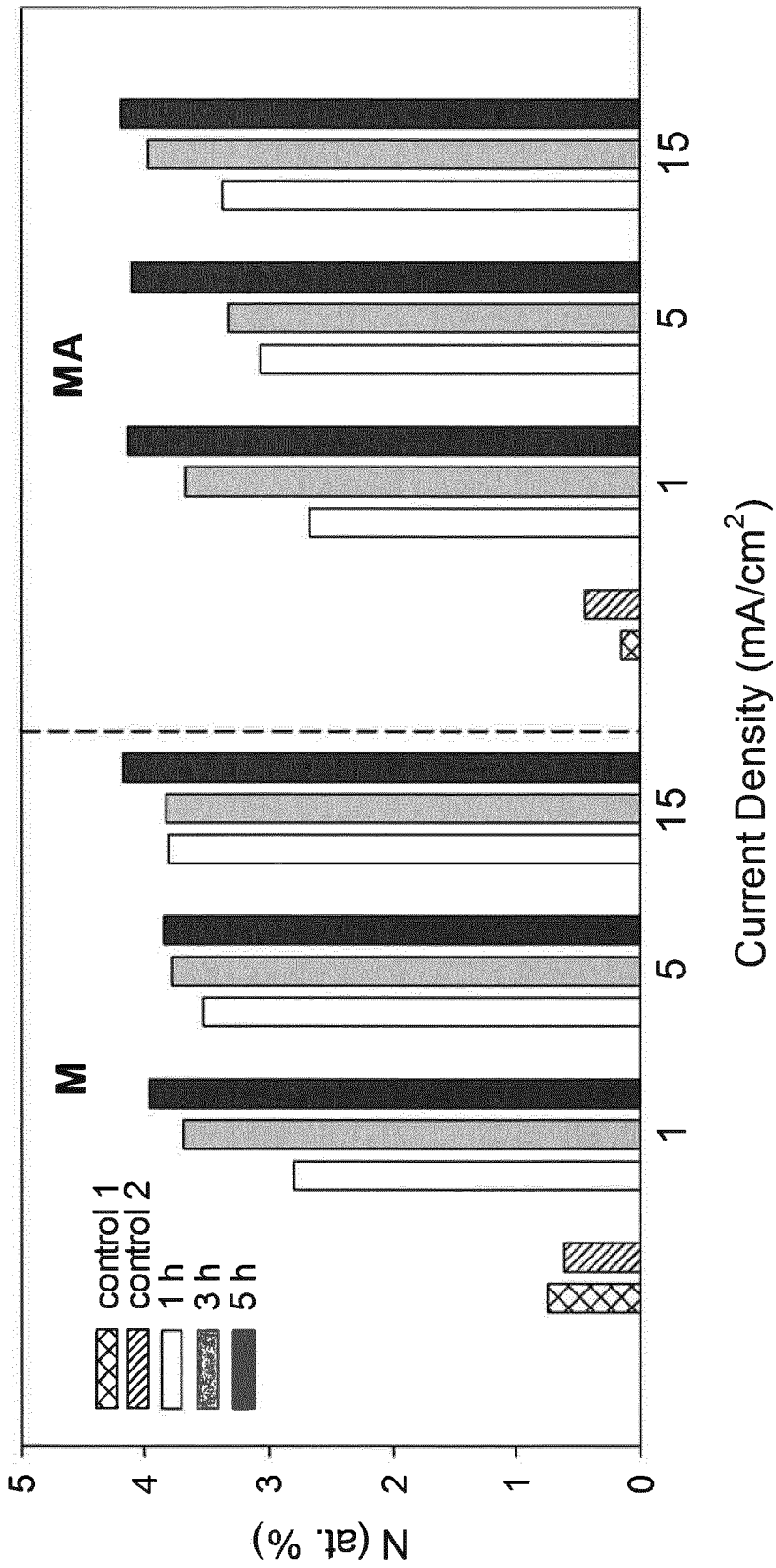

N (at. %)
0
1
2
3
4
5
M
MA
1
5
15
1
5
15
Current Density (mA/cm^2)
control 1
control 2
1 h
3 h
5 h

Fig.11

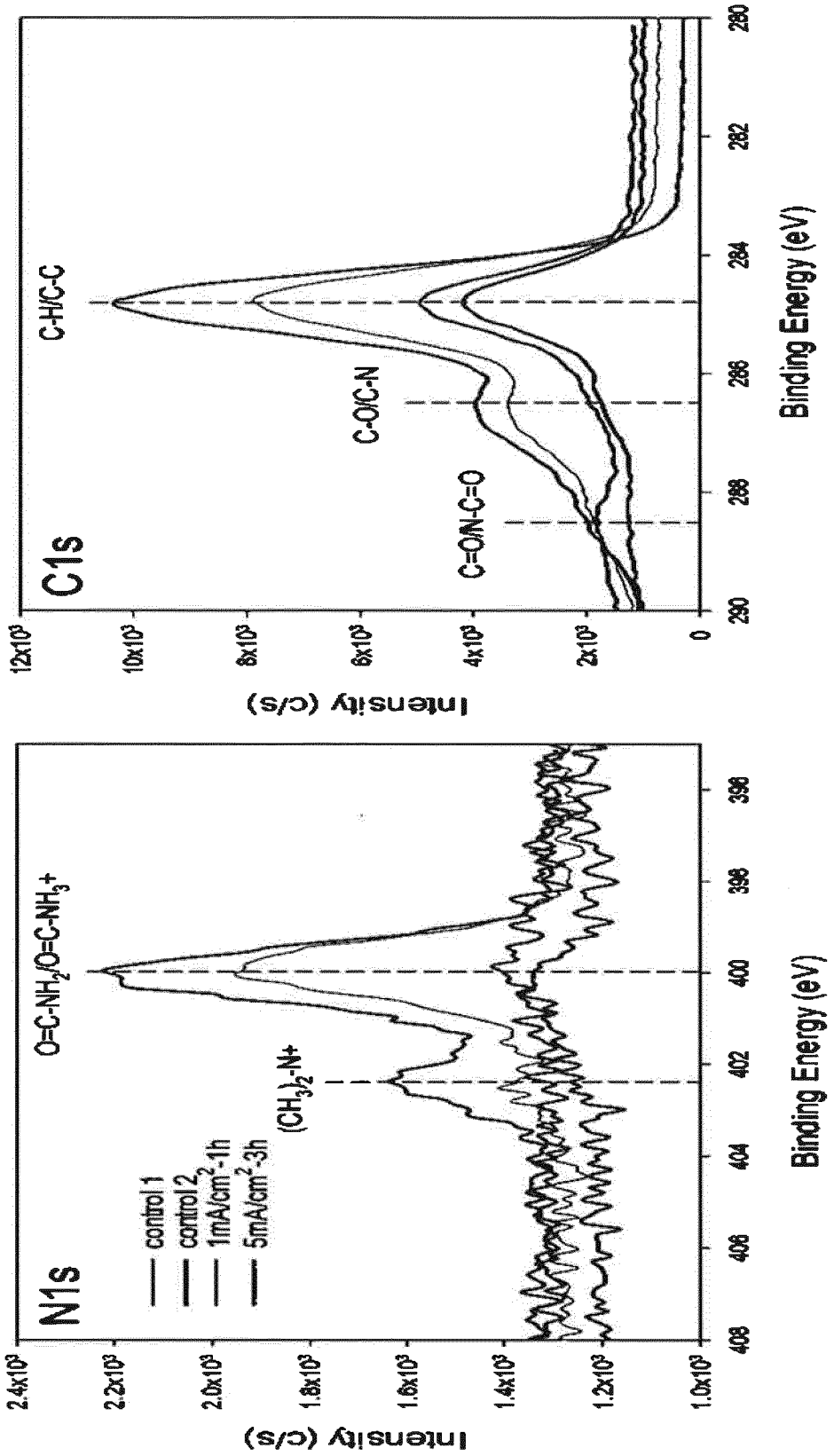

C1s
C-H/C-C
C-O/C-N
C=O/N-C=O
Intensity (C/s)
Binding Energy (eV)
N1s
O=C-NH2/O=C-NH3+
(CH3)2-N+
control 1
control 2
1mA/cm2-1h
5mA/cm2-3h
Intensity (C/s)
Binding Energy (eV)

Fig. 12A

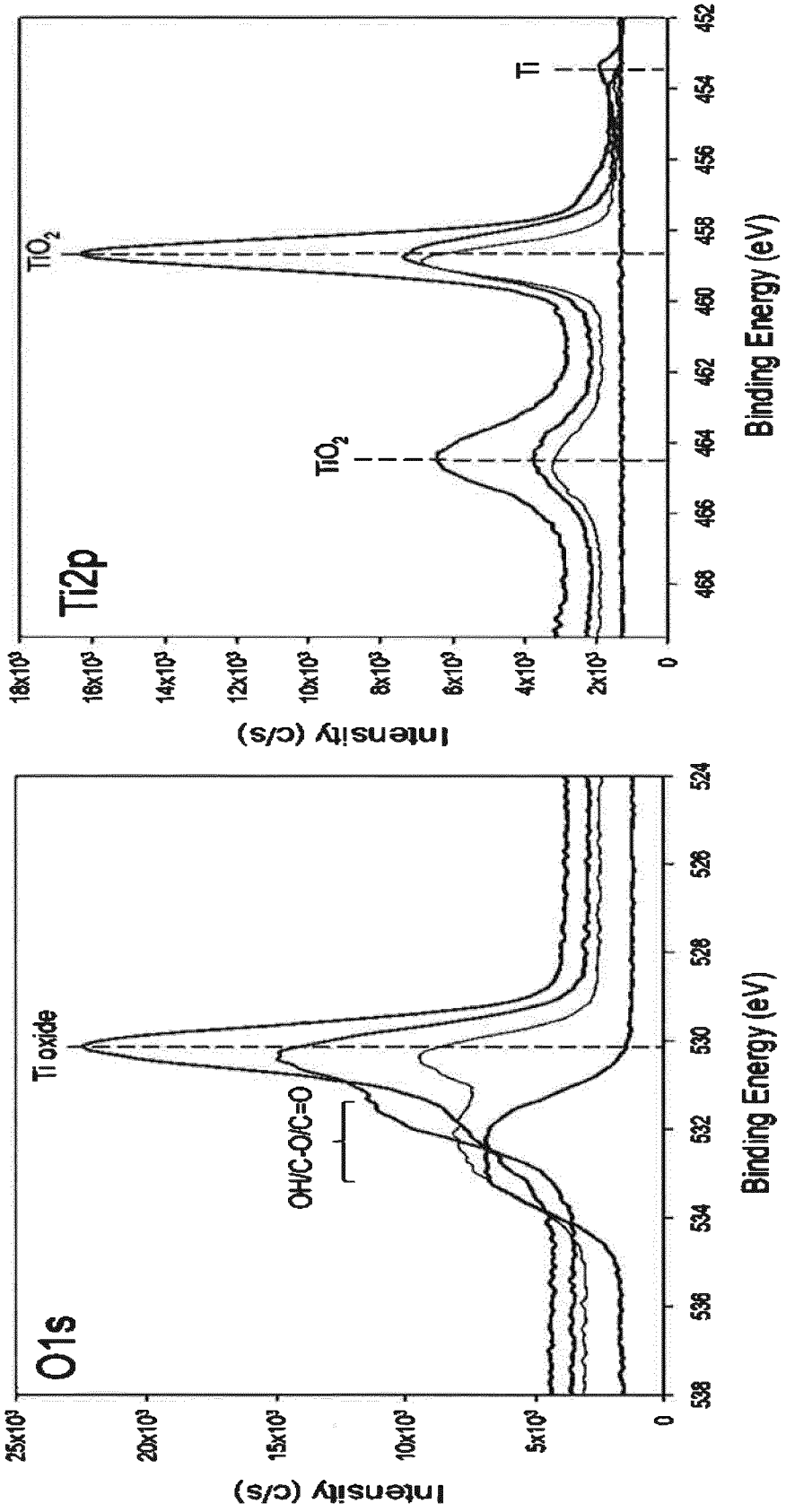

Ti2p
TiO_2
TiO_2
Ti
Intensity (c/s)
Binding Energy (eV)
O1s
Ti oxide
OH/C-O/C=O
Intensity (c/s)
Binding Energy (eV)

Fig. 12B

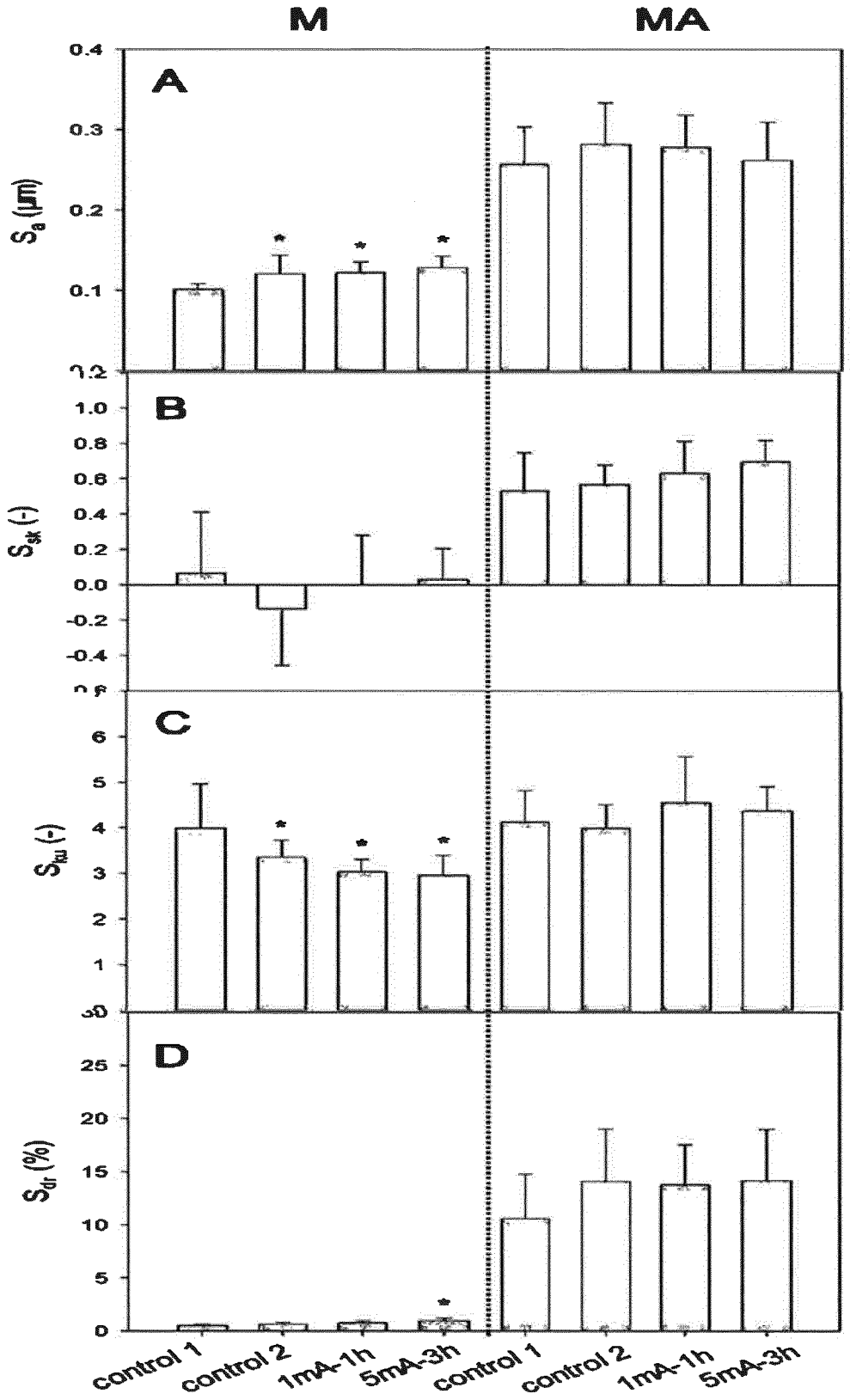

M
MA
A
B
C
D
S_a (µm)
S_{sk} (-)
S_{ku} (-)
S_{dr} (%)
control 1
control 2
1mA-1h
5mA-3h
control 1
control 2
1mA-1h
5mA-3h

Fig. 13

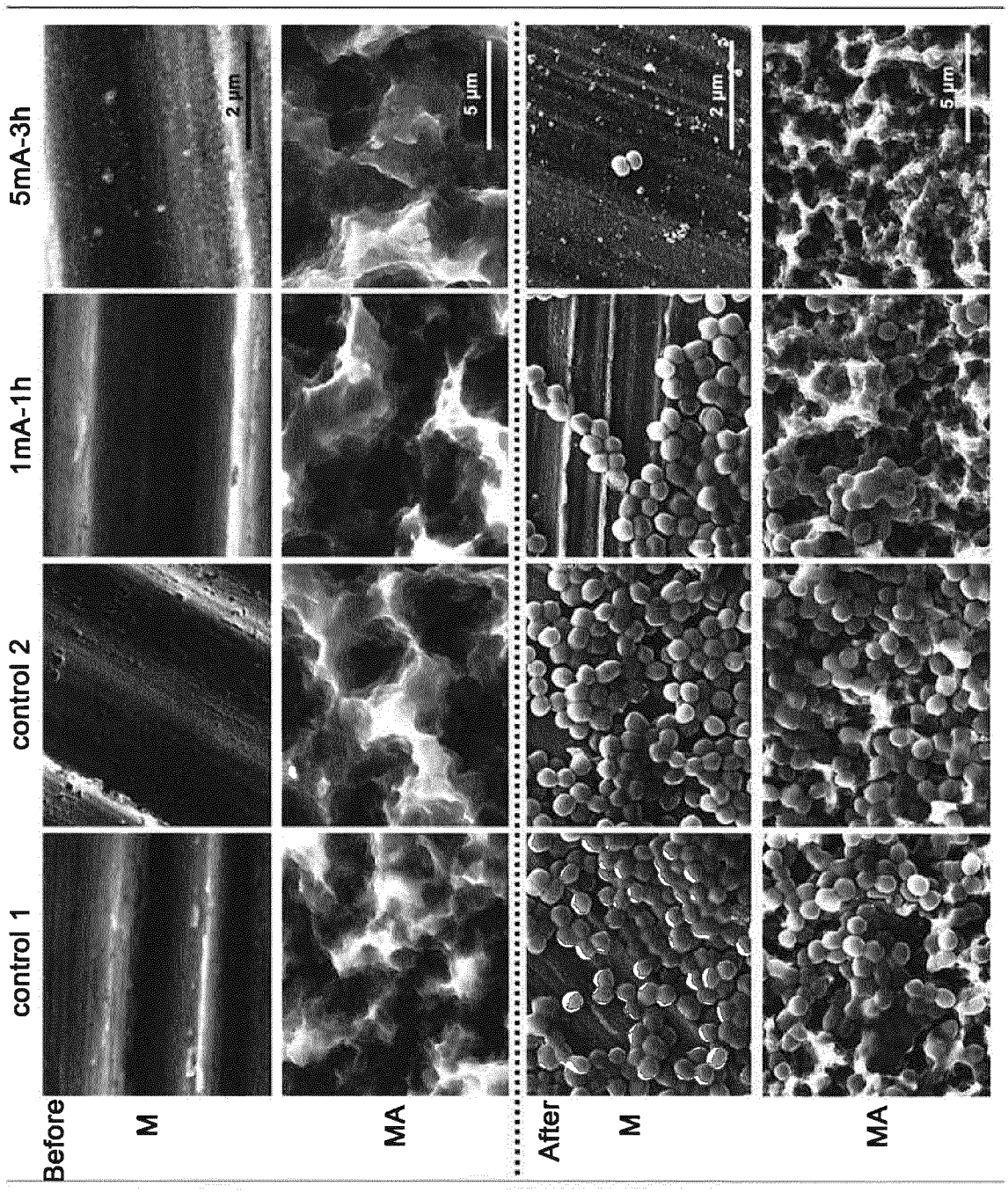

5mA-3h
1mA-1h
control 2
control 1
Before
M
MA
After
M
MA
2 µm
5 µm
2 µm
5 µm

Fig.14

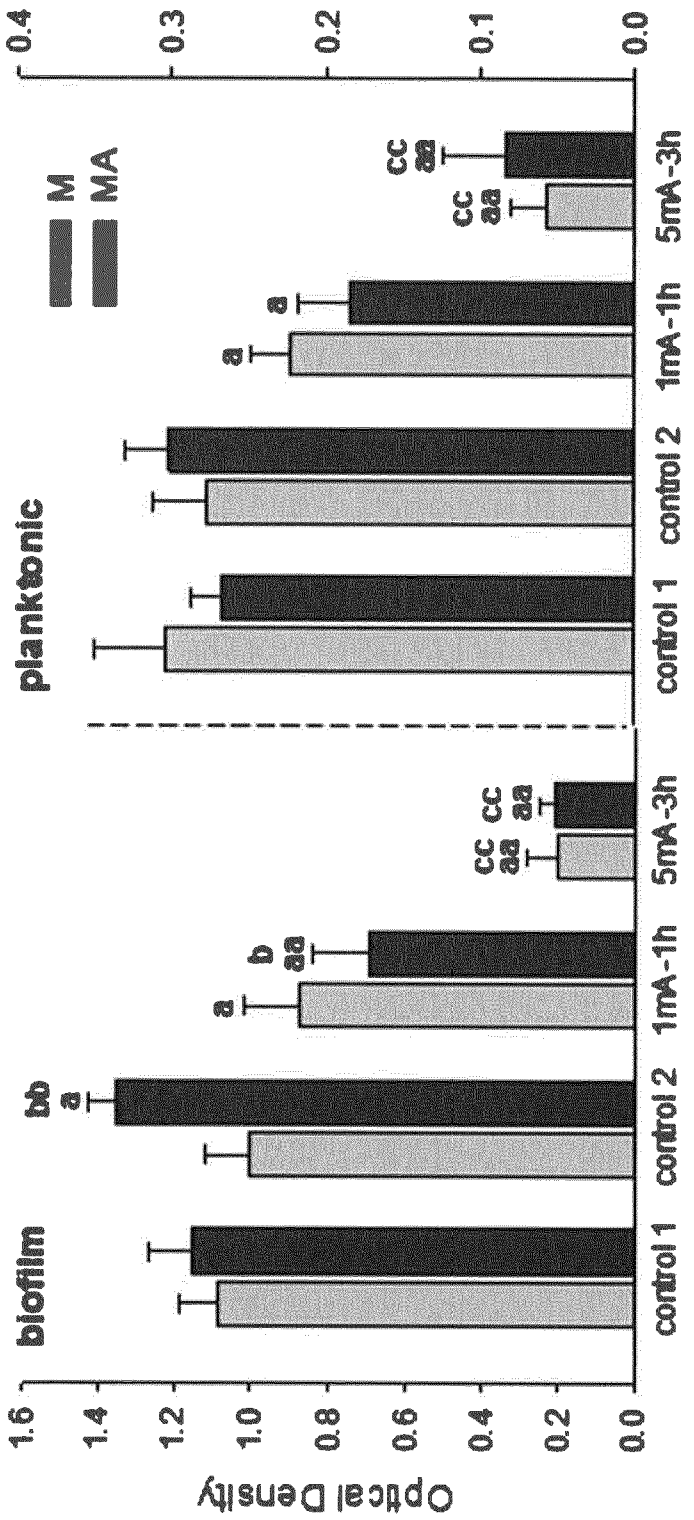

Optical Density
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
biofilm
planktonic
M
MA
control 1
control 2
1mA-1h
5mA-3h
a
b
aa
bb
cc

Fig.15

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/060929

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61L27/04 A61L27/06 A61L27/30 A61L27/54
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 2007/093894 A1 (DAROUICHE RABIH O [US]) 26 April 2007 (2007-04-26) paragraphs [0029], [0036], [0042], [0043], [0044], [0046], [0080]; claims -----	1,8-11, 13,15,16 2-7,12, 14
Y	WO 02/45764 A1 (BIOTI AS [NO]; LYNGSTADAAS STAALE PETTER [NO]; ELLINGSEN JAN E [NO]) 13 June 2002 (2002-06-13) page 1, line 30 - page 2, line 10 page 9, line 14 - line 20 page 11, line 20 - line 33; claims; examples ----- -/--	2-7,12, 14

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 September 2014	12/09/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Espinosa y Carretero

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/060929

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2012/170975 A1 (US OF AMERICA AS REPRESENTRD BY THE SECRETARY OF THE NAVY [US]; DESILV) 13 December 2012 (2012-12-13) paragraphs [0004], [0023], [0034]; claims	1,8-11, 13,15,16
A	WO 2012/003432 A2 (GILES PATTY-FU [US]) 5 January 2012 (2012-01-05) claims	1-16
A	US 2004/241619 A1 (ALLRED PETER M [US] ET AL) 2 December 2004 (2004-12-02) paragraphs [0016], [0020]; claims	1-16

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2014/060929

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 17
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [X] Claims Nos.: 18-21
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.1

Claims Nos.: 17

Claim 17, Rule 39.1(iv) PCT - Method for treatment of the human or animal body by surgery or therapy. The present application contains 21 claims, of which 4 are independent. There is no clear distinction between the independent claims because of overlapping scope. There are so many claims, and they are drafted in such a way that the claims as a whole are not in compliance with the provisions of clarity and conciseness of Article 6 PCT, as it is particularly burdensome for a skilled person to establish the subject-matter for which protection is sought. The non-compliance with the substantive provisions is to such an extent, that the search was performed taking into consideration the non-compliance in determining the extent of the search (PCT Guidelines 9.19 and 9.25). The search was based on the subject-matter that, as far as can be understood, could reasonably be expected to be claimed later in the procedure, and the corresponding claims, namely claims 1-16

Continuation of Box II.2

Claims Nos.: 18-21

The present application contains 21 claims, of which 4 are independent. There is no clear distinction between the independent claims because of overlapping scope. There are so many claims, and they are drafted in such a way that the claims as a whole are not in compliance with the provisions of clarity and conciseness of Article 6 PCT, as it is particularly burdensome for a skilled person to establish the subject-matter for which protection is sought. The non-compliance with the substantive provisions is to such an extent, that the search was performed taking into consideration the non-compliance in determining the extent of the search (PCT Guidelines 9.19 and 9.25).
The search was
based on the subject-matter that, as far as can be understood, could reasonably be expected to be claimed later in the procedure, and the corresponding claims, namely a craniofacial and/or dental prosthetic device or implant containing a metal material comprising Doxycycline.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

out during examination before the EPO (see EPO Guidelines C-IV, 7.2), should the problems which led to the Article 17(2) declaration be overcome.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2014/060929

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2007093894 A1	26-04-2007	US 2007093894 A1	26-04-2007
		WO 2007050565 A2	03-05-2007
WO 0245764 A1	13-06-2002	AT 271887 T	15-08-2004
		AU 2095502 A	18-06-2002
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		BR PI0413288 A	10-10-2006
		EP 1659976 A2	31-05-2006
		JP 2007501671 A	01-02-2007
		US 2004241619 A1	02-12-2004
		WO 2005016167 A2	24-02-2005