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Synergic antibacterial coatings combining titanium nanocolumns and tellurium nanorods

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List of abbreviated terms

Ar: argon
CFU: colony forming units
Cm: centimeter
CO₂: Carbon dioxide
D: Continuous Ti disk/thin film
DCT: Tellurium-functionalized short Ti columns
DMSO: dimethylsulphoxide
EDTA: Ethylenediaminetetraacetic acid
EDX: Energy Dispersive X-Ray Analysis
H₂DCFDA: 2',7'-dichlorodihydrofluorescein diacetate
LB: Luria Bertani
LC: long Ti columns
LCT: Tellurium-functionalized long Ti columns
MS-GLAD: glancing angle deposition technique by magnetron sputtering
MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
Nm: nanometer
OD: Optical density
PBS: Phosphate-buffered saline
PVP: Polyvinylpyrrolidone
ROS: Reactive oxygen species
SC: short Ti columns
SEM: Scanning Electron Microscopy
Ti: titanium
Te: tellurium

Legend for figures and tables in the main manuscript

Table 1. Designation of samples

Fig. 1: SEM images (top view on the left and cross section on the right) of the nanocolumnar coatings with short nanocolumns (top) and long nanocolumns (bottom), named SC and LC respectively.

Fig. 2: SEM images (left column with secondary electrons and right column with back-scattered electrons; top view on the first row and cross section on the second row) of Te-functionalized nanocolumnar coatings with short nanocolumns, named SCT.

Fig. 3: SEM images (left column with secondary electrons and right column with back-scattered electrons; top view on the first row and cross section on the second row) of Te-functionalized nanocolumnar coatings with long nanocolumns, named LCT.

Fig. 4: EDX spectra on an area centered at a bright nanorod (top) and on an area centered at a bare Ti nanocolumn (bottom).

Fig. 5: *S. aureus* colonies on the different samples after 16h. N=3. * $p < 0.005$ versus control, ** $p < 0.01$ versus control.

Fig. 6: *E. coli* colonies on the different samples after 16h. N=3. * $p < 0.005$ versus control, ** $p < 0.01$ versus control.

Fig. 7: Cytotoxicity assays with osteoblasts cells. N=3 No significant statistical difference found compared to the control.

Fig. 8: ROS analysis for the different metallic surfaces. N=3 * $p < 0.005$ versus control.

Legend for figures and tables in Supplementary material

Fig. S1: SEM images (left column with secondary electrons and right column with back-scattered electrons; top view on the first row and cross section on the second row) of Ag NPs-functionalized nanocolumnar coatings with long nanocolumns.

Fig. S2. *E. coli* colonies on the different samples after 16h.. N=3. * $p < 0.01$ versus control, ** $p < 0.005$ versus control.

Fig. S3. *S. aureus* and *E. coli* colony forming assays prior and after sonication experimental process. D1 and D2 are the samples before and after the ultrasounds treatment, respectively. N=3. No significant statistical difference compared to the control.

Fig. S4. Cytotoxicity assays with osteoblasts cells. N=3. No significant statistical difference found compared to the control. N=3. No significant statistical difference compared to the control.

Fig. S5. XPS wide scan of Te nanoparticles on Ti columns.

Fig. S6. (a) Te 3d, (b) Ti 2p, (c) C 1s, (d) O 1s and (e) N 1s core level spectra.

Fig. S7. Contact angle measurements for the different samples.

Abstract.

Nanocolumnar titanium coatings have been fabricated in two sputtering systems with very different characteristics (a laboratory setup and semi-industrial equipment), thus possessing different morphologies (150 nm long columns tilted 20° from the normal and 300 nm long ones tilted 40°, respectively). These coatings exhibit similar antibacterial properties against Gram positive (*Staphylococcus aureus*) and Gram negative (*Escherichia coli*) bacteria. When a synergic route is followed and these coatings are functionalized with tellurium (Te) nanorods, the antibacterial properties are enhanced, especially for the long nanocolumns case. The biocompatibility is preserved in all the nanostructured coatings.

KEYWORDS: Antibacterial properties, Biocompatibility, Nanostructured coatings, Magnetron sputtering.

Introduction

Bacteria are the oldest living organisms on Earth and mankind has been living between them for a long time. From this interaction, several pathologies have been developed, as well as symbiosis mechanisms. Most of these bacteria are not a cause for concern, but there are a substantial number which have the ability to infect and sicken humans.

One of the major concerns related to bacterial infection is found in implants [1, 2]. Although orthopedic implants have improved their quality properties and enhanced the life of millions of patients, they are not free from the action of bacteria. Therefore, one of the most serious complications of prosthetic devices, in terms of morbidity, mortality and medical costs, is the incidence of infection [3]. Most of these infections are caused by common bacteria, such as *Staphylococcus aureus*, *Staphylococcus epidermitis* or *Escherichia coli*, showing enhanced activity within metallic and biocompatible surfaces used for implants [4, 5, 6]. Once the bacteria, in planktonic growth mode (moving cells), reach the implant, an exponential growth ends with the formation of a biofilm, a polymeric-like matrix with millions of bacterial cells that will spread all over the metallic surface, providing both mechanic stability and antibiotic resistance, avoiding the penetration of antibacterial agents [7, 8].

In fact, one of the milestones in this research field consists of the development of implant surfaces that diminish the bacterial adherence and the formation of a biofilm [9]. There have been several attempts to reduce the bacterial adhesion to metallic surfaces by using corrugation at the nanoscale [10, 11, 12, 13, 14, 15, 16]. A recent study showed the successful preparation of

nanocolumnar coatings made of titanium (Ti) on typical metallic surfaces used in orthopedic devices that preserve the biocompatibility towards human cells while the adherence and proliferation of the bacteria *Staphylococcus aureus* dramatically decreased [17]. Such coatings were prepared by the so-called glancing angle deposition technique by magnetron sputtering (MS-GLAD) [18, 19], a scalable and environmentally friendly technique that allows the production of nanostructured coatings in large areas and with a large variety of morphologies. However, all the in vitro tests were carried out with coatings possessing nanocolumns with similar characteristics, so in this new work our first goal is testing the response of different columns, to check the robustness of this approach. Moreover, up to now only Gram-positive bacteria (*Staphylococcus aureus*) were used. Since producing nanostructures effective in preventing bacterial proliferation for both gram-positive and gram-negative bacteria without using antibiotics is a workhorse for implant technologies, this new work also addresses the behavior with Gram-negative bacteria such as *Escherichia coli*.

Finally, the last goal of this new work is the fabrication of synergic coatings that benefit from the combination of nanostructured metallic surfaces with metallic nanoparticles, which enhances the antibacterial properties of such coatings.

Despite novel techniques, bacteria always find a way to adapt to the situation. Therefore, they still show certain growth on almost all surfaces. In order to overcome this limitation and improve the antibacterial effect of the metallic surfaces, nanostructures can offer a suitable answer [20, 21]. For years, nanoparticles have been created with different sizes, morphologies and compositions using quick, facile and smooth reactions [22]. Several metallic nanoparticles, such as gold [23], silver [24], copper [25], zinc [26] and selenium [27] nanoparticles, have been tested as antibacterial agents with successful results, allowing for the inhibition of bacterial growth.

One important factor when dealing with nanoparticles is the synthetic process. Traditional chemical synthesis has been used for long time, building protocols from the base of both chemistry and physics. Although quick and easy-to-follow, traditional synthetic approaches are full of drawbacks, such as the use of harsh and corrosive chemicals, expensive procedures and more disturbingly, the production of toxic by-products, a real threat for the environment [28, 29, 30]. Therefore, new approaches are needed, and nature can provide a suitable answer to overcome the limitations mentioned above.

Among all the synthetic approaches, hydrothermal methods are very promising because they can be easily implemented and offer high throughput [31] due to the use of supercritical water. Hydrothermal techniques allow for the combination with organic and natural compounds [32], as well as with biocompatible polymers [33] that can act as a coating and stabilizer agent for the generation of stable metallic nanoparticles.

Taking into account all of the above, the combination of metallic nanoparticles with bactericidal effects with nanostructured metallic surfaces that inhibited bacterial adhesion can be a promising strategy in order to create a synergic interaction that enhances the antibacterial properties of the final coating. This interaction is the main aim for the present article, where tellurium (Te) nanorods have been synthesized on top of nanocolumnar titanium coatings using a hydrothermal and environmentally-friendly approach. It is first shown here that two nanocolumnar titanium coatings, with short or long nanocolumns, exhibit similar antibacterial properties against both Gram negative and Gram-positive bacteria. Secondly, it is shown that such behavior is enhanced when Te nanorods functionalized with Polyvinylpyrrolidone (PVP) are synthesized on top of them, especially in the case of long nanocolumns. Finally, it is determined that all of the coatings remain biocompatible for human cells.

Methods

Fabrication of the nanocolumnar coatings

Magnetron sputtering was used to fabricate the nanocolumnar coatings. It is a sustainable method to manufacture thin films for multiple applications, since it is physical technique in vacuum (so no aggressive waste are produced and no recycling problems are associated) and the coating takes place with a small number of steps usually at room temperature (i.e., low energy consumption). Manufacturing nanocolumnar coatings instead of thin films only requires minor modifications, mainly using oblique deposition. Two different kinds of coatings were fabricated at room temperature onto ultrasonically cleaned Si(100) substrates using two set-ups.

On the one hand, Ti coatings with short columns (150 nm length) were deposited onto $\sim 1 \text{ cm}^2$ square substrates in the laboratory setup described in detail in ref. [17], which has a magnetron source with a circular Ti target (5 cm diameter) and a cylindrical collimator (length: 9 cm), the distance between the target and substrate being 22 cm. The parameters used were as follows: argon (Ar) as sputter gas at 0.15 Pa pressure, deposition time = 70 min, power = 300 W, and 75° inclination of the substrate with respect to the target (tilt angle). These samples will be labelled in the following as SC –for short columns–.

On the other hand, a semi-industrial sputtering system that operates at the company Nano4 Energy has been used to prepare Ti coatings with long columns (300 nm length). The target is rectangular ($20 \times 7.5 \text{ cm}^2$) and long substrates ($7 \times 1 \text{ cm}^2$) placed at a 10 cm distance but facing

only one of the long sides of the racetrack were used and subsequently cut into square pieces ($\sim 1 \text{ cm}^2$). The parameters were in this case: Ar as the sputter gas at 0.36 Pa pressure, deposition time = 25 min, power = 285 W, and tilt angle = 77.5° . We will denote these samples as LC –for long columns-. For the sake of comparison, continuous thin films with about 300 nm thickness were also fabricated in a standard configuration (i.e., without a tilt angle) with an 8.5 min deposition time.

Functionalization of the nanocolumnar coatings with PVP-Te nanorods

Tellurium nanorods coated with PVP were synthesized on top of the two types of nanocolumnar coatings using a novel hydrothermal reduction method, reducing sodium tellurate (Na_2TeO_4) to elemental tellurium (Te^0) in the form of nanorods that were attached to the surface.

Briefly, 40 mL of 20 g/L PVP (Sigma Aldrich, St. Louis, MO) in deionized water were mixed with 1.0 gram of L-ascorbic acid (Sigma Aldrich) and enough sodium tellurate to reach a final concentration of 0.5 mM (Alfa Aesar, Haverhill, MA). This solution was stirred for 10 minutes at room temperature. The solution was then transferred into a sealed reaction vessel. Carefully, different samples with nanocolumnar coatings were placed inside, with the coated side facing the top. The reactor was then placed into a Thermo Scientific Heratherm™ General Protocol Oven at 90°C for 20 hours. The vessel was then removed from the oven and allowed to cool naturally to room temperature. The dark liquid was transferred and the metallic pieces were carefully removed from inside and, once placed in a 6-well plate Falcon, they were cleared and washed twice with ethanol and di-water to remove impurities and sterilize the surfaces. As a result of this process, both SC and LC samples were covered by Te nanorods, and the resulting ones were labeled SCT and LCT, respectively, for Te-functionalized short and long Ti columns. Finally,

these SCT and LCT pieces were transferred to another 6-well plate and kept at room temperature for further experiments. Prior experiments, samples were sterilized with ethanol.

It is worth mentioning that the functionalization with Te nanorods of the continuous Ti thin films (that were also sputtered for the sake of comparison, see above) was also tried, but without success: firstly, only a small amount of Te nanorods was produced on top of the films, and secondly, and in clear contrast to what happens onto the Ti nanocolumns, the Te nanorods were easily detached from the surface of the thin films when washing with ethanol and di-water. Thus, Te nanorods alone cannot be used as stable coatings.

Instruments and characterization

Characterization of the samples was accomplished using Scanning Electron Microscopy (SEM) Energy Dispersive X-Ray Analysis (EDX) analysis and X-ray photoelectron spectroscopy (XPS). The morphology has been studied with a FEI Verios 460 Field Emission scanning electron microscope using selective secondary/backscattered electrons detection. EDX spectroscopy analysis was performed using a dedicated EDX detector coupled with a Hitachi S-4800 SEM. Samples were placed into an aluminum pin mount. An accelerating voltage of 10.0 kV was used to obtain an elemental spectrum for the surfaces. For XPS, the chamber has a base pressure of 10^{-10} mbar and is equipped with a hemispherical electron energy Analyzer (SPECS Phoibos 100 spectrometer) and a $\text{MgK}\alpha$ (1253.6 eV) X-ray source. The angle between the hemispherical analyzer and the plane of the surface was kept at 60° . Wide scan spectra were recorded using an energy step of 0.5 eV and a pass-energy of 40 eV while specific core levels spectra (Te 3d, Ti 2p, O 1s and C 1s) were recorded using an energy step of 0.1 eV and a pass-

energy of 20 eV. Data processing was performed with CasaXPS software (Casa software Ltd, Cheshire, UK).

Bacterial cultures

Strains of both gram negative and gram positive bacteria were used in this study to determine the antibacterial activity of the nanocolumnar coating samples, both with and without PVP-Te nanorods. For the sake of comparison, the sputtered Ti thin films as well as commercial Ti disks from Goodfellow (thickness: 0.5 mm, code: 303-115-36) were also used: no significant differences between them were observed, so their results were averaged and jointly labeled D. *Escherichia coli* (strain K-12 HB101; Bio-Rad, Hercules, CA) and *Staphylococcus aureus* (subsp. aureus Rosenbach, ATCC® 12600™; ATCC, Manassas, VA) bacteria were used. Prior to inoculation, the bacterial cultures were maintained on agar plates at 4 °C. Bacteria were introduced into 6 mL of sterile Luria-Bertani (LB) (bioPLUS, bioWORLD) medium in a 15 mL Falcon centrifuge tube and incubated at 37 °C/200 rpm for 24 hours. The optical density (OD) of the bacterial cultures was measured at 600 nanometers (nm) using a spectrophotometer (SpectraMax M3, Molecular Devices, Sunnyvale, CA). The bacterial suspension was then diluted to a concentration of 10^6 colony forming units per milliliter (CFU mL⁻¹) and stored at 4 °C until use.

Antibacterial studies

For the antibacterial assay, a bacterial concentration of 10^6 CFU/mL in LB media was placed inside 6-well plates together with the different samples (Table 1), and they were placed inside an incubator for 16 hours at 37 °C. Right after this time, samples were taken out and the bacterial media was removed. Samples were rinsed twice with Phosphate-buffered saline (PBS) and each of them was transferred inside a 15 mL Falcon tube with 5 mL of PBS. The tubes were then placed inside an ultrasound water bath and sonication was applied for around 5 minutes. Afterwards, a suitable volume of each solution was extracted with micropipettes and diluted in sterile PBS to obtain serial dilutions of 100x, 1000x, and 10,000x. Then, 10 μ L of each sample were placed onto a labeled agar plate and incubated for about 12 hours at 37 °C. The total number of colonies for each sample was counted post-incubation. Colony counting experiments were performed in triplicate with N = 3 for each concentration and dilution.

In vitro cytotoxicity assays

Cytotoxicity assays were performed with Human Fetal Osteoblastic cells (HFOB) (PromoCell, Heidelberg, Germany). Samples with different coatings were placed individually into the wells of a 24-well plate. Before cell seeding, the samples were rinsed twice with PBS to remove any possible debris. Osteoblasts cells were cultured to 80-90% confluence, rinsed twice with PBS and trypsinized using 0.25% trypsin–EDTA (Sigma). Released cells were centrifuged to form a pellet and then resuspended in cell growth medium at a density of 95,000 cells/mL. 1 mL of this solution was added to each well. Samples were incubated under standard culture conditions (37 °C in a humidified incubator with a 5% carbon dioxide (CO₂) atmosphere) for 4 h; then the medium was aspirated from each well and each sample was rinsed with PBS and washed twice. Samples were carefully transferred to a brand new, clean 24 well plate and 1 mL of cell growth

media was added to each sample along with 200 μ L of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) dye (Promega, Madison, WI). Samples were placed back into the incubator for 4 h to allow the MTS to completely react with the metabolic products of adherent cells, and then 200 μ L of the solution from each well was transferred to a 96 well plate in triplicates. The 96 well plate was placed into a SpectraMax M3 microplate reader (Molecular Devices) and the absorbance of the MTS solution in each well was measured at a wavelength of 490 nm. The number of cells adherent to each metallic sample was determined by comparing the resulting absorbance values for each one of the pieces. Standard curves were prepared for conversion between OD values and cells/mL, and then convert to cells/cm² using appropriate software and measurements of the samples.

Reactive oxygen species (ROS) analysis of samples

For ROS quantification, 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) was used. HFOB cells were seed in a 6 well-plate at a concentration of 3×10^5 cells/mL in presence of each one of the four metallic samples –Te-coated and uncoated-, as well as in a control without any metallic surfaces. The cell were cultured under standard culture conditions -37 °C in a humidified incubator with a 5% carbon dioxide (CO₂) atmosphere- for 24 hours before the experiment. Briefly, the ROS indicator was reconstituted in anhydrous dimethylsulphoxide (DMSO) to make a concentrated stock solution that was kept and sealed. The growth media was then carefully removed and a fixed volume of the indicator in PBS was added to each one of the wells at a final concentration of 10 μ M. The cells were incubated for 30 minutes as optimal temperature and the loading buffer was removed after. Fresh media was added and cells were allowed to recover for a short time. The baseline for fluorescence intensity of a sample of the loaded cells period

exposure was determined. Positive controls were done stimulating the oxidative activity with hydrogen peroxide to a final concentration of 50 μ M. The intensity of fluorescence was then observed by flow cytometry. Measurements were taken by an increase in fluorescence at 530 nm when the sample is excited at 485 nm. Fluorescence was also determined in negative control – untreated loaded with dye cells maintained in a buffer-.

Statistical analysis

All experiments were done in triplicate (N=3) to ensure reliability and replicability of the results. Experimental results were assessed for statistical significance using a Student's t-test ($p \leq 0.01$ being considered significant). All data are presented as the mean $\pm$ standard deviation.

Results

Morphology of the nanocolumnar coatings

Samples were organized using the particular designation shown in Table 1.

Table 1. Designation of samples

Sample	Meaning
D	Continuous Ti disk/thin film
SC	Short Ti columns
LC	Long Ti columns
SCT	Short Ti columns with tellurium rods
LCT	Long Ti columns with tellurium rods

As can be seen in Figure 1, the short nanocolumns, about 150 nm in length and labeled SC fabricated with the laboratory set-up, are more vertical than the longer ones (300 nm long and

labeled LC) obtained with the semi-industrial equipment, the inclination of the columns with respect to the normal to the substrate being about 20° and 40°, respectively.

Fig. 1: SEM images (top view on the left and cross section on the right) of the nanocolumnar coatings with short nanocolumns (A, B) and long nanocolumns (C, D), named SC and LC respectively.

Synthesis and purification of the tellurium-functionalized nanocolumnar coatings

Characterization via SEM,EDX and XPS

After purification and cleaning, the Te-functionalized nanocolumnar coatings were characterized using both SEM and EDX not only to determine the composition and nature of the nanorod functionalizing the surfaces, but also to demonstrate that the synthetic approach respected the integrity and morphology of the nanocolumnar coatings.

Figure 2 shows representative SEM images obtained on a Te-functionalized nanocolumnar coating sample with short nanocolumns, labeled SCT. As the signal collected in the images formed with back-scattered electrons depends on the atomic number Z , the Te nanorods ($Z=52$) can be clearly distinguished with brighter contrast than the Ti nanocolumns ($Z=22$) on those images (right column of Fig. 2). It can be observed, especially in the cross section image, that the majority of the Te nanorods lie on the top of the Ti nanocolumns and only a few penetrate in the interspace between columns. In the top view image, two different kinds of Te nanorods can be differentiated, big and small, the former being more frequent. From the statistical analysis of several SEM images and about a hundred nanorods, the big nanorods have a length= 297 ± 78 nm and a diameter= 27 ± 8 nm whereas the small ones have a length= 125 ± 33 nm and a diameter= 10 ± 2 nm. Moreover, it can also be deduced that about 14% of the image area is covered with Te.

Fig. 2: SEM images (A, C with secondary electrons and B, D with back-scattered electrons) of Te-functionalized nanocolumnar coatings with short nanocolumns, named SCT.

Figure 3 shows representative SEM images obtained on a Te-functionalized nanocolumnar coating sample with long nanocolumns, labeled LCT. As it happened in sample SCT, the majority of the Te nanorods lie over the Ti nanocolumnar coating, and again two kinds of nanorods can be distinguished, although the big ones dominate. The statistical analysis indicates that their sizes are: length= 267 ± 86 nm and diameter= 27 ± 5 nm for the big nanorods, length= 90 ± 23 nm and diameter= 8 ± 2 nm for the small ones. Both sizes are quite similar to those observed in SCT. However, a clear difference can be observed: the number of nanorods is now much higher, and as a result about 32% of the LCT sample surface is covered with Te. We attribute this difference to the number of structural defects in the Ti nanocolumns that can act as nucleation centers for the production of Te nanorods: the deposition rate in the semi-industrial

equipment used to fabricate LC is higher than that in the laboratory set-up used to produce SC, thus more defects are expected.

Fig. 3: SEM images (A, C with secondary electrons and B, D with back-scattered electrons) of Te-functionalized nanocolumnar coatings with long nanocolumns, named LCT.

In order to check the composition of the nanorods, EDX measurements were performed with the beam centered in two different positions: on top of a bright nanorod in the back-scattered electrons image and on top of a naked Ti nanocolumn. This characterization confirmed that the bright nanorods were composed of tellurium as determined by specific tellurium peaks (see Figure 4). Significant oxygen, carbon and nitrogen peaks are also seen, indicating the presence of PVP within the nanorods. The presence of silicon and aluminium peaks can be attributed to the substrate and the sample holder, respectively.

Finally, a complete XPS analysis of sample LCT have been included in the Supplementary Information. As XPS is a surface sensitive technique, and as the majority of the Te nanorods lie over the Ti nanocolumnar coating, the Te nanorods were clearly detected, whereas only the top part of the Ti nanocolumns could be detected but with a signal one order of magnitude smaller than that from Te. Besides C, N and O were also detected.

Fig. 4: EDX spectra on an area centered at a bright nanorod (A) and on an area centered at a bare Ti nanocolumn (B).

Antibacterial activity against both *Escherichia coli* and *Staphylococcus aureus*

Antibacterial studies by means of colony counting unit assays were performed on nanostructured samples (short and long nanocolumns) both coated and uncoated with tellurium nanorods, comparing the data with a plain metallic surface. Colony forming assays were performed using *Escherichia coli* and *Staphylococcus aureus*. A control experiment –colony forming assay- was conducted with the bacteria before and after sonication for the same experimental conditions to which the metallic samples were subjected, with the aim to study how the ultrasounds would affect the bacteria (Figure S3).

Figure 5 shows the results for *Staphylococcus aureus*. Firstly, it can be stated that the nanocolumnar coatings show an antibacterial effect as compared to the metallic control.

Fig. 5: *S. aureus* colonies on the different samples after 16h. N=3. *p<0.005 versus control, **p<0.01 versus control.

Figure 6 summarizes the results obtained in the experiments with *Escherichia coli*.

Fig. 6: *E. coli* colonies on the different samples after 16h. N=3. *p<0.005 versus control, **p<0.01 versus control.

In vitro cytotoxicity with osteoblast cells

To determine the possible toxicity on mammalian cells of the nanocolumnar coatings as well as of the synergic coatings functionalized with PVP-coated tellurium nanorods, *in vitro* cytotoxicity assays were performed with human osteoblast cells. As it can be seen in Figure 7, neither the Ti nanocolumnar coatings nor those functionalized with Te rods exhibit less osteoblast cells attached to the surface.

Fig. 7: Cytotoxicity assays with osteoblasts cells. N=3 No significant statistical difference found compared to the control.

Cytotoxicity analysis was also conducted with Te nanorods alone, with the aim to elucidate the behavior of the osteoblast cells in presence of the nanostructures (Image S4)

Reactive oxygen species (ROS) analysis

The ROS generation study is summarized in Figure 8. A slight increase of ROS production was observed in the Ti nanocolumnar coatings (SC and LC), while a much higher release of these oxygen active species was noticed for the synergic samples with Te nanorods on top of Ti nanocolumns (SCT and LCT).

Fig. 8: ROS analysis for the different metallic surfaces. N=3 *p<0.005 versus control.

Discussion

Morphology of the nanocolumnar coatings.

The morphology of the Ti coatings is the outcome of the shadowing mechanisms at the atomic level (which are enhanced with the percentage of ballistic atoms, the culmination degree and the tilt angle) and the relatively high kinetic energy of the sputtered atoms [18]. The particular morphology in each case depends on the geometry of the targets and the deposition chambers as well as on the deposition parameters that were used. As can be seen in Figure 1, the short nanocolumns, about 150 nm in length and labeled SC fabricated with the laboratory set-up, are more vertical than the longer ones (300 nm long and labeled LC) obtained with the semi-industrial equipment, the inclination of the columns with respect to the normal to the substrate being about 20° and 40°, respectively.

Synthesis and purification of the tellurium-functionalized nanocolumnar coatings and characterization via SEM, EDX and XPS

As it has been proved by SEM and EDX, a simple, safe method was developed for the synthesis of tellurium nanorods on top of the nanocolumnar coatings employing a hydrothermal approach as an environmentally-friendly approach. The presence of the reducing agent, L-ascorbic acid, in

addition to the polymeric material, PVP, within the solvent, assisted in the reduction of metallic ions to form nanoparticles that grew within the surface of the metal. The temperature gradient within the reactor also assisted in the formation of the nanostructures [34, 35]. Both L-ascorbic acid and PVP are readily available, environmentally benign, and odorless. Furthermore, the Food and Drug Administration (FDA) has approved PVP for use as a food additive and in pharmaceutical applications. Because these environmentally safe materials were used, no toxic byproducts were produced and the liquid remaining after the experiments was used for obtaining single nanorods (no attached) for further experiments.

Concerning the XPS experiments, significant facts can be deduced, especially when combined with the results provided by the ROS analysis. Half of the detected Te is metallic and the other half is in oxide form. Taking into account the kinetic energy of the photoemitted electrons, the estimated thickness of the Te oxide layer can be deduced: it is about 3nm. Although the mechanism responsible for the bactericidal activity of TeNPs is not yet completely understood, the production of ROS is the most accepted mechanism [36]. Thus, it could be expected that the Te nanorods provide bactericidal activity, which is confirmed in the ROS generation experiments summarized in Fig. 8 where samples SCT and LCT exhibit enhanced ROS production.

On the other hand, for Ti, in spite of the weak signal, it can be inferred that the thickness of the titanium oxide measured is at least 2 nm. This is in agreement with our previous work [22] in which grazing incidence XRD patterns revealed the presence of a very thin titanium oxide layer formed as a result of dry passivation in ambient conditions, a few monolayers thick (6 monolayers is about 2.2nm). Besides, in that previous work with Ti nanocolumns only, we did

not observe bactericidal effects, but the adhesion of *S. aureus* was strongly impeded. In the new ROS experiments, samples SC and LC only showed a slight increase of ROS production.

Antibacterial activity against both *Escherichia coli* and *Staphylococcus aureus*

Data from experiments with *Staphylococcus aureus* show that the nanocolumnar coatings show an antibacterial effect as compared to the metallic control. This could be expected after Ref. [17], where a notable decrease in *S. aureus* adhesion was observed for Ti nanocolumnar coatings using not only collection strains but also clinical ones. The novelty now is that both coatings, with short and long columns, exhibit the same effect. Taking into account that these two coatings have been fabricated in two sputtering systems with very different characteristics (a laboratory setup with a small circular target and a semi-industrial equipment with a large rectangular target) and that the resulting coatings have different morphologies (150 nm long columns tilted 20° from the normal and 300 nm long ones tilted 40°, respectively), it can be stated that the proposed strategy to achieve antibacterial effects is robust: the key point is to produce nanocolumns, which offer a limited number of anchoring points for the adhesion of the bacteria. Secondly, it is clear that the functionalization with tellurium nanorods leads to an enhanced antibacterial activity of the metallic coatings, with even a lower number of bacteria growing on top of the surface. Such enhancement in this synergic route combining Ti nanocolumns and Te nanorods is especially efficient in the case of long nanocolumns (LCT), something that can be ascribed to the higher amount of tellurium nanorods for such coatings, as it has been explained in section 3.2.

The observed behavior for *Escherichia coli* is similar than that obtained with *S. aureus*: i) the two coatings, with short and long columns, exhibit an antibacterial effect with similar intensity;

and ii) such an antibacterial effect is enhanced when the synergic approach with Te nanorods is considered, especially in the case with long Ti nanocolumns. It is worth mentioning that Gram-negative bacteria have a second outer lipid membrane in the bacterial cell wall, which gives them a special resistance to the conventional treatment with antibiotics. Thus, the antibacterial effect obtained with these coatings even with Gram-negative bacteria gives them added value, boosting its potential use in permanent and temporary implants susceptible to infection.

It is also important to mention that Te nanostructures themselves have shown bactericidal effects against bacteria [37]. Thus, the combination here studied, a nanocolumnar surface that is able to reduce the adhesion of bacteria with metallic nanorods that exhibit a bactericidal effect, seems to be a synergetic combination with suitable applications in biomedicine.

It has been reported that the detachment of bacteria from metallic surfaces using sonication for a short period of time can cause cell lysis and a lost in viability [38]. Colony forming assays were performed using bacteria solutions before and after ultrasonication at the same experimental conditions that the metallic samples, in order to elucidate possible effects on the cells (see Supplementary Information). Data show that there is no significant difference between the number of cells before and after the experiments. Therefore, the ultrasounds, at the experiment conditions, have a bacteriostatic effect, but no bactericidal. As a consequence, it is possible to conclude that the bacterial inhibition showed is due to the coating and the surfaces themselves, not to the sonication process conducted prior experiments.

Considering all things together, and following the schematics proposed by Sjollemma and co-workers to classify antimicrobial surfaces [39] we can say that the Te nanorods mainly provide

contact-killing (bactericidal effect) whereas the Ti nanocolumnar template promoted non-adhesivity. It may be argued that wherever there is a bunch of Te nanorods, the Ti nanocolumns are hidden, modifying the non-adhesive behavior. Contact angle measurements (see Supplementary Information) point in that direction, since the initial flat surface is hydrophilic (sample D), the nanocolumnar Ti coatings are hydrophobic (samples SC and LC), and the synergic coatings with Te nanorods (samples SCT and LCT) become hydrophilic again. This may indicate that the non-adhesive behavior decreases, but as the Te rods add contact-killing, the overall effect is an enhancement of the antibacterial properties, as shown in Figs. 5 and 6.

The antibacterial effect of the tellurium nanorods alone was reported in a previous study [40], showing a significant delay in the bacterial growth after exposure of both bacterial strains to different concentrations of the nanorods.

In vitro cytotoxicity with osteoblast cells

Data from cytotoxicity experiments showed an agreement with a previous study [17] in which Ti nanocolumnar surfaces exhibited good osteoblast adhesion and proliferation, since these cells are much bigger (tens of micrometers) than the diameter and separation between nanocolumns (about 50 and 100 nm, respectively) and can be anchored easily. Actually, it seems that the adhesion in the coatings containing tellurium nanorods is slightly enhanced when compared to the other samples.

For the sake of comparison, cytotoxicity analysis of Te nanorods alone (i.e. in solution and without Ti nanocolumns) in presence of osteoblasts cells showed no significant cytotoxic effect

for these cells at nanorods concentrations up to 50 $\mu\text{g/mL}$, and with a slight decay in the cell proliferation at concentrations up to 100 $\mu\text{g/mL}$, but not reaching less than 65% of cell viability (see Supplementary Information).

In summary, all the coatings remain biocompatible for human cells and allow for their proliferation, with a slight enhancement when the tellurium nanorods are present in the surface of the samples.

Reactive oxygen species (ROS) analysis

ROS are chemically reactive agents and free radicals containing oxygen, such as hydroxyl ($\text{OH}^\cdot$) or superoxide ($\text{O}_2^\cdot$) groups. Although these species are normally formed as natural byproducts of oxygen metabolism, exposure of cells to metallic nanoparticles can increase ROS concentrations to induce cell toxicity [41,42]. There is a slight increase in the ROS production compared to the control for both SC and LC samples, which can be explained due to the presence of a small layer of titanium oxide covering some parts of the structure. It has been reported that the presence of titanium oxide is related to the production of ROS [43]. The increase of the production of ROS when Te nanorods are added, samples SCT and LCT, can be ascribed to the presence of tellurium oxide in these samples, as was deduced in the XPS experiments. Therefore, it is shown that the presence of PVP-coated tellurium nanorods increases the formation of ROS.

Therefore, as conclusions of the project, nanocolumnar titanium coatings exhibit antibacterial properties against both Gram negative and Gram positive bacteria. It has been verified that the antibacterial activity is similar for two types of coatings with short and long nanocolumns, which allows us to state that the strategy is robust. Such antibacterial properties are enhanced when tellurium nanorods are added, especially for the long columns case, and remarkably for Gram negative bacteria. Finally, it has been proved that the biocompatibility is preserved in all the nanostructured coatings.

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Graphical abstract

The synergetic combination of tellurium nanostructures prepared using a green-synthesis approach and metallic titanium surfaces have been accomplished resulting in an enhancement of antimicrobial properties –against both Gram negative and positive bacteria- and the biocompatibility effect –on osteoblasts- respect to the systems alone, resulting in a successful combination of materials that can be used for biomedical purposes.

ACCEPTED MANUSCRIPT

NANO_1923:Table 1

Table 1. Designation of samples

Sample	Meaning
D	Continuous Ti disk/thin film
SC	Short Ti columns
LC	Long Ti columns
SCT	Short Ti columns with tellurium rods
LCT	Long Ti columns with tellurium rods