Original Article

Biocompatibility and antibacterial properties of TiCu(Ag) thin films produced by physical vapor deposition magnetron sputtering

Saqib Rashid[a,#], Gian Marco Vita[b,#], Luca Persichetti[b], Giovanna Iucci[b], Chiara Battocchio[b], Rostislav Daniel[c], Daniela Visaggio[b], Martina Marsotto[b], Paolo Visca[b], Edoardo Bemporad[a], Paolo Ascenzi[b,d], Giovanni Capellini[b], Marco Sebastiani[a*], Alessandra di Masi[b*]

[a]*Università degli Studi Roma Tre, Engineering Department, via della Vasca Navale 79, 00146 Rome, Italy*

[b]*Università degli Studi Roma Tre, Department of Science, Viale Guglielmo Marconi, 446, 00146 Rome, Italy*

[c]*Department of Materials Science, Montanuniversität Leoben, Franz-Josef-Straße 18, Leoben, 8700, Austria*

[d]*Accademia Nazionale dei Lincei, Via della Lungara 10, 00165 Rome, Italy*

# These Authors contributed equally to this work

Running title: Biocompatibility and antibacterial properties of TiCu(Ag) thin films

***Corresponding authors:**

Marco Sebastiani, e-mail: marco.sebastiani@uniroma3.it

Alessandra di Masi, e-mail: alessandra.dimasi@uniroma3.it

Abstract

Mechanical robustness, biocompatibility, and antibacterial performance are key features for materials suitable to be used in tissue engineering applications. In this work, we investigated the link existing between structural and functional properties of TiCu(Ag) thin films deposited by physical vapor deposition magnetron sputtering (MS-PVD) on Si substrates. Thin films were characterized by X-ray diffraction (XRD), nanoindentation, atomic force microscopy (AFM), and X-ray photoelectron spectroscopy (XPS). The TiCu(Ag) films showed complete amorphous structure and improved mechanical properties in comparison with pure Ti films. However, for contents in excess of 20% Ag we observed the appearance of nanometric Ag crystallite. The TiCu(Ag) thin films displayed excellent biocompatibility properties, allowing adhesion and proliferation of the human fibroblasts MRC-5 cell line. Moreover, all the investigated TiCu(Ag) alloy display bactericidal properties, preventing the growth of both *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Results obtained from biological tests have been correlated to the surface structure and microstructure of films. The excellent biocompatibility and bactericidal properties of these multifunctional thin films opens to their use in tissue engineering applications.

Keywords: bactericidal; biocompatibility; copper; human fibroblast; physical vapor deposition (PVD); silver; titanium; thin films.

1. Introduction

The aging of world population drives an increasing demand of tissue and organ replacements [1]. More than 10 million transplantations are performed annually, with a yearly increase of about 6% and an overall cost of more than $500 billion per year [2]. However, tissue and organ transplantations present two major limitations: the low availability of donors and/or risk of disease transmission and immune rejection [3-5].

Tissue engineering (TE) is an emerging and promising alternative approach of biomedicine to treat or to replace damaged tissues and organs. TE combines materials science, chemistry, physics, and cell biology to allow tissue and organ repair or reconstruction. TE is often based on nanoscaffolds that enables cell adhesion, migration, proliferation, and differentiation [6-9]; the properties of the scaffold mainly depends upon the types of biomaterial and fabrication techniques [8]. In particular, the size, the shape, and patterning of adhesion sites are crucial elements in the design of effective scaffold surfaces. Moreover, nano-scaffolds must be biocompatible and can be combined with organic and inorganic materials to mimic the structure and function of the natural extracellular matrix (ECM), which allows cells to accomplish the biochemical and biophysical functions related to tissue and/or organs regeneration [8, 10]. Cells grown on nanoscaffolds can generate biocompatible, immunocompatible, and biofunctional tissues inside the body, counteracting the drawbacks associated with autologous grafting and allograft tissue transplantation, thereby alleviating the risk of rejection [8-9].

Another critical issue of nanoscaffolds is their capability to prevent microbial growth. Antimicrobial capability is conventionally obtained by means of biochemical approaches relying on nanoscaffold coating with biocidal substances such as silver and antibiotics. Nonetheless, chemical toxicity, antimicrobial durability, and microbial resistance remain critical problems [11].

In this complex framework, surface engineering and development of nanostructured thin films is gaining importance, especially for applications where a combination among surface hardness (or wear resistance), biocompatibility, and antibacterial performance are desired. Recently, multi-element thin films, such as Zr-based thin films (*e.g.*, Zr-Cu, Zr-Cu-Ag, ZrCN, Zr/ZrCN multilayer) [12, 13] and Ti-based thin films (*e.g.*, TiN, TiCu, Ti-Zr-Si) have emerged as a new class of nano-engineered thin films, featuring an excellent combination of high mechanical strength and biocompatibility. Moreover, these films are promising systems for biocompatible coating deposition. Indeed, the use of physical vapor deposition (PVD) for their growth allows a fine control of the material nanostructure, which leads to increased hardness and wear resistance [14, 15].

The desired combination of mechanical strength, biocompatibility, and antimicrobial activity can also be achieved by constructing multi-layers. Recent studies have confirmed the potential antibacterial behavior of Au, Cu, Zn, Ag additions to Ti-based films [16, 17]. The biocidal performance of Cu is linked to the release of Cu^{+1} and Cu^{+2} ions, as observed in TiCu [18, 19]. Recently, Cu-based systems have also been proposed to engineer surfaces with antiviral properties, also in the framework of the COVID-19 pandemic [20]. Very recent examples include Cu-coated touch surface fabricated by cold-spray technology, as well as antiviral Cu_xO/TiO_2 photo catalyst thin films with photo-activated anti-viral properties [21, 22]. Consequently, the TiCu systems are of particular interest for the generation of material systems, featuring both anti-infective properties and surface hardness, as required in human implants and/or touch surfaces [23, 24].

Here, we report the development of antibacterial metallic TiCu(Ag) PVD sputtered thin films combining biocompatibility with relatively high surface hardness. By using high-resolution surface chemical and morphological characterization, combined with cell growth

studies and antibacterial tests, a significant biocompatibility and antibacterial properties have been observed, and correlated to TiCu(Ag) film structure and surface properties.

2. Materials and methods

2.1. Thin film deposition

TiCu(Ag) thin films were deposited on 21×7 mm^2 coupons extracted from 4" Si (100) undoped wafers by means of direct current (DC) magnetron sputtering in a deposition system equipped with three unbalanced magnetrons. The choice of a silicon substrate eases the study of the process-structure-property correlations for the Ti-Cu-Ag system. Nonetheless, we plan to use other substrates of commoner use in the biomedical realm. We have employed for the deposition Ti, Cu, and Ag targets with a 3" diameter and featuring 99.99% purity. The Si substrates were cleaned in ultrasonic bath and ethanol for 10 minutes before mounting them on the substrate holder. An Ar^+ sputtering step (powered by radio frequency (RF) power supply at 50 KHz, at the Ar pressure of 1.2 Pa and a discharge power of 0.03 KW) was performed to clean and activate the Si surface immediately prior to metal deposition. The distance between the substrates and targets was 70 mm, while the substrate was kept in rotation at 80 rpm. All the depositions were performed at a 0.52 Pa Ar pressure (chamber base vacuum of 1.0×10^{-5} Pa), with no intentional substrate heating. By applying different DC-power to the targets for 40 min, 4 sample sets were obtained, always keeping the Ti:Cu ratio equal to ~1. Deposition conditions, thickness, and composition are listed in Table 1.

2.2. Characterization of thin films

Crystallographic structure of the thin films was carried out by X-ray diffraction (XRD), using a θ-2θ Bruker D8 Advanced system with Cu Kα radiation (λ = 0.154 nm). Diffraction scans were performed by using grazing incident angle of 0.75 deg with time step of 0.02˚/sec.

The composition of the thin films was estimated via energy dispersive X-ray spectroscopy (EDX, Oxford instrument INCA), using built-in sensitivity factors for calibration. The film thickness was measured by using a white light optical profilometer with a Leica DCM 3D software package via automatic step measurement of the coated and the uncoated parts of the substrate.

The elastic modulus (E) and hardness (H) values were determined using nano-indentation testing method using a KLA-Nanomechanics G200 fitted with a Berkovich diamond indenter operating in continuous stiffness measurement mode, hence allowing to obtain both E and H as a continuous function of the depth from a single indentation experiment [25]. A standard fused silica sample was tested before and after a batch of measurements to calibrate the tip, so to ensure the reliability of the results. A least 25 indentations were performed on each sample. Calculations were made by the Oliver and Pharr method from the load-displacement curve using 10% of the film thickness at the maximum indentation depth [26].

2.3. X-ray Photoelectron Spectroscopy (SR-XPS)

X-ray Photoelectron Spectroscopy (SR-XPS) measurements were performed at the materials science beamline (MSB) of the Electra synchrotron radiation source (Trieste, Italy). The UHV end station, with a base pressure of 2×10^{-10} mbar, is equipped with a SPECS PHOIBOS 150 hemispherical electron analyzer and a dual-anode Mg/Al X-ray source, an ion gun, and a sample manipulator with a K-type thermocouple attached to the rear side of the sample. Al Kα radiation at 1486.6 eV photon energy impinging at 60˚ was used to analyze the Cu2p, Ti2p, Ag3d, C1s, N1s and O1s core levels on the respective samples. Photoelectrons were detected at normal emission geometry.

149 Calibration of the energy scale was made referencing the spectra to the C1s core level
150 signal of aliphatic C atoms (285.0 eV). Curve-fitting analysis of the experimental spectra was
151 carried out using Gaussian curves as fitting functions. The $Ti2p_{3/2,1/2}$ core level were fitted
152 using a spin−orbit splitting of 5.7 eV and a ($2p_{3/2}/2p_{1/2}$) branching ratio of 2; the $Cu2p_{3/2,1/2}$
153 doublets were fitted using a spin-orbit splitting of 19.8 eV and a ($2p_{3/2}/2p_{1/2}$) branching ratio
154 of 2. For the $Ag3d_{5/2,3/2}$ doublets, a splitting of 6.0 eV and a branch $3d_{5/2}/3d_{3/2}$ ratio of 1.5 were
155 used. When different species were identified in a spectrum, the same Full Width at Half
156 Maximum (FWHM) value was set for all individual photoemission peaks. Atomic ratios were
157 calculated from peak intensities by using Scofield's cross section values.
158
159 *2.4. Surface sterilization*
160 Thin films were rinsed in 70% ethanol in sterile deionized water and then flamed with a
161 Bunsen burner. Sterilization was performed under biosafety cabinets with installed HEPA
162 filters to avoid contamination. After sterilization, films were air-dried, and structural and
163 mechanical properties were evaluated in order to verify stability prior to testing for
164 biocompatibility and antibacterial properties.
165
166 *2.5. Human cells culture*
167 MRC-5 human lung fibroblasts were cultured in Dulbecco's Modified Eagle Medium
168 (DMEM) (Corning, VA, USA) complemented with 10% fetal bovine serum (FBS) (BioWest,
169 Nuaillé, France), 100 mg/mL penicillin and streptomycin (Merck KGaA, Darmstadt,
170 Germany), and 2.0×10^{-3} M L-glutamine (Merck KGaA). Cells were grown at 37 °C and 5%
171 CO_2. Before seeding, cells were counted using the BLAUBRAND® counting chamber (Brand
172 GMBH, Wertheim, Germany).
173

174 *2.6. Evaluation of surfaces biocompatibility*

175 To correlate MRC-5 cell density to the relative luminescence units (RLU) value, a
176 calibration curve was set up. With this aim, MRC-5 cells were plated in triplicate in opaque
177 96-well plates at a density of 2.5×10^3, 5.0×10^3, 1.0×10^4, 2.0×10^4, and 4.0×10^4 cells/well. On
178 the basis of the results obtained from the calibration curve, 1.0×10^4 MRC-5 fibroblasts were
179 seeded on sterile TiCu surfaces coated with 0%, 10%, 20%, and 30% Ag thin films placed
180 into opaque 96-well plates. Cell growth and proliferation was assessed by incubating MRC-5
181 fibroblasts for 10 min with the Cell Titer-Glo® Luminescent reagent (Promega, Madison, WI,
182 USA) added in a 1:1 ratio with the complete cell culture medium. Luminescence was measured
183 using the Tecan Spark 10M plate reader (Tecan, Männedorf, Switzerland). Background
184 luminescence was measured in the complete culture medium without cells, and subtracted
185 from each experimental value.

186

187 *2.7. Testing of the antibacterial properties of TiCu(Ag) thin films*

188 Bacteria were routinely grown in Nutrient Broth (NB) No. 2 (# CM0067B; Thermo
189 Scientific™, Waltham, Massachusetts, USA). The day before the experiment, glycerol stocks
190 of *S. aureus* ATCC 25923 or *P. aeruginosa* ATCC 15692 (strain PAO1) were streaked on NB
191 supplemented with 15% agar (NA) plates and incubated at 37 °C for 24 h. By using sterile
192 inoculating loops, bacterial colonies were transferred in 1 mL NB diluted 1:500 ($NB_{1:500}$) in
193 deionized sterile water and the bacterial concentrations was adjusted to ~5.0×10^7 colony
194 forming unit (CFU)/mL.

195 The plate count method was conducted according to a minor modification of the
196 International Standard ISO 22196 protocol [27]. Briefly, each sterilized TiCu and TiCu(Ag)
197 surface, as well as the glass control surface, was placed into a Petri dish (⌀ 3 cm). Then, 0.005
198 mL of a suspension of either *S. aureus* or *P. aeruginosa* at a concentration of ~ 5.0×10^7

CFU/mL was dripped onto the surfaces and samples were incubated at 37 °C overnight (ON) at 99% relative humidity. After incubation, each surface was placed into 1 mL $NB_{1:500}$ at room temperature for 15 min, and then vortexed for 1 min to allow the detachment of bacteria from the surface. The bacterial suspension was appropriately diluted and plated on NA for CFU counts. To determine the CFU at time 0 h, suspensions of either *S. aureus* or *P. aeruginosa* (presumptive concentration ~5.0×10^7 CFU/mL) were appropriately diluted in saline and plated onto NA. At least two samples were assessed for each bacterial strain. The antibacterial activity (BA) was calculated by the following formula:

$$BA = [(N_{0\,h} - N_{24\,h}) / N_0] \times 100\%$$

Where, $N_{0\,h}$ and $N_{24\,h}$ are the CFU average numbers counted at 0 h and 24 h, respectively, for each type of surface.

2.8. Ag and Cu ions release from TiCu and TiCu(Ag) thin films in agar

An agar diffusion assay was performed to detect the release of bacterial growth inhibitors. A suspension of either *S. aureus* or *P. aeruginosa* (OD_{600} = 0.1) was uniformly spread onto NA plates using a sterile cotton swab. TiCu and TiCu(Ag) surfaces were placed on the NA inoculated plates. The glass surface was used as negative control, whereas antibiotic discs (*i.e.*, erythromycin, E 15 µg; and amikacin, AK 30 µg) were used as positive control of bacterial inhibition. After 16 h incubation at 37 °C, the release of antibacterial factors by TiCu(Ag), glass surfaces, and antibiotic discs was visually assessed by the presence of the inhibition zone around the sample.

2.9. Ag and Cu ions release from TiCu and TiCu(Ag) thin films in solution

The concentrations of Ag and Cu ions released by TiCu and TiCu-30% Ag thin films were measured by inductively coupled plasma optical emission spectrometry (ICP-OES) using an ICP-OES 710 Varian Spectrometer (Agilent Technologies, Santa Clara, CA, USA). Briefly, TiCu and TiCu-30% Ag thin films were immersed in 5 mL $NB_{1:500}$ supplemented with 0.02% (*w*/*v*) sodium azide for 24 h and 7 days at 37 °C. Collected medium were mixed with 5% HNO_3, heated for 1 h at 90 °C, and filtered through a Millipore membrane (pore size 0.45μm) prior to ICP-OES analysis. Concentration of Ag and Cu ions released for each thin film was normalized to the volume of bacterial suspension used in the antibacterial assay.

2.10. Testing of the antibacterial activity of $AgNO_3$ and $CuSO_4$

S. aureus ATCC 25923 and *P. aeruginosa* ATCC 15692 were incubated for 24 h at a density of ~5.0×10^7 CFU/mL in a final volume of 2 mL of $NB_{1:500}$ or $NB_{1:500}$ supplemented with 4 μg/mL $AgNO_3$, or 228 μg/mL $CuSO_4$, or both salts. After 24 h, 0.05 mL aliquot of the bacterial suspension were diluted in saline and plated on NA for CFU counts. To determine the CFU at time 0 h, suspensions of either *S. aureus* or *P. aeruginosa* (presumptive concentration ~5.0×10^7 CFU/mL) were appropriately diluted in saline and plated onto NA. The BA of $AgNO_3$ (μg/mL) or $CuSO_4$ (μg/mL) was calcuted by the following formula.

$$BA = [(N_{0\ h} - N_{24\ h}) / N_0] \times 100\%$$

Where, $N_{0\ h}$ and $N_{24\ h}$ are the CFU average numbers counted at 0 h and 24 h, respectively, for each condition assayed.

2.11. Morphological characterization

The surfaces morphology was characterized by AFM and optical microscopy; the results obtained were compared to control samples represented by a suspension of bacteria poured

directly on the TiCu surface. AFM measurements were performed using a Dimension ICON AFM (Bruker, Santa Barbara, CA) operating in peak-force mode. The AFM was equipped with a ScanAsyst-Air Bruker silicon probe featuring a nominal cantilever elastic constant of 0.4 N m^{-1} and a tip with a nominal radius of 2 nm. For each measurement, height sensor and peak force error images were recorded simultaneously. The AFM images were analyzed and processed with the software Gwyddion [28], applying a first-order flattening. Surface roughness was obtained by measuring the root-mean-square deviation of surface heights on 20×20 μm^2 images. Optical microscopy images were acquired using a Nikon Eclipse ME600 microscope equipped with Nikon DXM1200 digital camera (Nikon, Tokio, Japan).

3. Results

3.1. Structural, morphological, chemical, and mechanical characterization of substrates

The X-ray rocking curves of the as-deposited thin films (see Table 1) are shown in Figure 1. The diffraction pattern of the TiCu samples shows only a broad band in the [38-45°] 2θ-range, pointing to an amorphous structure [29-30].

For Ag concentration exceeding 10%, we observed the appearance of a second feature, whose angular position is compatible with the Ag(111) reflection, showing an increasing intensity and a narrowing for increasing Ag content. This point out to a Ag precipitation phenomenon with the formation of Ag nanocrystallites at Ag contents > 20%. By using the Scherrer's equation on XRD spectra, an Ag crystallite size of the order of 3-5 nm for the film with 20% Ag addition was estimated. No impact on the XRD spectra has been observed flaming with alcohol.

The AFM analysis revealed crack-free smooth surfaces for all thin films with an increase in the average roughness as the Ag content increased from 10 to 30% as shown in Figure 2. The amorphous TiCu and TiCu(Ag) thin films with the Ag content below 20% exhibited an

average surface roughness well below 0.5 nm. For TiCu(Ag) film featuring Ag content > 20%, we observed the appearance of nanoclusters, that, in agreement with the XRD evidence above discussed, was attributed to the diffusion and segregation of Ag and Cu atoms, leading to the formation of Ag nano-crystalline precipitates. Consequently, the surface roughness increases up to ~1 nm in the Ag-richest sample (see Fig.3). It is worth noting that the surface clusters observed by AFM have a size larger than that obtained by the XRD analysis. This is not in contrast with our hypothesis, since the surface cluster might be formed by the assembly of different nanocrystallites of a lesser size.

Thin films with such a low average surface roughness are in general very favorable for antibacterial and biomedical applications [31], especially if antimicrobial agents such as Cu and Ag are added into the protective thin film, which induces a release of metallic ions after exposure to a humid environment, as we shall furter discuss in the following sect. 3.4 [32]. The elastic modulus E and hardness H of thin films were calculated as a function of Ag contents by the nanoindentation method. The TiCu film exhibited the highest E = 124.3 GPa and H = 7.83 GPa values. As we can observe in Figure 3A and 3B, the addition of Ag into the TiCu(Ag) thin films induced a decrease of both modulus and hardness, with the lowest values of modulus (109.25 GPa) and hardness (6.45 GPa) observed in the sample containing 30% of Ag. The very good adhesion of the coating layer has been addressed in a previous article [33].

After the flame sterilization test, no change was observed in the mechanical properties of thin film, consistent with a previous report [33]. All the samples were analyzed by XPS spectroscopy before and after flaming with alcohol (flamed samples will be labelled (F) in the following text). The measured binding energies (BE, ±0.2 eV), FWHM, and atomic ratios calculated from peak areas for all the analyzed samples are reported in Table S1.

In Figure 4 is reported as an example the Ti2p, Cu2p, and Ag3d spectra and the relative curve-fitting analysis for the sample TiCu-30%Ag (F). The measured BE value of the $Ti2p_{3/2}$

signal (458.7 eV) corresponds to the expected value for TiO_2 [34]: indeed, when exposed to air, Ti is always oxidized to titania in the outmost surface layer [35]. The $Cu2p_{3/2}$ signal results from two components peaks located at 933.0 eV and 935.0 eV that have been attributed to metallic (Cu) and oxidized (CuO) copper, respectively. Moreover, the presence of a shake-up satellite, evident in the spectrum at about 943.5 eV, is a distinctive feature of Cu in the +2 oxidation state [36]. Finally, the $Ag3d_{5/2}$ main peak position at 368.5 eV is typical of metallic Ag; a small higher BE component, about 10% of the main component peak, located at about 369.9 eV can also be observed in the spectrum, and can be attributed to oxidized, positively charged silver atoms, indicated as Ag_2O in Table S1 [37]. In all samples, Ti was completely oxidized to Ti(IV), Cu was partially oxidized to Cu(II), and most Ag was predominantly in the unoxidized metallic state, consistent with the different reactivity towards oxygen of the three metals (Table S1). Since XPS spectroscopy probes a 1-5 nm thin outer layer of the surface, the appearance of O-related signal can be attributed to surface contamination during the sample transport from the growth reactor to the XPS analysis chamber at ELETTRA (not connected *in-vacuo*).

Peak areas have been used to calculate atomic ratios between the oxidized and metallic components and between the three elements present on the sample surface (Table S1). The measured atomic ratios between copper and titanium disagree with the expected value of 1:1, possibly because XPS is a surface-sensitive technique with a sampling depth of approximately ~5 nm. Therefore, we argue that the discrepancy observed between measured and expected elemental composition is relative only to the outmost sample surface. The effect could be due to the induced segregation between the two metals, given by their difference in density.

For TiCu samples, the Cu content of the sample surface was lower than expected. On the other hand, the Ag:Ti atomic ratio on the surfaces of TiCu(Ag) thin films was higher than those of the targeted bulk values, with a slight saturation effect has been observed at 30% Ag

concentration. We suggest that, when Ag was introduced in the mixture, Cu and Ag form an alloy and migrate together to the outmost sample surface, with Ag forming clusters as evidenced by AFM and XRD analysis (see Figures. 1 and 2). We notice that the flaming procedure does not affect the sample surface composition and the oxidation state of the three elements.

3.2. Biocompatibility studies of the TiCu(Ag) surfaces

The TiCu(Ag) thin films biocompatibility has been evaluated by measuring human MRC-5 fibroblasts viability based on adenosine triphosphate (ATP) production under aerobic conditions, which reflects the presence of metabolically active cells (Figure 5A). With this purpose, we first determined the correlation between the number of viable cells and the emitted luminescence (relative luminescence unit, RLU) (Figure 5B). Basing on obtained results, we next seeded 1.0×10^4 MRC-5 cells on polystyrene cell culture plates (control) and on sterile TiCu surfaces coated with 0%, 10%, 20%, and 30% Ag (Figure 5C). As shown, the RLU value after 24 h from seeding was comparable among control, TiCu, and TiCu(Ag) thin films, thus indicating that the tested surfaces did not affect cell viability and proliferation. Further, an unaltered morphology of MRC5 cells grown on TiCu and TiCu(Ag) thin films was observed, thus supporting the excellent biocompatibility of the developed thin films (data not shown).

Next, we evaluated if the trypsin-mediated detachment of cells affected the biocompatibility of TiCu and TiCu(Ag) thin films. Results obtained indicated that trypsinization did not alter the capability of the TiCu(Ag) thin films to allow MRC-5 growth, supporting their possible use for tissue culture studies (Figure 5D).

3.3. XPS analysis of TiCu(Ag) surfaces after human MRC-5 cells trypsinization

To evaluate the possibility of re-use the thin films for biocompatibility test after human cells detachment by trypsinization, TiCu(Ag) surfaces were analyzed by XPS and C1s, N1s, O1s, Ag3d, Ti2p and Cu2p core levels were investigated. XPS spectra and data (BE, FWHM, and atomic ratios) are reported in Figure 6 and Table S2. As already evidenced for the pristine samples, the Ti2p spectra are typical of TiO_2. The Cu2p spectra revealed the presence of both metallic and oxidized (CuO) copper, while the Ag3d signal is typical of metallic silver with a very small component related to oxidized silver (spectra not shown). No relevant change were evidenced on the oxidation state of metals on the sample surface. C1s, O1s and N1s spectra revealed the presence of organic molecules, particularly peptides, deposited on the TiCu(Ag) film surfaces.

The curve fitting analysis of C1s core level spectra (Figure 6A) showed four components: (i) the peak at 285.0 eV indicating aliphatic C−C carbons; (ii) the peak at ~286.5 eV, related to C−N and C−O carbons of peptide backbones; (iii) the peak at 288.3 eV due to O=C−N peptide carbons; and (iv) the peak at 290.0 eV due to COOH carbons. The N1s spectrum (Figure 6B) comprised a peak at 399.0 eV due to C=N nitrogens, a main peak at 400.4 eV related to peptide nitrogens, and a peak at about 402.5 eV due to protonated nitrogens [32, 38]. The O1s spectra (Figure 6C) showed four component peaks: (i) the peak at 530.1 eV assigned to the oxygens of titania; (ii) the peak at about 532.0 eV, assigned to O=C oxygens of the peptide backbone; (iii) the peak at 533.5 eV assigned to C-O oxygens; and (iv) the peak at nearly 535.0 eV, related to physiosorbed water [32].

In summary, the XPS data analysis points to the presence of peptide residues on the samples after human cells detachment, possibly representing cells residues of previous MRC-5 cell growth not completely removed by trypsin from the thin films, and/or trypsin residues adsorbed on the sample surface. However, the presence of these peptide residues did not influence MRC-5 adhesion and proliferation, as reported in Figure 5D.

3.4. Evaluation of the bactericidal properties of the TiCu(Ag) surfaces

The bactericidal property of TiCu and TiCu(Ag) thin films was evaluated by testing the growth of two well-known nosocomial pathogens, *i.e. S. aureus* and *P. aeruginosa*. The experimental protocol is illustrated in Figure 7A. Briefly, *S. aureus* and *P. aeruginosa* were dispersed on either a glass surface or TiCu thin films coated with 0%, 10%, 20%, and 30% Ag. Bacteria were incubated for 24 h at 37 °C in a controlled 99% humidity chamber prior to mechanical detachment and CFU counting. For both species, limited or no loss of bacterial viability was observed on glass after 24 h (Figure 7B). Conversely, no colony growth was observed when bacteria were dispersed on TiCu and TiCu(Ag) surfaces, indicating a strong bactericidal activity (BA) (Figure 7B).

To evaluate the diffusion of antibacterial agent(s) in solid media, a suspension of *S. aureus* or *P. aeruginosa* was spread over NA plates, and then TiCu(Ag) surfaces were placed onto the inoculated plates. The glass surface was used as the negative control (no growth inhibition), whereas antibiotic discs (*i.e.*, erythromycin, E 15 µg, and amikacin, AK/30 µg) were used as the positive control (growth inhibition due to antibiotic diffusion around the discs). No growth inhibition was observed around both glass and TiCu(Ag) surfaces, indicating no diffusion of inhibitory agent(s) as opposed to the large inhibition halo around the antibiotic discs (Figure 7C).

To exclude incomplete detachment of *S. aureus* and *P. aeruginosa* from the $NB_{1:500}$ washed TiCu surfaces, both optical microscopy (Figure 8A) and AFM analyses (Figure 8B) were performed. Microscopy results were compared with positive control samples in which the same number of bacteria was directly dispensed onto the TiCu surfaces, without subsequent washing step. A complete detachment of bacterial cells from TiCu surfaces was observed for both strains. Indeed, the large-scale optical microscopy images showed that on

control samples many "coffee-stain" clusters were present, while no clusters were observed on washed TiCu surfaces (Figure 8A). This was confirmed by acquiring several images across the surfaces. By AFM characterization, the clusters on control samples were univocally identified as bacterial aggregates (Figure 8B). Notably, *S. aureus* bacteria showed a round shape, with an average diameter and height of 950 nm and 530 nm, respectively. The *P. aeruginosa* bacteria were instead shallower with an average height of 230 nm and showed an elongated shape, often with flagella, having an average long-axis length of 1300 nm. The tendency of bacteria towards clustering in control samples (Figure 7B) was observed for similar deposition conditions and attributed to the capillary flow induced by the evaporation of the drop deposited [39, 40]. Conversely, the TiCu surfaces analyzed after bacteria detaching showed morphological features with heights below 30 nm (thus more than one order of magnitude lower than typical bacteria heights), likely to be attributed to residual of the nutrient medium used for the growth. The root mean square (Rms) surface roughness, as evaluated on 20×20 μm^2 images, was 1.71 nm and 1.87 nm after detachment of *S. aureus* and *P. aeruginosa*, respectively, while it exceeded 50 nm for the control surfaces with bacteria adhered. This comparative morphological analysis confirms that: (*i*) bacteria were completely detached with the experimental protocol used for the plate count experiments and, (ii) TiCu(Ag) surfaces were bactericidal for both *S. aureus* and *P. aeruginosa*.

To demonstrate that the antibacterial activity of TiCu and TiCu(Ag) thin films was due to the release of Ag and Cu ions into the medium, thin films were immersed for either 24 h or 7 days in $NB_{1:500}$ and the concentration of each metal was determined by ICP-OES. Results obtained indicated that after 24 h the presumptive concentration of Cu ions released in the medium from TiCu and TiCu-30% Ag thin films was 228.80±18.67 μg/mL and 227.93±16.59 μg/mL, respectively (Figure 9A). The presumptive concentration of Ag ions released in the medium after 24 h incubation by the TiCu-30% Ag was 4.07±0.47 μg/mL (Figure 9A).

Interestingly, the concentrations of both metals in the medium did not increase after 7 days incubation (Figure 9A). To correlate the antibacterial properties of TiCu(Ag) thin films with the presumptive concentration of released metals, 5.0×10^7 CFU/mL of either *S. aureus* or *P. aeruginosa* were incubated for 24 h in $NB_{1:500}$ and in $NB_{1:500}$ supplemented with 4 μg/mL $AgNO_3$, or 228 μg/mL $CuSO_4$ or both salts. The addition of $AgNO_3$, or $CuSO_4$, or both salts to the medium caused the 100% killing of both *S. aureus* and *P. aeruginosa* (Figure 9B). These results demonstrated that the antimicrobial activity of TiCu and TiCu(Ag) thin films is due the release of Ag and Cu ions into the medium, in agreement with previous suggestions [32].

4. Discussion

In this article, the biocompatibility and the antibacterial properties of TiCu and TiCu(Ag) thin films produced by PVD magnetron sputtering method are reported. All the TiCu(Ag) thin films allow adhesion and growth of human MRC5 fibroblast, together with a strong antibacterial activity against both *S. aureus* and *P. aeruginosa*, here considered as prototypes of nosocomial bacterial pathogens. Results are extremely significant for TE applications as biocompatibility and antibacterial performance represent key design parameters for biomaterials. In fact, tissue and implant-associated bacterial infection are a growing problem responsible for increased morbidity and mortality, together with enormous economic losses to the public health system. Bacteria can tightly adhere to the biomaterial surface, and the formation of biofilm can help bacteria to escape the host immune system and antibiotics. In turn, this causes the emergence of bacterial resistance to antibacterial drugs and finally determines implantation failures [41]. Therefore, implanting materials that combine the capability to favor eukaryotic cells adhesion and proliferation for tissue regeneration with strong antibacterial properties are urgently needed. Metallic elements (*e.g.*, Au, Ag, Cu, and Zn) have been proven to exert antibacterial activity by surfaces coating or doping [42].

The XPS analysis shows that TiO_2 represents the main oxidation status for titanium, which allows an excellent growth of eucaryotic cells and explains the observed biocompatibility. At the same time, all films show the presence of a combination between metallic and oxidized copper, with an increase of Cu/Ti ratio after addition of Ag. In addition, Ag is shown to be predominantly present in the metallic state with low levels of Ag_2O on the surface. These observations allow to explain the relevant antibacterial performance of the films. In fact, the presence of a single-phase film with homogeneous surface distributions of Cu and/or Ag can be very effective in protecting the surface against bacteria, while maintaining a high biocompatibility thanks to the concurrent homogenous distribution of TiO_2.

The biocompatibility of TiCu(Ag) thin films here reported agrees with the fact that Ag displays a very low cytotoxicity when tested in human blood, adenocarcinomic alveolar basal epithelial cells, liver cancer cells, breast cancer cells, and gastric cancer cells [43-45]. On the other side, Cu is a transition metal and is an essential micronutrient in humans. Indeed, Cu is involved in many biosynthetic and metabolic processes, being a cofactor of many redox enzymes and playing a role in iron metabolism, as well as in immunity [46]. It has been reported that Cu exerts a very low cytotoxicity in human cells (TC_{50} value of 344±4.4 μM in human gingival fibroblast [47]). Here, we tested the TiCu(Ag) thin films biocompatibility using MRC-5 human fibroblasts, a cell line widely used to perform cytotoxicity assays [48-56]. Human fibroblasts are responsible for synthesizing and depositing ECM components, mediate epithelial-mesenchymal interactions allowing other cells to settle and migrate along a three-dimensional support and thereby generating an organ-specific architecture. Noteworthy, fibroblast cells contain high levels of fibronectin and fibrin, which generates rapid and secure fibroblast adhesion to the Ti substrate [53]. Therefore, studying fibroblast adhesion and proliferation on nanosurfaces may be used with the aim of increasing tissue repair in several conditions like healing of acute and chronic wounds [49].

Additionally, we demonstrated that the anti-bacterial activity of TiCu and TiCu(Ag) thin films is due to the release of Ag and Cu ions into the medium. Noteworthy, several data support the anti-bacterial, anti-viral, anti-biofilm, and anti-inflammation activity of Ag, especially at nanoscale [43-45, 57, 58]. The antibacterial property of Ag relies on its ability to form pores and penetrate the bacterial wall by reacting with the peptidoglycan component. Indeed, owing to electrostatic attraction and affinity to sulfur proteins, silver ions can adhere to the cell wall and the cytoplasmic membrane. In turn, adhered ions can enhance the permeability of the cytoplasmic membrane. Once into the bacterial cells, silver ions can inhibit cellular respiration, resulting in the generation of reactive oxygen species (ROS), and interrupting metabolic pathways by inhibition of ATP production. ROS can also induce cell membrane disruption and DNA oxidation. Since sulfur and phosphorus are important components of DNA, the interaction of silver ions with DNA can impair its replication, hence cell duplication, ultimately causing bacterial death. Finally, Ag ions can inhibit the synthesis of proteins by denaturing ribosomes in the cytoplasm [59-61]. While low concentrations of Cu are essential for bacteria metabolism, high concentrations, cause cell growth inhibition or even cells death [62, 63]. Therefore, Cu represent an optimal metal to prepare antibacterial titanium alloys [42, 64, 65].

5. Conclusion

In this paper, the surface structural and functional properties of amorphous TiCu(Ag) thin film have been investigated. The increase of Ag content is accompanied by the appearance of Ag-nanocrystallites and by a decrease of both elastic modulus and hardness of the thin films. TiCu(Ag) thin films allowed a very good adhesion and growth of fibroblast MRC5 cells irrespective to different Ag content.

Based on the multi-technique characterization and cellular studies, it can be concluded that binary TiCu and TiCu-10% Ag showed the best mechanical properties with amorphous glassy structure combined to excellent biocompatibility and antibacterial activity. On the contrary, ternary TiCu(Ag) thin films with 20% Ag content showed moderate mechanical properties, although they display excellent biocompatibility and antibacterial properties.

Although many coatings and modified surfaces provide similar antibacterial activity, such surfaces functionalisation can have a detrimental effect on tissue biocompatibility, impairing the integration of the implants into the surrounding tissue. The excellent biocompatibility and bactericidal properties of our multifunctional thin films opens to their use in TE applications. Moreover, these types of thin films could be also used to coat surgical tools and hospital furnishing. For the future, it might be of interest to study these thin films for the biocompatibility of mesenchymal stem cells, one of the most studied stem cells in the TE field due to their great potential to enhance tissue regenereation thanks to the capability to differentiate into cartilage, bone, fat, muscle, tendon, skin as well as hematopoietic-supporting stroma and neural tissue [66]. For the future, it could be also tested if these kinds of thin films could be suitable for applications against SARS-CoV-2 infection [20], since Cu and its oxide have been demonstrated to act as efficient antiviral agents [20, 21, 67].

Funding

This work was supported by the grant "Piano straordinario di sviluppo della ricerca di Ateneo – Azione 4" from Roma Tre University to P. A. The Grant of Excellence Departments, MIUR (ARTICOLO 1, COMMI 314 – 337 LEGGE 232/2016) to the Department of Science, University Roma Tre is also gratefully acknowledged. Moreover, the Authors acknowledge the European Commission for support through the H2020 project OYSTER, g.a. n. 760827.

Acknowledgements

Authors gratefully acknowledge the experimental support received form Dr. Giorgio Contini and Dr. Stefano Franchi in the XPS measurements, and Dr. Jacopo Albanesi for validating luminescence experiments. Nanomechanical testing activities were performed at the interdepartmental laboratory of electron microscopy (LIME) at Roma Tre university.

References

[1] J.C. Dayoub, F. Cortese, A. Anžič, T. Grum, J.P. de Magalhães, The effects of donor age on organ transplants: A review and implications for aging research, Exp. Gerontol. 110 (2018) 230-240.

[2] http://www.transplant-observatory.org/

[3] B.M. Frey, S.M. Zeisberger, S.P. Hoerstrup SP, Tissue engineering and regenerative medicine - New initiatives for individual treatment offers, Transfus. Med. Hemother. 43 (2016) 318-319.

[4] C. Hennessy, G. Lewik, A. Cross, J. Hester, F. Issa, Recent advances in our understanding of the allograft response, Fac. Rev. 10 (2021) 21.

[5] S. Miyairi, W.M 3rd Baldwin, A. Valujskikh, R.L. Fairchild, Natural killer cells: critical effectors during antibody-mediated rejection of solid organ allografts, Transplantation 105 (2021) 284-290.

[6] M.J. Lysaght, J. Crager, Origins, Tissue Eng. Part A 15 (2009) 1449-1450.

[7] A.J. Salgado, J.M. Oliveira, A. Martins, F.G. Teixeira, N.A. Silva, N.M. Neve, N. Sousa, R.L. Reis, Tissue engineering and regenerative medicine: past, present, and future, Int Rev Neurobiol 108 (2013) 1-33.

[8] N. Ghosh, E.R. Banerjee, Nanoscaffolds in tissue engineering, in: E.R. Banerjee (Ed.), Nanomaterials and biomedicine, Springer, Singapore, 2020, pp. 57-66.

[9] F. Han, J. Wang, L. Ding, Y. Hu, W. Li, Z. Yuan, Q. Guo, C. Zhu, L. Yu, H. Wang, Z. Zhao, L. Jia, J. Li, Y. Yu, W. Zhang, G. Chu, S. Chen, B. Li, Tissue engineering and regenerative medicine: achievements, future, and sustainability in Asia, Front. Bioeng. Biotechnol. 8 (2020) 83.

[10] T. Lu, Y. Li Y, T. Chen, Techniques for fabrication and construction of three-dimensional scaffolds for tissue engineering, Int. J. Nanomedicine 8 (2013) 337-350.

[11] J.J. Swartjes, P.K. Sharma, T.G. van Kooten, H.C. van der Mei, M. Mahmoudi, H.J. Busscher, E.T. Rochford, Current developments in antimicrobial surface coatings for biomedical applications, Curr. Med. Chem. 22 (2015) 2116-2129.

[12] S. Korkmaz, İ.A. Kariper, Glass formation, production and superior properties of Zr-based thin film metallic glasses (TFMGs): a status review, J. Non Cryst. Solids 527 (2020) 119753.

[13] W. Wenbo, M. Hesham, D. Wahyu, P. C. Jinn, L. Lin, C. Wenjun, Effects of nanoscale chemical heterogeneity on the wear, corrosion, and tribocorrosion resistance of Zr-based thin film metallic glasses, Surf. Coat. Technol. 402 (2020) 126324.

[14] S. Ketov, X. Shi, G. Xie, G. Xie, R. Kumashiro, A. Y. Churyumov, A.I. Bazlov, N. Chen, Y. Ishikawa, N. sao, H. Wu, D.V. Louzguine-Luzgin, Nanostructured Zr-Pd metallic glass thin film for biochemical applications, Sci. Rep. 5 (2015) 7799.

[15] J.F. Correa, W. Aperador, J.C. Caicedo, N.C. Alba, C. Amaya, Structural, mechanical and tribological behavior of TiCN, CrAlN and BCN coatings in lubricated and non-lubricated environments in manufactured devices, Mater. Chem. Phys. 252 (2020) 123164.

[16] Mitsunori Yada, Yuko Inoue, Tomohiro Morita, Shintaro Imamura, Toshio Torikai, Takanori Watari, Synthesis and antibacterial activity of a silver nanoparticle/silver titanium phosphate–nanocomposite nanobelt thin film formed on a titanium plate, Thin Solid Films 628 (2017) 184-189.

[17] L. Somlyai-Sipos, P. Baumli, A. Sycheva, G. Kaptay, E. Szőri-Dorogházi, F. Kristály, T. Mikó, D. Janovszky, Development of Ag nanoparticles on the surface of Ti powders by chemical reduction method and investigation of their antibacterial properties, Appl. Surf. Sci. 533 (2020) 147494.

575 [18] B.C. Sousa, C.J. Massar, M.A. Gleason, D.L. Cote, On the emergence of antibacterial
576 and antiviral copper cold spray coatings, J. Biol. Eng. 15 (2021) 8.
577 [19] J. Yang, H. Qin, Y. Chai, P. Zhang, Y. Chen, K. Yang, M. Qin, Y. Zhang, H. Xia, L.
578 Ren, B. Yu, Molecular mechanisms of osteogenesis and antibacterial activity of Cu-
579 bearing Ti alloy in a bone defect model with infection *in vivo*, J. Orthop. Translat. 27
580 (2021) 77-89.
581 [20] Z. Sun, K (Ken) Ostrikov, Future antiviral surfaces: Lessons from COVID-19 pandemic,
582 Sustain. Mater. Techno. 25 (2020) e00203.
583 [21] M. Miyauchi, K. Sunada, K. Hashimoto, Antiviral effect of visible light-sensitive
584 Cu_xO/TiO_2 photocatalyst, Catalysts 10 (2020) 1093.
585 [22] M. Janczarek, M. Endo, D. Zhang, K. Wang, E. Kowalska, Enhanced photocatalytic and
586 antimicrobial performance of cuprous oxide/titania: the effect of titania matrix, Materials
587 11 (2018) 2069.
588 [23] H. Ji, M-C. Zhao, B. Xie, Y.-C. Zhao, D. Yin, C. Gao, C. Shuai, A, Atrens, Corrosion
589 and antibacterial performance of novel selective-laser-melted (SLMed) Ti-xCu
590 biomedical alloys, J. Alloys Compd. 864 (2021) 158415.
591 [24] W. Zhang, S. Zhang, H. Liu, L. Ren, Q. Wang, Y. Zhang, Effects of surface roughening
592 on antibacterial and osteogenic properties of TiCu alloys with different Cu contents, J.
593 Mater. Sci. Technol. 88 (2021) 158-167.
594 [25] W.C. Oliver, G.M. Pharr, An improved technique for determining hardness and elastic
595 modulus using load and displacement sensing indentation experiments, J. Mater. Res. 7
596 (1992) 1564-1583.
597 [26] W.C. Oliver, G.M. Pharr, Measurement of hardness and elastic modulus by instrumented
598 indentation: Advances in understanding and refinements to methodology. J. Mater. Res.
599 19 (2004) 3.

600 [27] https://www.iso.org/standard/54431.html
601 [28] D. Nečas, P. Klapetek, Gwyddion: an open-source software for SPM data analysis, Cent.
602 Eur. J. Phys. 10 (2012) 181-188.
603 [29] A. Ewald, S.K. Glückermann, R. Thull, U. Gbureck, Antimicrobial titanium/silver PVD
604 coatings on titanium, Biomed. Eng. Online 5 (2006) 22.
605 [30] A. Etiemble, C. Der Loughian, M. Apreutesei, C. Langlois, S. Cardinal, J.M. Pelletier,
606 J.F. Pierson, P. Steyer, Innovative Zr-Cu-Ag thin film metallic glass deposed by
607 magnetron PVD sputtering for antibacterial applications, J. Alloys Compd. 707 (2017)
608 155-161.
609 [31] D.Campoccia, L. Montanaro, C.R. Arciola, A review of the biomaterials technologies
610 for infection-resistant surfaces. Biomaterials 34 (2013) 8533-8554.
611 [32] E. Dauvergne, C. Mullié, Brass alloys: copper-bottomed solutions against hospital-
612 acquired infections, Antibiotics (Basel) 10 (2021) 286.
613 [33] S. Rashid, M. Sebastiani, M. Zeeshan Mughal, R. Daniel, E. Bemporad, Influence of the
614 silver content on mechanical properties of TiCuAg thin films, Nanomaterials, 11 (2020):
615 435.
616 [34] J.F. Moulder, W.F. Stickle, P.E. Sobol, K.D. Bomben, Handbook of X-ray photoelectron
617 spectroscopy, (J. Chastain, Ed.) Perkin-Elmer Corporation, Physical Electronics
618 Division, Eden Prairie, Minnesota, 1992.
619 [35] S. Franchi, V. Secchi, M. Santi, M. Dettin, A. Zamuner, C. Battocchio, G. Iucci,
620 Biofunctionalization of TiO_2 Surfaces with self assembling oligopeptides in different pH
621 and ionic strength conditions: charge effects and molecular organization, Mater. Sci.
622 Eng. C 90 (2018) 651-656.

[36] D.A. Svintsitskiy, T.Kardash, A.I. Boronin. Surface dynamics of mixed silver-copper oxide AgCuO2 during X-rayphotoelectron spectroscopy study, Appl. Surf. Sci 463 (2019) 300-309.

[37] I. Schiesaro, L. Burratti, C. Meneghini, I. Fratoddi, P. Prosposito, J. Lim, C. Scheu, C. Venditti, G. Iucci, C. Battocchio, Hydrophilic silver nanoparticles for Hg(II) detection in water: direct evidence for mercury-silver interaction, J. Phys. Chem. C 124 (2020) 25975-25983.

[38] G. Beamson, D Briggs, High resolution XPS of organic polymers: the Scienta ESCA 300 Database, J. Chem. Edu. 70 (1993) A25.

[39] D. Mampallil, H.B. Eral, A review on suppression and utilization of the coffee-ring effect, Adv. Colloid Interface Sci 252 (2018) 38-54.

[40] S. Bashiri, M. Lucidi, D. Visaggio, G. Capecchi, L. Persichetti, G. Cincotti, P. Visca, G. Capellini, Growth phase- and desiccation-dependent *Acinetobacter baumannii* morphology: an atomic force microscopy investigation, Langmuir 37 (2021) 1110-1119.

[41] C.R. Arciola, D. Campoccia, L. Montanaro, Implant infections: adhesion, biofilm formation and immune evasion, Nat. Rev. Microbiol. 16 (2018) 397-409.

[42] Z. Lin, X. Sun, H. Yang, The role of antibacterial metallic elements in simultaneously improving the corrosion resistance and antibacterial activity of magnesium alloys, Mater. Des. 198 (2021) 109350.

[43] B. Liu, Y.F. Zheng, Effects of alloying elements (Mn, Co, Al, W, Sn, B, C and S) on biodegradability and in vitro biocompatibility of pure iron. Acta Biomater. 7 (2011) 1407-1420.

[44] Y. Huang, X. Ding, Y. Qi, B. Yu, F.J. Xu. Reduction-responsive multifunctional hyperbranched polyaminoglycosides with excellent antibacterial activity, biocompatibility and gene transfection capability. Biomaterials 106 (2016) 134-144.

[45] K. Saravanakumar, R. Chelliah, D. MubarakAli, D.H. Oh, K. Kathiresan, M.H. Wang, Unveiling the potentials of biocompatible silver nanoparticles on human lung carcinoma A549 cells and *Helicobacter pylori*. Sci. Rep. 9 (2019) 5787.

[46] Y. Issa, P. Brunton, C.M. Waters, D.C. Watts, Cytotoxicity of metal ions to human oligodendroglial cells and human gingival fibroblasts assessed by mitochondrial dehydrogenase activity, Dent. Mater. 24 (2008) 281-287.

[47] G. Faúndez, M. Troncoso, P. Navarrete, G. Figueroa, Antimicrobial activity of copper surfaces against suspensions of *Salmonella enterica* and *Campylobacter jejuni*, BMC Microbiol. 4 (2004) 19.

[48] M. Könönen, M. Hormia, J. Kivilahti, J. Hautaniemi, I. Thesleff, Effect of surface processing on the attachment, orientation, and proliferation of human gingival fibroblasts on titanium, J. Biomed. Mater. Res. 26 (1992) 1325-1341.

[49] T. Wong, J.A. McGrath, H. Navsaria H, The role of fibroblasts in tissue engineering and regeneration, Br. J. Dermatol. 156 (2007) 1149-1155.

[50] C. Wiegand, U.C. Hipler, Evaluation of biocompatibility and cytotoxicity using keratinocyte and fibroblast cultures. Skin Pharmacol. Physiol. 22 (2009) 74-82.

[51] H. Okawachi, Y. Ayukawa, I. Atsuta, A. Furuhashi, M. Sakaguchi, K. Yamane, K. Koyano, Effect of titanium surface calcium and magnesium on adhesive activity of epithelial-like cells and fibroblasts, Biointerphases 7 (2012) 27.

[52] F.Y. Teng, C.L. Ko, H.N. Kuo, J.J. Hu, J.H. Lin, C.W. Lou, C.C. Hung, Y.L. Wang, C.Y. Cheng, W.C. Chen, A comparison of epithelial cells, fibroblasts, and osteoblasts in dental implant titanium topographies, Bioinorg. Chem. Appl. 2012 (2012) 687291.

[53] D.W. Lee, J.G. Kim, M.K. Kim, S. Ansari, A. Moshaverinia, S.H. Choi, J.J. Ryu, Effect of laser-dimpled titanium surfaces on attachment of epithelial-like cells and fibroblasts, J. Adv. Prosthodont. 7 (2015) 138-145.

673 [54] J. Markhoff, M. Krogull, C. Schulze, C. Rotsch, S. Hunger, R. Bader, Biocompatibility
674 and inflammatory potential of titanium alloys cultivated with human osteoblasts,
675 fibroblasts and macrophages, Materials (Basel) 10 (2017) 52.
676 [55] A. Smargiassi, J. Bertacchini, M. Checchi, F. Cavani, M. Ferretti, C. Palumbo,
677 Biocompatibility analyses of Al_2O_3-treated titanium plates tested with osteocyte and
678 fibroblast cell lines, Biomedicines 5 (2017) 32.
679 [56] B.-H. Huang, Y.-J. Lu, W.-C. Lan, M. Ruslin, H.-Y. Lin, K.-L. Ou, T. Saito, H.-Y. Tsai,
680 C.-H. Lee, Y.-C. Cho,T.-S. Yang, C.-M. Liu, P.-J. Hou, Surface properties and
681 biocompatibility of anodized titanium with a potential pretreatment for Biomedical
682 applications, Metals 11 (2021) 1090.
683 [57] C.N. Lok, C.M. Ho, R. Chen, Q.Y. He, W.Y. Yu, H. Sun, P.K. Tam, J.F. Chiu, C.M.
684 Che, Proteomic analysis of the mode of antibacterial action of silver nanoparticles, J.
685 Proteome Res. 5 (2006) 916-924.
686 [58] O. Choi, Z. Hu, Size dependent and reactive oxygen species related nanosilver toxicity
687 to nitrifying bacteria, Environ. Sci. Technol. 42 (2008) 4583-4588.
688 [59] Y. Yakabe, T. Sano, H. Ushio, T. Yasunaga, Kinetic studies of the interaction between
689 silver ion and deoxyribonucleic acid, Chem. Lett. 9 (1980) 373-376.
690 [60] W.K. Jung, H.C. Koo, K.W. Kim, S. Shin, S.H. Kim, Y.H. Park, Antibacterial activity
691 and mechanism of action of the silver ion in *Staphylococcus aureus* and *Escherichia*
692 *coli*, Appl. Environ. Microbiol. 74 (2008) 2171-2178.
693 [61] J.R. Morones-Ramirez, J.A. Winkler, C.S. Spina, J.J. Collins, Silver enhances antibiotic
694 activity against gram-negative bacteria, Sci. Transl. Med. 5 (2013) 190ra181.
695 [62] L. Zhu, J. Elguindi, C. Rensing, S. Ravishankar, Antimicrobial activity of different
696 copper alloy surfaces against copper resistant and sensitive *Salmonella enterica*, Food
697 Microbiol. 30 (2012) 303-310.

[63] F. Heidenau, W. Mittelmeier, R. Detsch, M. Haenle, F. Stenzel, G. Ziegler, H. Gollwitzer, A novel antibacterial titania coating: metal ion toxicity and in vitro surface colonization, J. Mater. Sci. Mater. Med. 16 (2005) 883-888.

[64] E. Zhang, F. Li, H. Wang, J. Liu, C. Wang, M. Li, K. Yang, A new antibacterial titanium-copper sintered alloy: preparation and antibacterial property, Mater. Sci. Eng. C Mater. Biol. Appl. 33 (2013) 4280-4287.

[65] A.J. Rosenbaum, D.A Grande, J.S. Dines, The use of mesenchymal stem cells in tissue engineering: a global assessment, Organogenesis 4 (2008) 23-27.

[66] N. Hutasoit, B. Kennedy, S. Hamilton, A. Luttick, R.A.R. Rashid, S. Palanisamy, Sars-CoV-2 (COVID-19) inactivation capability of copper-coated touch surface fabricated by cold-spray technology, Manuf. Lett 25 (2020) 93-97.

Figure captions

Figure 1. **X-ray diffraction pattern**. Diffraction scans of the TiCu and TiCu(Ag) thin films.

Figure 2. Characterization of the surface morphology. Atomic force microscopy (AFM) images of the TiCu and TiCu(Ag) thin films with an Ag content of 10%, 20%, and 30%.

Figure 3. Comparison of elastic modulus and surface average roughness (A) and of hardness and surface average roughness (B) as a function of Ag content in TiCu thin films.

Figure 4. XPS spectra. (A) Ti2p, (B) Cu2p, and (C) Ag3d core level spectra of sample TiCu-30%Ag and related peak fitting analysis.

Figure 5. Evaluation of the biocompatibility of the TiCu and TiCu(Ag) surfaces. (A) Overview on the cell survival assay used to evaluate MRC5 cells grown on the TiCu surfaces. The Cell Titer-Glo® reagent added to the cells generates a luminescent signal (relative light unit, RLU) that is proportional to the ATP present, which in turn is directly proportional to the number of metabolically active cells grown on the TiCu surface. (B) Set up of the assay sensitivity using MRC5 cells grown on TiCu surfaces. Cells were seeded in triplicate at a density of 2.5×10^3, 5.0×10^3, 1.0×10^4, 2.0×10^4, and 4.0×10^4 cells/well. (C) Cell viability assay measured 24 h after from seeding 1.0×10^4 MRC5 cells in triplicate on the 96-well plastic surface (Ctrl) or on the TiCu surfaces coated with 0%, 10%, 20%, and 30% Ag. (D) Cell viability assay measured after 24 h from seeding 1.0×10^4 MRC5 cells in triplicate on the TiCu surfaces coated with 0%, 10%, 20%, and 30% Ag, which were either previously treated with trypsin or not. Background luminescence was measured in well containing the medium

without cells and subtracted from experimental values. Data are reported as means ± SD (Student's t test, $p<0.05$).

Figure 6. XPS spectra after human cells detachment by trypsinization. C1s (A), N1s (B) and O1s (C) XPS spectra of TiCu(Ag) films after human MRC-5 fibroblasts detachment with trypsin and related peak fitting analysis.

Figure 7. Bactericidal properties of the TiCu and TiCu(Ag) surfaces. (A) Schematic representation of the protocol used to test the bactericidal activity of the TiCu(Ag) surfaces. (B) Bactericidal effect of TiCu(Ag) surfaces determined after 24 h incubation at 37°C with 99% humidity of *S. aureus* and *P. aeruginosa* cells on TiCu(Ag) surfaces. The glass surface was used as negative control for bacterial killing. After incubation, aliquots of the bacterial suspension were diluted in saline and plated on agar for colony forming unit (CFU) counts. (C) Plate test for release of antibacterial agents from TiCu(Ag) surfaces. *S. aureus* and *P. aeruginosa* were used as test species. Antibiotic discs and glass were used as positive and negative controls of bacterial inhibition.

Figure 8. Surface morphology of TiCu samples after bacteria detachment. (A) Optical microscopy images and (B) AFM topographic (top panels) and peak-force error (bottom panels) images of control (Ctrl) and TiCu surfaces on which *S. aureus* or *P. aeruginosa* were grown. Images were acquired after bacteria detaching and were compared to control samples where a suspension of each bacteria strain, containing the same number of bacteria as in the experiment, was poured directly on the TiCu surface. In the insets shown in panels B, 3D zoom ups are displayed.

Figure 9. Ag and Cu ions release from TiCu and TiCu-30% Ag thin films and bactericidal activity. (A) The concentration of Ag and Cu ions released from TiCu and TiCu-30% Ag thin films, immersed for either 24 h or 7 days in the standard test medium ($NB_{1:500}$), was determined by inductively coupled plasma optical emission spectrometry (ICP-OES). The reported final concentrations of Ag and Cu ions released for each thin film were normalized to the volume of bacteria used in the antibacterial assay. The standard test medium ($NB_{1:500}$) was used as control. ND, not detectable. (B) Bactericidal effects of the presumptive concetration of Ag and Cu ions released from the thin films. *S. aureus* and *P. aeruginosa* cells were incubated in $NB_{1:500}$ or $NB_{1:500}$ supplemented with 4 μg/mL $AgNO_3$, or 228 μg/mL $CuSO_4$, or both salts. After 24 h incubation, aliquots of the bacterial suspension were diluted in saline and plated on agar for colony forming unit (CFU) counts.

Table 1. Composition of the TiCu(Ag) thin films.

Film composition	**Ti:Cu ~ 1**	**Power at Ti (W)**	**Power at Cu (W)**	**Power at Ag (W)**	**Thickness of the film (μm)**
TiCu	48:52	150	29	0	1.48
TiCu-10% Ag	43:47	143	27	3	1.44
TiCu-20% Ag	38:42	128	24	5	1.43
TiCu-30% Ag	33:37	113	21	9	1.46