

Beneficial Effect of TaON-Ag Nanocomposite Titanium on Antibacterial Capacity in Orthopedic Application

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Chih-Chien Hu^{1,4}
Chih-Hsiang Chang^{1,3}
Yuhan Chang^{1,3}
Jang-Hsing Hsieh^{5,6}
Steve Wen-Neng Ueng^{1,3}

¹Bone and Joint Research Center, Chang Gung Memorial Hospital, Kweishan, Taoyuan 33305, Taiwan; ²Department of Orthopedic Surgery, Chang Gung Memorial Hospital, Kweishan, Taoyuan 33305, Taiwan; ³College of Medicine, Chang Gung University, Kweishan, Taoyuan 33302, Taiwan; ⁴Department of Orthopedic Surgery, Xiamen Chang Gung Hospital, Xiamen, Fujian, People's Republic of China; ⁵Department of Materials Engineering, Ming Chi University of Technology, Taishan, Taipei 24301, Taiwan; ⁶Center for Thin Film Technologies and Applications, Ming Chi University of Technology, Taishan, Taipei 24301, Taiwan

Purpose: In this study, a novel oxygenated nanocomposite thin film, TaON-Ag, was investigated in vitro and in vivo to evaluate its biocompatibility and antibacterial ability.

Material and Methods: The antibacterial ability of TaON-Ag nanocomposite-coated titanium (Ti) was evaluated using the Kirby-Bauer disk diffusion susceptibility test. The effects of TaON-Ag nanocomposite-coated metal on osteogenesis were further evaluated in an in vitro osteogenic culture model with rat marrow-derived mesenchymal stem cells (rMSCs). Furthermore, titanium rods coated with TaON-Ag were implanted into a rat femur fracture model either with or without osteomyelitis to investigate the effects of TaON-Ag in osteogenesis.

Results: The TaON-Ag-coated Ti exhibited an effective antibacterial effect against *Staphylococcus aureus*, coagulase-negative *Staphylococcus*, and the Gram-negative strains *Escherichia coli* and *Pseudomonas aeruginosa*. Using an osteogenic culture with rMSCs and a rat femoral fracture model, the TaON-Ag-coated Ti did not interfere with the ossification of rMSCs in vitro or during fracture healing in vivo. Field-emission scanning electron microscopy (FE-SEM) revealed that coating with TaON-Ag could inhibit pathogen adhesion and biofilm formation in both *Staphylococcus aureus* and *Escherichia coli*.

Conclusion: Using the proposed novel oxygenation process, TaON-Ag nanocomposite-coated Ti yielded robust biocompatibility and antibacterial ability against common microorganisms in orthopedic infections, thereby demonstrating potential for use in clinical applications.

Keywords: silver nanoparticles, ossification, antibacterial ability, biofilm

Introduction

Silver nanoparticles (AgNPs) are increasingly being used in various fields, including medical, health care, consumer, and industrial purposes, because of their unique physical and chemical properties. Their applications include use as antibacterial agents; in industrial household- and healthcare-related products, consumer products, medical device coatings, orthopedics, and drug delivery; and even as anticancer agents.^{1,2} The antibacterial activity of the majority of metal coatings is closely linked to their ionic or nano form, rather than the bulk material. Despite extensive research, coating implants with a thin layer of metal is still not a standard practice.^{3,4} This has been reported in devices that are predominantly temporary, permanent orthopedic devices, and vascular prostheses. Antimicrobial effects have been observed when AgNPs are used in trauma implants, tumor prostheses, bone cement, and when combined with hydroxyapatite coatings. Although promising results have been obtained for in vitro and in vivo studies, there are a limited

Correspondence: Steve Wen-Neng Ueng
Department of Orthopedic Surgery,
Chang Gung Memorial Hospital, No. 5,
Fu-Hsin St, Kweishan, Taoyuan 33305,
Taiwan
Tel +88633281200-2420
Fax +886 33278113
Email wenneng@adm.cgmh.org.tw

number of clinical studies.^{5,6} Based on the results of in vitro studies, it was revealed that a Ag plasma-modified hierarchical TiO₂ film can be fabricated on the surface of Ti via acid etching to produce micropits, followed by hydrothermal treatment to generate TiO₂ nanorods, and subsequent plasma immersion ion implantation to impregnate Ag onto the surface of TiO₂. The antimicrobial activity, bioactivity, and cytocompatibility were systematically evaluated, demonstrating enhanced bioactivity and bacteriostatic effects.^{7,8} The addition of Ag demonstrated a minor effect on the bioactivity. It had no adverse effect on and even promoted the MG63 cell function, including adhesion, spread, and proliferation. Devlin-Mullin et al reported 3D Ti structures fabricated using selective laser melting and Ag nanolayer coating using atomic layer deposition on either methicillin-resistant *Staphylococcus aureus* (MRSA) or *Staphylococcus epidermidis*. The results revealed significant reductions in both bacterial recovery and biofilm formation. Ag nanolayer coatings on Ti implants significantly reduce pathogenic in vitro biofilm formation, facilitating in vivo vascularization and osseointegration.⁹ As such, it is a promising technique for clinical orthopedic applications. However, most of these reported materials are not yet appropriate for clinical application. The majority of the limited clinical usage of these Ag nanoparticle materials has the potential for adverse health effects as a result of prolonged exposure at varying ion concentration levels in humans, and their environment effects have not yet been established.^{5,10}

In our previous study, TaN-(Ag,Cu) thin films prepared using reactive co-sputtering with a Ta target in plasma formed using Ar, N, and O exhibited in vitro antibacterial activity.^{11–13} Theoretically, even at the doping level, the incorporation of oxygen into a nitrogen framework causes specific changes in the physical and chemical properties, which can affect the release of Ag and Cu ions. Therefore, variation in the O/N atomic ratio affects the mechanical, electrochemical, and bio-related properties.¹⁴ The current study was designed to evaluate the in vitro and in vivo applications of this proposed oxygenated nanocomposite thin-film metal with respect to biocompatibility and antibacterial ability.

Materials and Methods

Preparation of Nanocomposite Thin Films

TaN thin films were prepared using reactive co-sputtering with a Ta target in plasma formed using argon and

nitrogen. Previously, TaN thin films doped with soft metals (ie, TaN–Cu, TaN–Ag, and TaN–(Ag,Cu)) were prepared using a hybrid process that starts with reactive co-sputtering, followed by rapid thermal annealing (RTA) at temperatures higher than 350 °C. Ta metals and their alloys exhibit excellent biocompatibility. Therefore, TaN is an excellent choice for protective coatings in bio-related applications. Cu and Ag as doping elements are immiscible with TaN, which facilitates the synthesis of TaN–Ag or TaN–(Ag,Cu) nanocomposite thin films.¹³

Moreover, the oxynitrides of transition metals exhibit tunable optical, mechanical, electrical, and bio-related properties by varying the ratio of oxygen to nitrogen. Theoretically, even at a doping level, the incorporation of oxygen into a nitrogen framework causes specific changes in the physical and chemical properties. The oxygen flow rate can be varied from 0 sccm to 15 sccm.¹⁴ The sample with oxygen deposited at a rate of 1 sccm exhibited the highest roughness, resulting in high biocompatibility. According to our unpublished data, the sample with oxygen deposited at a rate of 1–3 sccm exhibited the best antibacterial efficiency against *Escherichia coli*. Under these process parameters, TaON-Ag thin films were prepared using reactive co-sputtering with an oxygen flow rate of 1 sccm. The material characteristics of the TaON-Ag-coated thin films, including those obtained using XRD, SEM, EDS, and AFM surface analysis, are presented in [Supplement Figures 1–4](#).

In vitro Antibacterial Ability Assay

To evaluate the antibacterial ability of various metal coatings on common pathogenic bacteria in muscle-skeletal infections, the antibacterial ability of TaN-Ag, TaN-(Ag,Cu), TaON-Ag, and TaN-coated Ti alloys on various common pathogenic bacteria was investigated using the Kirby-Bauer Disk Diffusion Susceptibility Test Protocol. The antibacterial effects of various coating samples were evaluated using selected strains, including methicillin-sensitive *Staphylococcus aureus* ATCC 25923 (MSSA), methicillin-resistant *Staphylococcus aureus* ATCC 43300 (MRSA), coagulase-negative *Staphylococcus* ATCC 12228 (CoNS), *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922. Metal specimens were sterilized in an autoclave at 121 °C for 40 min. Bacteria at a concentration of McFarland 0.5 (1×10^8) were inoculated onto a culture medium on a Mueller-Hinton (M-H) agar plate by the streaking method. Different coated samples were manually placed on an agar plate using forceps, and the M-Hagar

plates were incubated at 37 °C for 24 and 48 h. After incubation, bacterial activity was recorded according to the inhibition zone.

In vitro Osteogenic Assay

In the development of new materials for orthopedic applications, the osteogenetic properties should not be compromised. Thus, the various coated metals were co-cultured using rat-marrow-derived mesenchymal stem cells (rMSCs), and the effect of Ag ions on the ossification process was observed. The ossification capacities were evaluated by measuring the alkaline phosphatase (ALP) activity and the degree of calcification. rMSCs were obtained from the bone marrow of six-week-old male Sprague-Dawley (SD) rats (BioLASCO Taiwan Co., Ltd). The cells were suspended in MSC medium containing 10% FBS (Gibco) and 1% Pen-Strep, and seeded into 10-cm culture dishes. The dishes were maintained at 37 °C in a 5% CO₂ incubator for 1–2 weeks. (5×10^7 cells/dish). After 4 days at 37 °C in the incubator, the medium was replaced to remove non-adherent cells. rMSCs were sub-cultured to passage 3 (P3) for transduction and transplantation.¹⁵ The P3 cells were then cultured in an osteogenic induction medium (DMEM-LG with 10% FBS, 0.1 mM dexamethasone, 0.05 mM ascorbic acid-2-phosphate, and 2 mM β -glycerophosphate) for up to 21 days.¹⁶

The ALP profile in the culture medium was measured using an ALP substrate (p-nitrophenyl phosphate). The assay was performed according to the manufacturer's instructions. An adequate amount of a 1 mg mL⁻¹ solution of ALP substrate in a 50 mmol L⁻¹ glycine buffer and 1 mmol L⁻¹ MgCl₂·6H₂O was mixed with the waste culture medium collected from each microbioreactor. After 3 min, the solution was removed and transferred to a tube containing an equal volume of 1 mol L⁻¹ NaOH. The absorbance of the resulting solution was determined at 405 nm and compared with a standard curve generated from a series of dilutions of p-nitrophenol.¹⁶

In the calcium assay, calcium was extracted using 0.6 M HCl on day 20 of the osteogenic induction culture. Aliquots of the extract were mixed with a reagent from a commercial calcium assay kit (Biotron, Hemet, CA, USA), and the absorbance was determined at 575 nm. The calcium concentration was determined using the standard curve generated from a series of dilutions of CaCl₂.¹⁶ Calcium deposition was further evaluated using Alizarin red stain.

In vivo Osteogenic Assay Using a Rat Femoral Fracture Model

Twelve SD rats obtained from BioLASCO Taiwan Co. Ltd were used in this study, which was approved by the Institutional Animal Care and Use Committee (IACUC) at Chang Gung University, which follows the Animal Protection Law of the Council of Agriculture, Executive Yuan, R.O.C. After the administration of anesthesia following an approved protocol, the fur overlying the anterior distal femur was shaved and the skin was prepped with betadine prior to performing a 10-mm incision. A 1-mm-diameter Ti needle with or without a coating of TaON-Ag was inserted into the intercondylar notch after exposing the knee joint using the trephine technique. The nail was advanced through the intramedullary canal into the proximal femur. Following insertion of the needle, a closed mid-shaft fracture was generated using the method described by Nyman et al.¹⁷ The wound was then irrigated with sterile saline and closed with an absorbable suture. The fractures and needle positions were confirmed using anterior-posterior (AP) radiographs. Radiographic examination of the SD rats was performed at two-week intervals between 3 and 9 weeks post-fracture. Both the fractured and un-fractured contralateral femur specimens were harvested by performing joint disarticulations and clearing soft tissue without disturbing the fracture callus at nine weeks post-operation. The specimens were stored in a phosphate buffer solution-soaked gauze at 20 °C. The bones from different time points were investigated using radiography, micro-computed tomography (μ CT), and histological examinations.^{17,18}

In vivo Antibacterial Assay

The in vivo antibacterial assay of the Ti needle with or without coating with TaON-Ag was evaluated using the rat osteomyelitis model.¹⁹ Twelve Balb/c mice (BioLASCO Taiwan Co., Ltd) were used in this study, which was approved by the Institutional Animal Care and Use Committee (IACUC) at Chang Gung University. To evaluate the antibacterial activity of the test materials, the femur osteomyelitis model with MSSA ATCC 25923 and *Escherichia coli* ATCC 25922 infection was used. In summary, the needle was inserted into the right distal femoral bone cavity of mice simultaneously loaded with 5×10^5 CFU micro-organisms. To determine the time interval for which the surface of the TaON-Ag can hinder the adhesion of bacteria, animals were sacrificed on the 3rd, 7th, and

14th-day post-needle insertion. The adhesion status of the bacterial biofilm on the surface of the implant was examined using field-emission scanning electron microscopy (FE-SEM, JSM-6701F, JEOL, USA).²⁰

Results

Our data show that a TaON-Ag coating on the surface of titanium has an antibacterial effect on MSSA and MRSA, CoNS, *E. coli*, and *Pseudomonas* in vitro, as well as *Staphylococcus aureus* and *E. coli* in vivo, without fracture healing interference.

Antibacterial Ability

Based on the Kirby-Bauer Disk Diffusion Susceptibility Test Protocol, it was determined that the TaON-Ag-coated Ti exhibited the best antibacterial effect on MSSA, MRSA, CoNS, *Pseudomonas a.*, and *E. coli*, in comparison with TaN-Ag, TaN-(Ag,Cu), and the control TaN coating (Figure 1). Further examination of the surface of the sample using FE-SEM with high magnification (15,000X) revealed the formation of a biofilm by *E. coli* cells two days post in vitro co-culture with Ti alloy (Figure 2A). In contrast, abnormal geometry *E. coli* cell distortion and cytoplasmic membrane wrinkles were observed 24 h post-co-culture for the TaON-Ag-coated sample, leading to cyto-plasma leakage and cell lysis (Figure 2B).

In vitro Osteogenic Assay

Osteogenetic differentiation of rMSCs under various TaN coating profiles was assayed based on staining of the calcium deposition using alizarin red on day 20. Calcium mineralization was observed in all the study groups, and the density of calcium deposition did not differ significantly among coatings. To further confirm the osteogenetic induction effect of TaNs on rMSC differentiation, the ALP activity of rMSCs cultured with various types of TaN was assayed on day 10 (Figure 3). Although over-expression of ALP activity was observed in the TaN study group, a significant decrease in ALP expression for the TaN-(Ag, Cu) coating was also observed. Thus, by integrating the results for both the antibacterial disk diffusion test and the examination of osteogenetic ability, TaON-Ag was selected for additional in vivo studies, considering the optimal broad-spectrum antimicrobial ability and non-inferior osteogenesis potential.

In vivo Osteogenic Assay

Fractured rat femurs underwent sequential radiography at 3, 5, 7, and 9 weeks post-operation. Both the callus area and bone remodeling of the fracture were observed during follow-up. The TaON-Ag coating did not have an adverse effect on the fracture healing process. Thus, all fractures achieved union (Figure 4). Histological examination of fracture healing based on hematoxylin and eosin (H&E) staining revealed the presence of newly generated callus and bone in both the pure Ti and TaON-Ag study groups. In comparison with the control group (Ti alloy), TaON-Ag did not trigger significant immune reactions (Figure 5). After the SD rats were sacrificed at 9 weeks post-operation, all specimens were scanned after removal of the needle using an animal CT imaging system (BioScan, Washington, DC). (Figure 4C and D) The μ CT images were reconstructed using the accompanying software. Subsequent analysis was performed using third-party software (Amira, Carlsbad, CA) to calculate the bone volume and bone density (averaged Hounsfield units [HU] in the cylindrical volume of interest) in the region 10 mm above to 10 mm below the fracture site of the regenerated bone with 11 cuts in comparison with the un-fractured contralateral femur.¹⁷ There was no significant difference in the remodeled bone and contralateral femur between the TaON-Ag-coated and control groups. (Table 1) The augmentation rate of the callus and remodeled bone was 154.8% for the TaON-Ag group, which is almost equal to 154.5% for the Ti control group (augmentation rate: average R/average L).

In vivo Antibacterial Assay

After the rats were sacrificed, the bone needles were removed from the femur of the specimens, and possible bacterial adhesion on the surface of the TaON-Ag-coated and non-coated needles were assessed using FE-SEM. The relatively smooth surface of the Ti needles observed at 5000X magnification for the unstained bacterial control group at 7 and 14 days post-implantation served as the control (Supplement Figure 4). The non-coated Ti needles with MSSA revealed single to multiple cocci adhesion at 3 and 7 days onwards (Figure 6A and B), and biofilm formation after 14 days (Figure 6C). It was also determined that a few *E. coli* cells (red arrows) were present on the surface after 3 days (Figure 7A), followed by biofilm formation 7 and 14 days after implantation of the non-coated Ti needle (Figure 7B and C). In contrast, SEM

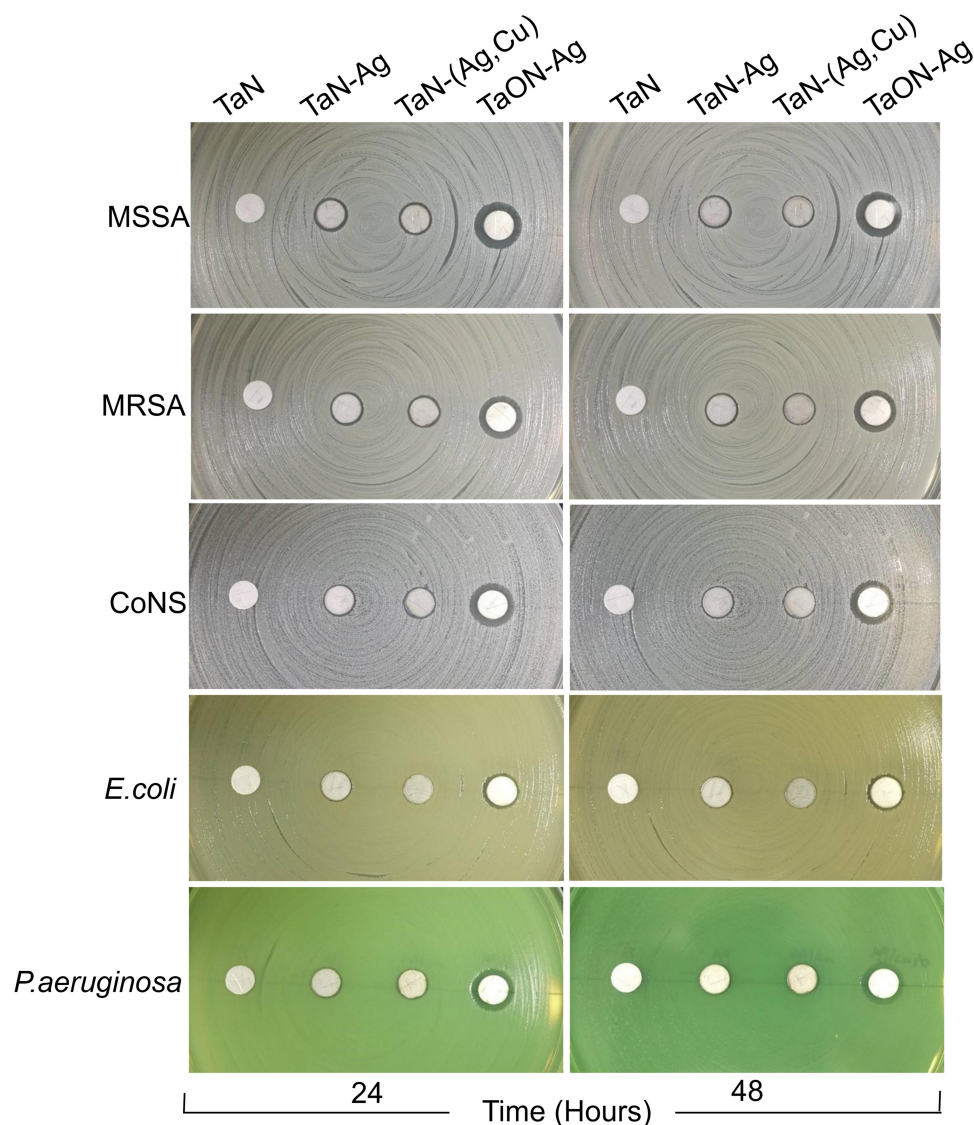


Figure 1 TaON-Ag exhibits excellent ability to inhibit bacterial growth in comparison with TaN, TaN-Ag, and TaN-(Ag,Cu). Antibacterial activity of Ti discs without (TaN) or with various coatings of TaN-Ag, TaN(Ag,Cu), and TaON-Ag using the Kirby-Bauer Disk Diffusion Susceptibility Test Protocol. The antibacterial effects of various coating samples were evaluated according to the width of the inhibition zone. The data revealed, in terms of the different inhibition behavior of the various test organisms (MSSA, MRSA, CoNS, *Escherichia coli*, and *Pseudomonas aeruginosa*), that the TaON-Ag-coated Ti exhibits the best antibacterial effect, as determined by the agar disk diffusion bioassay. The antibacterial activity could last up to 48 h without decline.

imaging did not reveal any bacterial adhesion for the TaON-Ag-coated needle infected with either *S. aureus* or *E. coli* until 14 days (Figures 6D–F and 7D–F).

Discussion

Based on the current data, the TaON-Ag nanocomposite thin film-coated Ti exhibits antibacterial activity without interfering with the osteogenic processes in either the *in vitro* or *in vivo* models. The commercial application of AgNP materials has been widely accepted for several decades. Ag ions and AgNPs are used as antimicrobials in a variety of industrial, healthcare, and domestic applications. Ag has been used in wound

dressings, and as an antimicrobial coating for medical devices to prevent biofilm formation.^{1,3,5,7,21,22} The clinical potential of Ag nanotechnology is of particular interest in the field of orthopedics, wherein the infection of implanted devices is a persistent threat.^{5,23–25} The biological activity of AgNPs depends on several factors, including surface chemistry, size, size distribution, shape, particle morphology, particle composition, coating/capping, agglomeration, dissolution rate, particle reactivity in solution, and efficiency of ion release. In addition, the type of reducing agents used for the synthesis of AgNPs is a crucial factor for the determination of cytotoxicity.^{1,26} Dissolved Ag cations are biochemically active

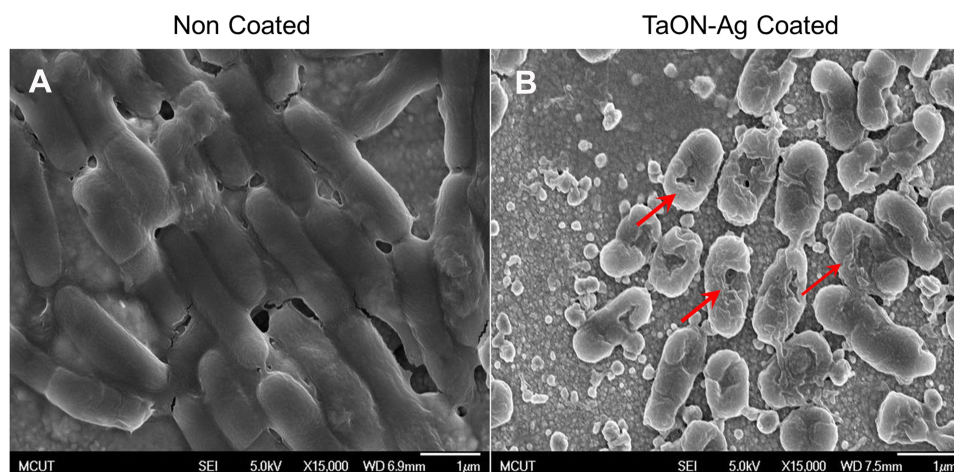


Figure 2 TaON-Ag coated Ti alloy not only inhibits the formation of bacterial biofilms but also causes bacterial lysis. FE-SEM at 15,000X magnification revealed *E. coli* biofilm formation two days post in vitro co-culture with non-coated Ti alloy (**A**). Conversely, FE-SEM revealed geometry of *E. coli* cells with abnormal distortion and cytoplasmic membrane wrinkle 24 h post-co-culture with TaON-Ag-coated Ti alloy, leading to cyto-plasma leakage and cell lysis (**B**).

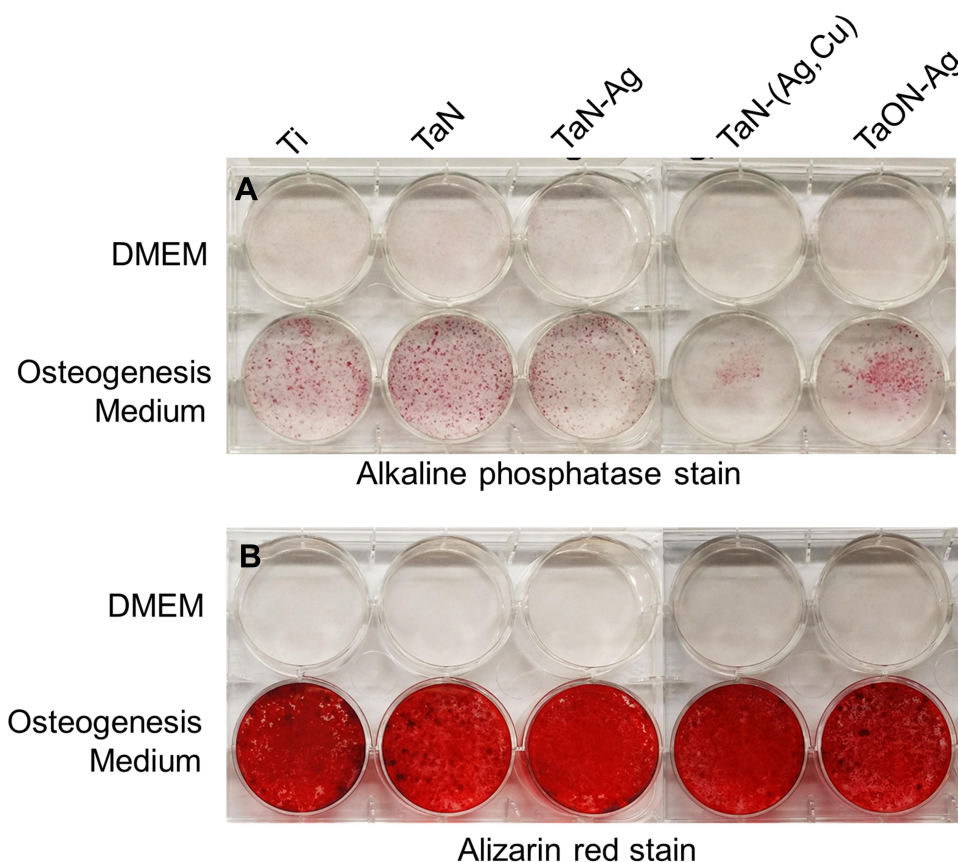


Figure 3 TaON-Ag coating did not inhibit the bone formation index, including the alkaline phosphatase and calcium oxalate crystals stains. Alkaline phosphatase (ALP) stain revealed suppressed expression >50% for the TaN-(Ag,Cu) coated sample compared with the control Ti alloy. In contrast, the TaN coating revealed an over-expression of approximately 150%. The TaN-Ag, TaON-Ag coating revealed almost the same expression of ALP stain compared with the control Ti alloy (**A**). Alizarin red staining for calcium mineralization quantification yielded over 90% expression levels for various coatings, including TaN, TaN-Ag, TaN-(Ag,Cu), and TaON-Ag, compared with the control Ti alloy (**B**).

agents that interfere with bacterial cell membrane permeability and cellular metabolism. Ag also contributes to the formation of reactive oxygen species and other mechanisms that

potentially affect prokaryotic cells. There is concern regarding the toxicity of Ag ions. However, most reports have documented acceptably low, and even an absence of, cytotoxicity based

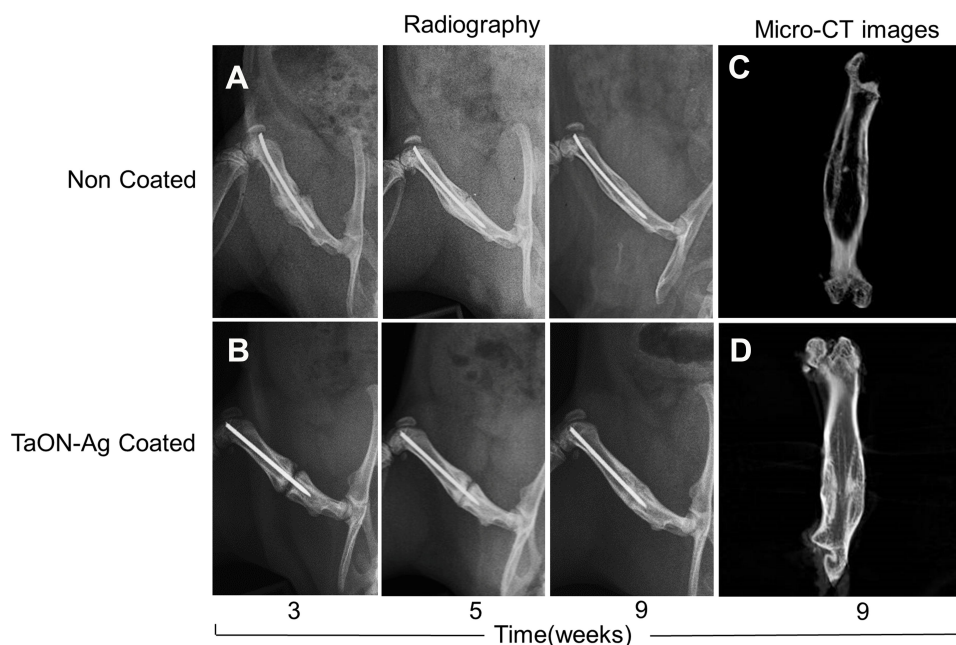


Figure 4 TaON-Ag coated Ti pin and non-coated Ti pin achieved solid bone union in femoral bone fractured rat. SD rat femoral fracture model with either TaON-Ag coated or non-coated Ti needle intramedullary fixation under sequential radiography examination 3, 5, and 9 weeks status post-surgery (**A**, **B**). Bony callus formation was observed from 3, 5 weeks s/p surgery, after which solid bone union was achieved at 9 weeks in both groups. Micro-CT image at 9 weeks status post needle fixation in non-coated group (**C**) and TaON-Ag coated group (**D**) after removal of the implant revealed solid union of the fracture site.

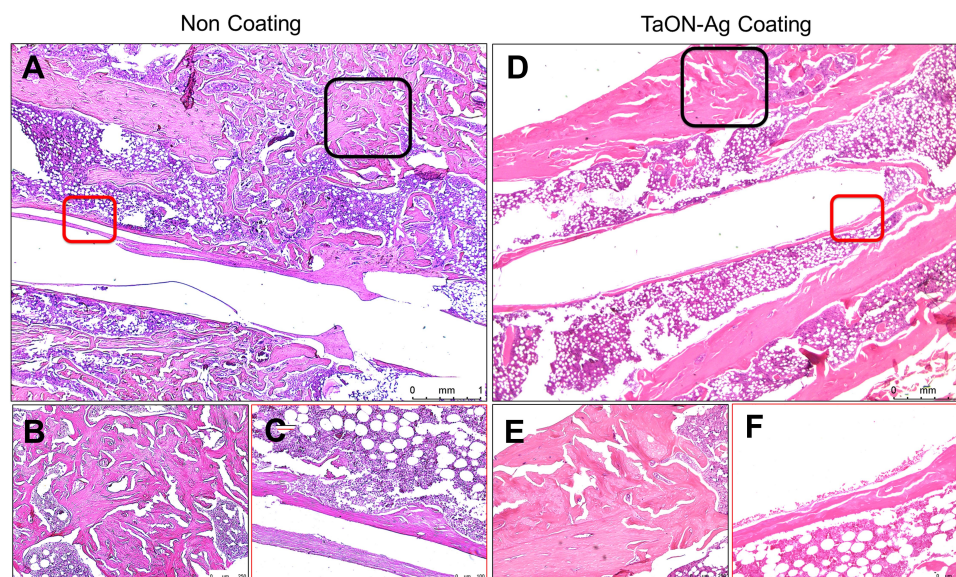


Figure 5 TaON-Ag coated Ti pin achieved bone union as well as the non-coated Ti needle under microscopic histology. Histological evaluation of a fractured femur based on hematoxylin and eosin (H&E) staining revealed trabeculae and callus formation in the non-coated and TaON-Ag-coated groups. The strip space over the center of the image revealed an intramedullary pin tract (**A**, **D**). Microscopic images of the peri-implanted bone section of both groups with higher magnification showed no evidence of advanced inflammation such as monocyte/eosinophil aggregation (**C**, **F**). A well-formed callus was also observed over the united fracture site with higher magnification in both groups without significant difference (**B**, **E**).

on in vitro and in vivo investigations with variable control release. A high concentration of Ag-containing coating was used, which revealed the dose-dependent manner of inferior biocompatibility.^{27,28} The development of green methods for nanomaterial synthesis is another important aspect of research

in the field of nanotechnology. The reported biosynthesis of $\text{Fe}_3\text{O}_4@\text{Ag}$ nanocomposite by *Spirulinaplantensis* cyanobacterium impacts the expression of efflux pump genes and also shows effective antibacterial activity against antibiotic-resistant ciprofloxacin-resistant *S. aureus* (CRSA) in medicine.²⁹

Table I Average Hounsfield Units (HU) of the Cylindrical Bone Volume of the Fractured Rat Femur (R) and the Contralateral Site Femur (L)

Non-Coated	R	L	TaON-Ag Coated	R	L
1	18.54	10.92	1	20.63	9.76
2	14.64	9.50	2	14.68	9.52
3	12.52	9.23	3	13.88	9.54
4	15.49	10.07	4	19.22	11.43
5	20.87	11.58	5	11.56	10.41
6	23.07	12.85	6	13.01	9.47
	16.52	10.69		15.51	10.02

Our data may serve as the basis for a new fabrication process for orthopedic materials. AgNPs seem to be alternative antibacterial agents, in comparison with traditional antibiotics, and they demonstrate the potential to address the problem of bacterial resistance. Among several promising nanomaterials, AgNPs may potentially be used as antibacterial agents because of their large surface-to-volume ratios and crystallographic surface structure.¹ In comparison with previously published reports on Ag ion materials, the proposed material exhibits relatively stable mechanical characteristics that are more suitable for orthopedic applications because of the controlled release of metal ions and high wear resistance.¹¹ If the antibacterial Ag or Cu nanoparticles are formed on a ceramic matrix, the constructed nanocomposite thin films exhibit varying antibacterial behaviors, depending on the metal particle size and the total exposed amount.^{7,22} According to our previous reports, it was determined that TaN-Ag is more effective against *E. coli*, whereas TaN-Cu is more effective against *S. aureus*. Therefore, Ag-Cu nanocomposite thin films have been proposed and used because of their potential for a wider spectrum of bactericidal effects.¹³ However, the results of the comparison of the manufacture of the different TaN-Ag, TaN-(Ag,Cu), and TaON-Ag coatings using the Kirby-Bauer Disk Diffusion Susceptibility Test Protocol to evaluate the broad-spectrum antibacterial ability confirm that the best effectiveness as well as the widest inhibition zone were achieved using the TaON-Ag nanocomposite thin film.

The improvement of the antibacterial capacity of TaN nanocomposite thin films using an appropriate oxygen/nitrogen atomic ratio has been investigated in only a few articles.^{8,30} Li et al reported Ag-embedded hierarchical TiO₂ films with excellent antimicrobial activity. TiO₂ can inhibit microbial adhesion because of its excellent photocatalytic activity. When excited by photons with a minimum energy equal to its bandgap, electron/hole (e⁻/h⁺) pairs are produced and promote strong reduction-oxidation (redox) reactions that kill the microbe cells. The release of

Ag ions was a result of the silver oxide in the aqueous solution. These Ag ions react with the hydroxide radicals to form unstable silver hydroxide, thereby decomposing the Ag ions in the aqueous solution. In this experiment, Ag-O ionic bonds were detected on the surface of the coated film. Once the Ag-O is in contact with the aqueous solution, a redox reaction releases Ag ions and free radicals. This results in the release of a large amount of Ag ions, demonstrating an excellent antibacterial effect. The greater inhibition zone revealed that Gram-positive or Gram-negative pathogens were not inhibited by the TaON-Ag coating material. This significant finding is consistent with the results presented in our previous reports: oxygen induction during fabrication results in the exchange of mechanical, electrochemical, and bio-related properties of the nanocomposite thin film.¹⁴ The biocompatibility of oxygen induction TaON-Ag nanocomposite thin films has been confirmed using an MTT assay, according to previous publications.^{14,31} However, the osteogenic ability of Ag composite thin-film materials has been investigated in only a few studies. Li et al reported a Ag-embedded hierarchical TiO₂ film, which exhibited excellent bioactivity and cytocompatibility, as a promising candidate for orthopedic and dental implants.⁸ Their data revealed that the embedded Ag caused minor degradation of the bioactivity and exhibited insignificant toxic side effects on MG63 cell function, based on an examination of the adhesion/morphology, proliferation, and vitality. Using immunofluorescence microscopy, it was determined that all cell functions were maintained or even promoted. Qing et al reported that AgNPs demonstrate the capacity to enhance mineralization and ALP expression in MC3T3-E1 cells at a concentration of 20 µg/mL.²³ When AgNPs were incorporated into TiO₂ nanotubes and cultured with MC3T3-E1 cells, favorable effects were observed for the promotion of cell spreading based on cell morphology assays. The current study using various metal coatings

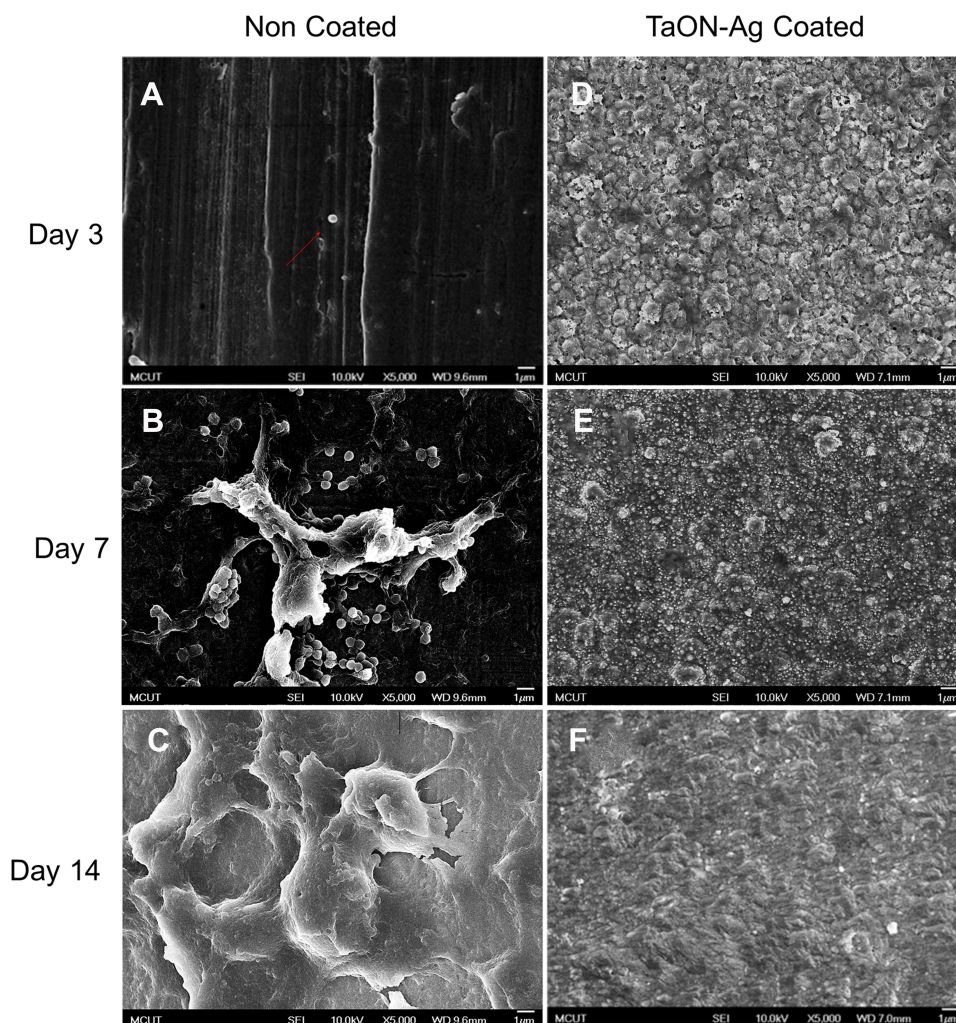


Figure 6 Ti pin with or without TaON-Ag coating has a distinct effect on *Staphylococcus aureus* adhesion and biofilm formation. FE-SEM (5000X) of non-coated Ti needle loaded with *Staphylococcus aureus* revealed only a single coccus (red arrow) adhesion on the surface 3 days after needle implantation (A), instead of multiple cocci adhesion 7 days post-surgery (B). Furthermore, thick biofilm formation occurred 14 days post non-coated Ti needle implantation (C). On the contrary, the TaON-Ag-coated needle loaded with *Staphylococcus aureus* did not exhibit any cocci adhesion for up to 14 days (D-F).

co-cultured with rMSCs under osteogenic induction revealed variable ALP staining performance. TaON-Ag exhibited non-inferior performance compared with the Ti material; however, its performance was much better than that of TaN-Ag and TaN-(Ag,Cu). The current data confirm that oxygen induction TaON-Ag results in improved biocompatibility, particularly for osteogenic induction.

To confirm the in vivo osteogenic induction ability for orthopedic applications, the femoral fracture healing model of the SD rat was adopted following the methodology described by Kevin R. O'Neill.¹⁸ The fracture healing process was evaluated by performing serial radiography, quantitative μ CT, and histological examinations. The results of our SD rat fracture model revealed no significant difference in callus formation and the fracture healing bone augmentation

ratio between the TaON-Ag-coated and control groups, which is consistent with our in vitro data. In our literature review, we found that only a few articles reported animal fracture model results for AgNPs. Zhang et al reported that AgNPs promote the proliferation and osteogenesis of MSCs, which improved fracture healing in mice.⁶ AgNPs are also considered to facilitate the promotion of the formation of fracture callus and the early closure of the fracture gap in vivo, based on the findings of Qing et al.²³ Moreover, we investigated an implant infection model based on a method described by Guan et al.²⁰ Their imaging and histopathology results revealed that the injected MRSA was killed by the Ag-TiO₂ coating. FE-SEM examination was utilized to identify the morphology and membrane integrity of both *E. coli* and *S. aureus* on AgNPs.^{8,20,32} These studies examined the in

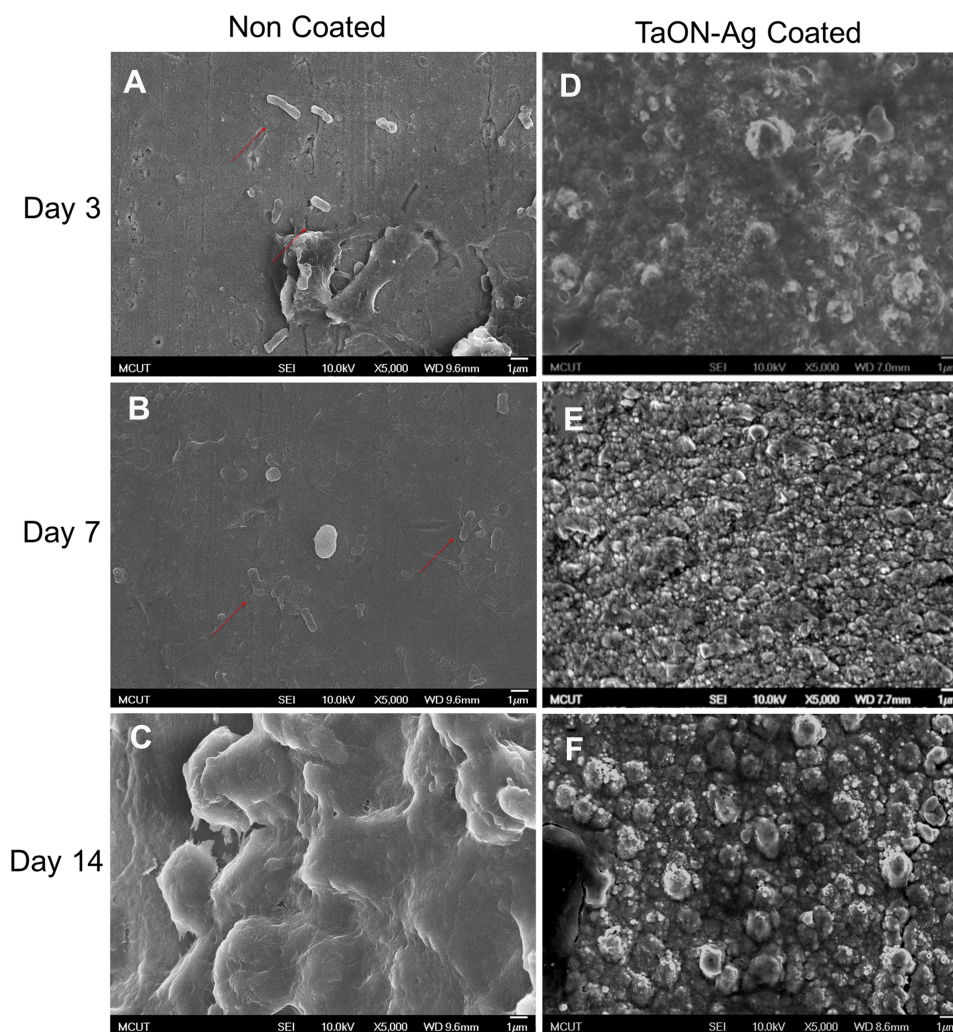


Figure 7 Ti pin with or without TaON-Ag coating has a distinct effect on *Escherichia coli* adhesion and biofilm formation. FE-SEM (5000X) of non-coated Ti needle loaded with *E. coli* revealed the adhesion of only a few bacilli (red arrows) on the surface 3 days after needle implantation (**A**), a smooth surface with mixed bacilli cells 7 days post-surgery (**B**), and a thick biofilm formation 14 days post-non-coated Ti needle implantation (**C**). The TaON-Ag coated needle loaded with *E. coli* revealed neither bacilli adhesion nor biofilm formation after 14 days (**D–F**).

vitro antibacterial ability of these particles, and morphological changes in AgNP-treated bacteria were observed. These findings are highly correlated with the present results.

Conclusion

TaON-Ag-coated titanium exhibits broad-spectrum antibacterial ability without interfering with in vitro and in vivo fracture healing. Further validation of its superior antibacterial durability, as well as its low cytotoxicity, may lead to additional clinical applications of this TaON-Ag nanocomposite thin film.

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Disclosure

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