Multi-layered composite coatings of titanium dioxide nanotubes decorated with zinc oxide and hydroxyapatite nanoparticles: Controlled release of Zn and antimicrobial property against *Staphylococcus aureus*

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ABSTRACT

This study aimed to decorate the surface of TiO_2 nanotubes (TiO_2 NTs) grown on medical grade Ti-6Al-4V alloy with an antimicrobial layer of nano zinc oxide particles (nZnO), and then determine if the antimicrobial properties were maintained with a final layer of nano hydroxyapatite (HA) on the composite. The additions of nZnO were attempted at three different annealing temperatures: 350, 450 and 550 ^{o}C. Of these temperatures, 350 ^{o}C provided the most uniform and nano-porous coating and was selected for antimicrobial testing. The LIVE/DEAD assay showed that $ZnCl_2$ and nZnO alone were more than 90% biocidal to the attached bacteria, and nZnO as a coating on the nanotubes resulted in around 70% biocidal activity. The lactate production assay agreed with the LIVE/DEAD assay. The concentration of lactate produced by the attached bacteria on the surface of nZnO coated TiO_2 NTs and ZnO/HA coated TiO_2 NTs were 0.13 ± 0.03 mM and 0.37 ± 0.1 mM respectively which was significantly lower than that produced by the bacteria on TiO_2 NTs alone, 1.09 ± 0.30 mM (Kruskal Wallis, $p < 0.05$, $n = 6$). These biochemical measurements were correlated with electron micrographs of cell morphology and cell coverage on the coatings. In conclusion, nZnO on TiO_2 NTs was a stable and antimicrobial coating, and most of the biocidal properties remained in the presence of nano HA on the coating.

Keywords: zinc oxide nanoparticles; TiO_2 nanotubes; hydroxyapatite; antimicrobial; *Staphylococcus aureus*

INTRODUCTION

Medical implants used in orthopaedics or dentistry should be sufficiently durable with mechanical properties that mimic the intended tissue.[1] They must also be safe for the patients in the long term and ideally show some antimicrobial properties to minimise the infection risk right after surgery. Unfortunately there is no single material with all these desirable properties, and in recent years attention has turned towards enhancing the properties of implants with coatings of nanomaterials.[2, 3] In orthopaedic and dental implants several types of nanocomposite coatings are employed including: diamond like carbon coatings on Co/Cr alloy,[4] nano collagen and calcium phosphate,[5] hydroxyapatite nanoparticles and polycaprolactone,[6] and carbon nanotubes (CNTs) reinforced with hydroxyapatite.[7] The purpose of such coatings has been mainly to improve biocompatibility and/or strengthen the respective implant material, rather than address antimicrobial properties.

Titanium dioxide nanotubes (TiO_2 NTs) have shown promise in such field in the past. The nanotubes are readily grown on medical grade titanium and they can resist mechanical stresses similar to those faced by bone.[8] They have also been shown to be biocompatible with bone cells, partly because they mimic the surface morphology of bone.[9] However, TiO_2 NTs alone are not antimicrobial and the development of infection around bone implants is a clinical concern. Indeed, the failure of two thirds of implants post-surgery is attributed to infection.[10] *Staphylococcus aureus* is one of the most common cause of infection in both polymer [11-13] and metallic implants.[14] To enhance antimicrobial properties of titanium implants, attempts have been made to coat TiO_2 NTs with antibiotics such as gentamicin [15] or vancomycin.[16] However, infections related to implants are normally caused by a consortium of microbes.[17] Individual antibiotics are inevitably only targeting at a few of the organisms present. There is also the concern that antibiotic resistance can develop during the treatment.[18] Alternatively, dissolved metallic elements such as silver, copper and zinc have been known for their antimicrobial properties for centuries. Their solubility and biological reactivity have restricted their applications to simple disinfectants in the past, but now nanoparticulate forms of these metals are available. Of these metals, silver nanoparticles are arguably the strongest biocide with minimum inhibitory concentrations of 3.25 mg/L to *Streptcoccus mutans*[19] and silver nanoparticles are also toxic to *S. aureus* when silver is presented as a filler in chitosan[20, 21] or a coating on medical grade titanium alloy.[22]

However, from a clinical safety perspective, silver remains a non-essential toxic element that should not normally be present in the human body. It is therefore more desirable

to use a nutritionally required metal, such as zinc, that is easily handled and excreted by the human body; but at the same time antimicrobial. Zinc oxide nanoparticles (nZnO) have antibacterial properties against both Gram positive and Gram negative bacteria. For example, nZnO was found to be an effective bactericide against *Escherichia coli*, as measured by Varaprasad *et al.* [23]. In the latter study the inhibition zone for the nano zinc oxide containing fibres was between 2.1 and 3.6 mm in an agar diffusion plate test. A minimum of 100 µg/mL of nZnO in suspension was found to be antibacterial against both Gram positive bacteria (*S. mutans and S. pyogenes*) and Gram negative bacteria (*Vibrio cholerae, Shigella flexneri* and *Salmonella typhii*) as measured by minimum inhibitory concentration assay (MIC) after 12 hours exposure in Muller-Hinton broth.[24] The effect of particle shape and size on toxicity is still being debated.[25] Apparently, it is the method of synthesis that determines the initial shape, size and morphology of zinc-containing nanoparticles.[26]

There are several techniques for growing nZnO on the surface of TiO_2 NTs. These include a hydrothermal method, electrodeposition, pyrolysis deposition, atomic layer deposition, self-assembled monolayers, and others.[27, 28] These methods give rise to nZnO of different shapes and dimensions, such as flower-like, hexagonal rod-like and spherical-like particles.[26] All of the various shapes have been shown to have antibacterial properties, with the smallest sizes generally exhibiting the highest antibacterial properties.[25] However, the challenge is to firmly attach the nZnO to the surface of the TiO_2 NTs such that the integrity of the composite is not compromised and so that the antimicrobial activity persists. In some cases, nZnO particles are formed with uneven coverage on the surface of the nanotubes.[29, 30] Other researchers were able to get uniformly distributed nZnO particles on the surface of TiO_2 NTs.[31, 32] Annealing can also affect the size of the nZnO particles on the nanotubes;[32] and higher annealing temperature tends to give improved stoichiometry of nZnO relative to other components in the composite.[33]

The biocompatibility of the external surface of the composite also needs to be considered in the context of the fibroblasts involved in wound healing and the osteoblasts that are critical to the osseointegration of the implant into the surrounding bone. There is evidence that nZnO can also have some toxicity to mammalian cells (epithelial cells, [34, 35] and so it may be desirable to moderate any direct contact of the nZnO with the human tissue. Hydroxyapatite (HA) is a bioceramic material which has a similar structure to bone and is well-known as a biocompatible material that promotes osseointegration.[7, 36] Nano forms of HA are also available in this regard.[37] This study aimed to develop a process to decorate TiO_2 NTs grown on Ti-6Al-4V alloy discs with a uniform coating of nZnO. The synthesis of the

nZnO coating was optimised by exploring different annealing temperatures. The composite coating was then made with a nano HA top coat. To demonstrate the antimicrobial properties, the resulting composite coatings were tested against *S. aureus*. This microbe is considered to be one of the main causes of infection in orthopaedic implants[38] and was hence used for testing the biocidal properties of the nZnO coatings. For these latter studies, the approaches included counting the proportions of live and dead bacteria on the coatings, monitoring microbial activity with a lactate production assay, as well as electron microscopy to observe coating integrity and the presence of any bacteria.

MATERIALS AND METHOD

The material fabrication process involved the synthesis of TiO_2 NTs by anodising the surface of medical grade titanium alloy that was subsequently doped with nZnO to incorporate some antibacterial properties. The nZnO was allowed to incorporate as crystal growing on the surface of the TiO_2 NTs. Then a final HA mineral was added to form composite coating. The composite coatings were characterised and then tested for their antibacterial properties against *S. aureus*.

Growth of TiO_2 NTs with nZnO and HA coating

A sheet of medical grade Ti-6Al-4V alloy of 1 mm thickness (William Gregor Ltd, London, UK) was initially laser cut into 15 mm discs (Laser Industries Ltd, Saltash, UK). The alloy was then polished with #400, #800 and #1200 grit silicon carbide paper (Elektron Technology Ltd, Torquay, UK). Subsequently, the discs were further polished with 6 micron and 1 micron diamond paste (Agar Scientific, Stansted, UK); after which they were cleaned by ultrasonication (12 MHz) in a mixture of NaOH (1 mol/L), $NaHCO_3$ (1 mole/L) and Na $C_6H_7O_7$ (1.5 mol/L) in a ratio of 1 : 1 : 1.5 respectively, for 10 minutes. The TiO_2 NTs were then grown on the cleaned surface of the Ti-6Al-4V alloy and then characterised following the optimised protocol described in the previous paper.[39] Briefly, TiO_2 NTs of an external diameter of 116.2 ± 6.4 nm (mean ± S.E.M., n = 54) were grown on the surface of the Ti-6Al-4V alloy by anodising. This involved immersing the alloy for 1 h in a mixture of 1 mol/L NH_4HPO_4 and 5 g/L NH_4F (0.5 g of NH_4F in 100 mL of ammonia solution). The solution was adjusted to pH 4 with 1 mol/L phosphoric acid. The samples were anodised at a voltage of 20 V, with an initial sweep rate of 0.5 V/sec, using a dual output programmable power supply (Metrix electronics limited, Tadley, UK). The Ti-6Al-4V discs with the freshly grown TiO_2 NTs were then annealed at 350 ºC for 2 h in a furnace (Carbolite RWF 1200, Carbolite

Engineering Services, Hope Valley, UK). Care was taken to provide a gradual increase in temperature, and gradual decrease back to room temperature during the annealing to ensure the final crystalline phase of the nanotubes was anatase.[40] Afterwards, the TiO_2 NTs were functionalised with –OH groups by treating them with 2 mol/L NaOH at 50 ºC for 2 minutes.[41] This provided a reactive surface for the next steps in the synthesis of the composite material.

A modified version of the protocol by Liu *et al*.[42] was used for the synthesis of nZnO on the TiO_2 NTs. In order to determine the appropriate concentration of chemicals required to grow nZnO, pilot trials were performed (Refer to the Appendix), cumulating in the following procedure. The Ti-6Al-4V discs with the functionalised TiO_2 NTs were immersed in a 1 : 2 mixture of 0.075 mol/L analytical grade $ZnNO_3$ (prepared in ultrapure deionised water) and 0.1 mol/L hexamethylenetetramine (prepared in dilute ammonia), with 2 mg analytical grade citric acid. The mixture was subsequently heated to 80 ºC, with continuous stirring on a magnetic hot plate. After 2 h in the mixture, the alloy discs of TiO_2 NTs now with the nZnO present, were sonicated in deionised water for 10 minutes to wash the coatings and remove any loosely bound materials and dissolved zinc.

The next step involved stabilising the crystalline structure of the nZnO onto the TiO_2 NTs (hereafter, called TiO_2-ZnO). Little is known about the formation of nZnO crystals on the surface of novel structures such as TiO_2 NTs and so this step was performed at three different annealing temperatures (350, 450 and 550 ºC) in order to explore the resulting material morphology, surface roughness and chemical composition. The annealing was performed in triplicate, by gradual heating of the samples to the required temperature in a furnace (Carbolite RWF 1200). The samples were maintained at the desired final temperature for 1 h, before being allowed to gradually cool to room temperature. The resulting coatings are hereafter termed as TiO_2-ZnO/350, TiO_2-ZnO/450 and TiO_2-ZnO/550 in relation to the annealing temperatures of 350, 450 and 550 ºC respectively. A control for the annealing treatment was the unheated TiO_2-ZnO discs for comparison. The resulting discs were examined for morphology and elemental composition of the surfaces (in triplicate) by scanning electron microscope (JEOL7001F SEM) coupled with energy-dispersive X-ray spectroscopy (EDS). The EDS composition was described using the AZtec analysis software supplied with the EDS attachment (Oxford Instruments, Oxford, UK). In addition, surface roughness was measured using an Olympus Laser Microscope LEXT OLS3100. The characterisation of all of the TiO_2-ZnO composites at the end of this step of the synthesis is shown in Figure 1.

The final step in the overall synthesis of the composite coating was to add hydroxyapatite. Each of the nZnO coated materials from the step above (from all annealing temperatures) were separately immersed in 3 times the normal concentration of a simulated body fluid (3SBF) which was prepared using a concentrated version of Kokubo's recipe (in mmol/L): Na^+ 426, K^+ 15.0, Mg^{2+} 4.5, Ca^{2+} 7.5, Cl^- 443.4, HCO_3^- 12.6, HPO_4^{2-} 3.0, SO_4^{2-} 1.5 mmol/L.[43] To make one litre of this concentrated SBF in ultrapure water the following salts were added: 7.996 g NaCl, 0.350 g $NaHCO_3$, 0.224 g KCl, 0.228 g $K_2HPO_4.3H_2O$, 0.305 g $MgCl_2.6H_2O$, 0.278 g $CaCl_2$, 0.071 g Na_2SO_4, 6.057 g $(CH_2OH)_3CNH_2$, 40 ml of 1 mol/L HCl. Fine adjustments were made with the same dilute HCl to achieve a final pH value of 7.4. The exposure was maintained at 37 ºC for 24 h with the aim of growing HA crystals on the surface of the samples.[43] After 24 h, the resulting HA-coated composites were removed, washed in deionised water, then air dried and examined by electron microscopy for morphology, and for surface roughness as above. The 3SBF media (n =15 in total) were retained for metal analysis to determine any losses of Zn from the discs and the expected decrease of Ca and P in the media during this final step of the HA synthesis. The spent 3SBF media were acidified with 1-2 drops of 70 % nitric acid and stored until required for trace metal analysis (see below).

Characterisation of the coatings

The morphology and chemical composition of the TiO_2 at each step of the synthesis (i.e., addition of nZnO and then HA) was found by scanning electron microscopy with energy dispersive spectroscopy (SEM/EDS) as shown in Figures 1 & 2. Figure 1 shows the surface morphology, prior to the HA additions. The growth of the TiO_2 NTs gave generally good coverage of the alloy. The material is known to consist of two different phases, the alpha-phase (α, the majority of the coating) and the beta phase (β, the depressions), which cause uneven TiO_2 NTs growth rate.[39] The additions of nZnO, regardless of the annealing temperature, gave complete coverage (Figures 1B-E); although there were some differences in surface roughness due to heat treatment temperature. Figure 1A illustrates the evenly distributed TiO_2 (before nZnO is added) and the EDS analysis confirmed the chemical composition as mainly Ti and O which is consistent with the presence of a majority of TiO_2. As shown in Figure 1B, TiO_2-ZnO had a nano-needle structure with a length of about 100 nm and width in the order of 10 nm and uniformly distributed over the surface of the nanotubes. The presence of zinc and oxygen in the EDS analysis confirmed the attachment of nZnO to the surface, although the nanostructure of the underlying TiO_2 NTs was visually discernible.

Figure 1C illustrates the uniformity of the coating on TiO_2-ZnO/350 with the nano-needle structure still present, but denser than those on TiO_2-ZnO. The EDS analysis showed a higher amount of Zn present on TiO_2-ZnO/350 than on TiO_2-ZnO. Similar observations were made for the TiO_2-ZnO/450 (Figure 1D). However at the highest annealing temperature of 550 ºC, although the coverage was good (> 90 % coverage), a few gaps were observed in the coating with respect to TiO_2-ZnO/550 (Figure 1E). The nZnO layer was denser than the other coatings and the underlying nanotubes were not visible. The amount of zinc present on the surface of the TiO_2-ZnO/550 was similar to TiO_2-ZnO/450 by EDS (Figure 1D).

The final completed composite with the HA added is shown in the lower row of Figure 2 (panels A-D). The HA formed on TiO_2 were present as micron-scale globules with nano-structured surfaces as shown in Figure 2A. TiO_2-ZnO, TiO_2-ZnO/350, TiO_2-ZnO/450 and TiO_2-ZnO /550 had HA grown on them after the overnight immersion in 3SBF as seen in Figure 2B-D. Similar observations to the nZnO coating were observed. With increasing annealing temperature used for nZnO, the HA coating became denser. TiO_2-ZnO-HA/550 (Figure 2E) had less gaps in the coating as compared to TiO_2-ZnO-HA/350 (Figure 2C). The HA coating on TiO_2-ZnO provided a full coverage so that the underlying nZnO was not exposed.

One of the concerns regarding the incubation of the partially made composite in 3SBF was that, while a HA layer might be evolved, this would be at the expense of considerable Zn leaching from the material surface. This was not the case as shown in Figure 3. In Figure 3A, the EDS measurements of the composite before and after incubation in the 3SBF media are shown. While there was a loss of some Zn from the surface as measured by EDS, this was only about 1/5 of the total Zn present regardless of the previous annealing temperature. In terms of total Zn metal lost to the external medium (Figure 3B, one-way ANOVA, $p < 0.05$), there was a clear relationship with the annealing temperature in the nZnO addition step; with the highest temperatures resulting in the least Zn leaching. The 3SBF media showed the expected trend of decreasing Ca and P concentrations following the incubation (Figure 3C), consistent with ion adsorption to the surface during HA formation on the composite. The samples at the highest annealing temperature for nZnO coating addition, resulted in the greatest decreases in Ca concentration in the 3SBF media (One-way ANOVA, $p < 0.05$).

The visual observations of the final surface morphology in Figure 1 were confirmed by surface roughness measurements (Figure 3D). The presence of nZnO on the coating increased the roughness, compared to the TiO_2 nanotubes coating alone (one-way ANOVA, $p < 0.05$). The annealing temperature at the nZnO addition step of the synthesis also influenced

the final outcome on surface roughness; with the greatest roughness values associated with the highest annealing temperatures (one-way ANOVA, $p < 0.05$). However, the final step of HA additions tended to decrease the surface roughness of each composite (one-way ANOVA, $p < 0.05$, Figure 3D). In this study, only the coatings were characterised with the aim of testing their antimicrobial activity. The effect of the coatings on the base alloy was not investigated. However, the total coating thickness is below 1 μm so no significant effect on mechanical properties is expected.

For the logistics of biological testing, one 'best' composite had to be selected for experimental work. After considering all the characterisation information, TiO_2-ZnO/350 and TiO_2-ZnO-HA/350 were chosen as the coated samples to be taken forward for further testing. This was selected on the basis that the nZnO coating was uniformly structured as well as covering the whole surface, and while the deposition of HA was also good, the gaps in the HA would allow some direct access to the biocidal nZnO coating. Subsequently, further batches of Ti-6Al-4V discs coated with the composite using the 350 ^{o}C annealing temperature were prepared. The composites were then sterilised under 36.42-40.72 kGy gamma radiation (Becton, Dickinson and company, Swindon, UK), as we have done previously with nano-coated Ti-6Al-4V alloys.[19]

Dialysis experiment and the release of dissolved metal

This experiment was conducted to aid the interpretation of the biological experiments with respect to the toxicity due to the presence of dissolved Zn, but also to inform on the stability of the coatings in the simulated body fluid. The dialysis experiments were conducted according to Besinis *et al*.[44] using the TiO_2-ZnO/350 and TiO_2-ZnO-HA/350 discs as selected for the biological experiments. A normal concentration of SBF was prepared in deionised water [43], with the pH adjusted to 7.4 with a few drops of 1mol/L HCl. Experiments were conducted in triplicate at room temperature in previously acid washed (5% nitric acid) and deionised glassware. Dialysis tubing (MW cut off, 12 000 Da, Sigma Aldrich, UK), was cut in 7 cm x 2.5 cm lengths and sealed at one end using a Mediclip; then filled with one of the coated discs as appropriate with 7 mL of SBF. The dialysis bag was closed with another Mediclip and the bag suspended in a 500 mL pyrex beaker containing 243 mL of SBF (i.e., total volume 250 mL). The beakers were gently stirred throughout and maintained at 37 ºC, and 4 mL aliquots of the SBF were collected from the external compartment of the beaker at 0, 0.5, 1, 2, 3, 4, 6, 8, 24 h. The SBF samples were acidified with a drop of 70 wt% nitric acid and stored for metal analysis (see below). At the end of the 24 h, the dialysis bags were also

carefully opened and 4 mL of the fluid therein collected for metal analysis. Dialysis curves were plotted using SigmaPlot 13.0 (Systat Software, Inc.), after deducting the background ionic concentrations of the SBF. A first order rectangular hyperbola function was used to fit dialysis curves to the raw data. The maximum initial slope of the curves informed on the maximum apparent dissolution rate of each substance.

Plate preparation and exposure to *S. aureus*

The experimental design involved exposing *S. aureus* to the coated samples of TiO_2-ZnO/350 and TiO_2-ZnO-HA/350 in 24-well, flat-bottom sterile polystyrene plates (Thermo Fischer Scientific, Loughborough, UK). TiO_2 NT-coated discs were used as a control for the coating effect. Zinc chloride was used as a metal salt control for any possible dissolved zinc effect from the nZnO. *S. aureus* was allowed to grow on its own as a negative control (ie., no biomaterials present). Nine replicate runs were conducted for each type of coated samples and the various controls (n = 6 for biochemical assays and n = 3 for SEM). Following the approach by Besinis *et al.*,[44] the materials were exposed to *S. aureus* for 24 h and the proportion of live to dead cells and the amount of lactate produced were evaluated (see below). The concentration of total dissolved zinc, calcium and phosphorus released from the coating in the SBF were also measured (see metal analysis, below).

S. aureus was chosen as it is considered to be one of the main causes of infection in orthopaedic and dental implants.[38, 45] *S. aureus* was cultured in brain heart infusion (BHI) broth (Lab M Ltd, Bury, UK) at 37 ºC. A bacterial suspension having optical density 0.018 at 595 nm absorbance (Spectrophotometer Genesys 20, Fisher Scientific, Loughborough, UK) was prepared in the BHI broth at a concentration of 1×10^7 cells/mL. For the experiments, 2 mL of the bacterial culture was pipetted in each well of a 24-well plate containing TiO_2 NTs, TiO_2-ZnO/350, TiO_2-ZnO-HA/350, $ZnCl_2$ (0.001M), and nZnO dispersed in ultrapure deionised water on their own (n = 9 replicates of each). A zinc concentration of 0.001M was used for the positive controls as this reflected the maximum amount of zinc released from the coatings. The 24-well microplates were then incubated at 37 ºC on a shaking table. At the end of the overnight exposure, six of the replicate plates were used for biochemistry. An aliquot (1 mL) of the supernatant from each well were collected for the LIVE/DEAD® kit and lactate production assays (see below). The remaining supernatant was acidified with 70 wt% HNO_3 and used for metal determination (see below). Then the remaining adherent bacterial were collected. Bacterial pellets were obtained using the same protocol as Besinis *et al*.[19] whereby the samples from the wells were sonicated (12 MHz) for 60 s in 2 mL of sterile

saline to remove the attached bacteria from the discs. Then, 1 mL of the resulting suspension were allowed to grow in 5 mL of BHI broth for 5 h at 37ºC on a shaking table with the aim of increasing the amount of live cells in order to readily measure them with the Live/Dead assay. The viability of the cells and the amount of lactate in the suspension was also assessed followed by the measurement of the ionic composition of the latter. For the remaining three replicates, the supernatant was removed and the samples were prepared for electron microscopy (see below).

CELL VIABILITY

The cell viability of *S. aureus* in both the supernatant and incubated cell suspension from all of the relevant treatments and controls were assessed using the L7012 LIVE/DEAD® Backlight™ Kit (Invitrogen Ltd, Paisley, UK). Briefly, 100 μL of the supernatant and 100 μL of the incubated homogenate from each replicate for the different treatments were transferred to a V-bottom 96-well microplates (Corning, UK). The microplates were centrifuged at 4000 rpm for 10 minutes in a 2040 Rotors microplate centrifuge (Centurion Scientific Ltd, Chichester, UK) with the aim of pelleting the bacteria; after which the pellets in each well were washed with 1 mL of sterile NaCl saline. The pellets were centrifuged again at 4000 rpm for another 10 minutes. The final washed pellets were re-suspended in 1 mL of saline. Then 100 μL of the final suspension from each well were pipetted into another 96 well plate flat bottom microplate for fluorimetry. Briefly, 100 μL of freshly prepared dyes from the LIVE/DEAD kit was added to those wells and mixed thoroughly. The microplate was incubated in the dark at room temperature for 15 min after which the fluorescence of the wells were immediately measured on a Cytofluor II fluorescence plate reader at an excitation wavelength of 485 nm and emission wavelengths of 530 nm and 645 nm respectively. The readings at 530 nm were divided by the readings at 645 nm in order to obtain the percentage of live to dead cells in the supernatant and the incubated cell suspension from the different samples and controls according to the kit instructions.

Lactate production

The metabolic activity of *S. aureus* was assessed by measuring the amount of lactate present in both the supernatant and incubated cell suspension from the treatments and appropriate controls in the experiment (6 replicates of each) using the approach utilised by Besinis *et al*.[44] The measurement of lactate would suggest the presence of metabolically

active bacterial cells. The lactate assay reagent was prepared by pipetting 1 μL of 1000 units/mL of lactate dehydrogenase (Sigma-Aldrich Ltd, UK) into wells in a flat bottom 96-well plate followed by 10 μL of 40 mmol/L nicotinamide adenine dinucleotide (NAD) (Melford Laboratories Ltd, UK) and 200 μL of 0.4 mol/L hydrazine prepared in a glycine buffer of pH 9. Then, 100 μL of the supernatant, or 100 μL of the incubated homogenate as appropriate, were transferred to a V-bottom 96-well microplate and were centrifuged at 2000 rpm for 10 minutes to generate a clean supernatant that could be measured for total lactate. Ten μL of these supernatants were added to the 211 μL of the lactate assay reagent mixture in the flat bottom 96 well plate described above. The microplate was then placed in an incubator at 37 ºC for 2 hours to allow lactate production to occur. The absorbance was then read at 340 nm against lactic acid as standards (0, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0 mmol/L).

Metal analysis following *S. aureus* exposure

The exposed broth and the detached bacteria were analysed for zinc, calcium and phosphorus composition. After the exposure to *S. aureus*, 1 mL of the broth or the detached bacteria were diluted with Milli-Q water to a final volume of 5 mL. Subsequently they were acidified with two drops of 70 wt% nitric acid to prevent Zn adsorption to the test tubes during storage. Total Zn concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS, Varian 725-ES Melbourne, Australia). Whereas the total Ca and P concentrations were analysed by optical emission spectrometry (ICP-OES, Thermo Scientific XSeries 2, Hemel Hempstead, UK). Calibrations for both ICP-OES and ICP-MS were performed with matrix-matched analytical grade standards. For ICP-MS the standards and samples contained internal references (0.5, 0.25 and 1% of iridium) for SBF, broth and any homogenates made from bacteria. In the complex matrix of broth and SBF, the detection limit was around 0.003 μg/L for zinc for ICP-MS, and 5 μg/L for calcium and 40 μg/L for phosphorus by ICP-OES.

Imaging of the attached *S. aureus*

The remaining 3 repeats of the control, TiO_2, TiO_2-ZnO/350, TiO_2-ZnO-HA/350, $ZnCl_2$ and nZnO alone were used for imaging under high resolution scanning electron microscope with the aim of visually confirm the attachment of *S. aureus* on the different surfaces. After the 24 h exposure to *S. aureus*, the supernatants from the 24-well plates were removed after which the plates were washed twice with sterile saline (0.85 wt% NaCl). Then 2 mL of 3 wt% glutaraldehyde in 0.1 mol/L cacodylate buffer was added to each well and

was allowed to stay overnight at 4 ºC. The next day, the glutaraldehyde was removed and the samples were washed with 0.1 mol/L cacodylate buffer. An increasing concentration of ethanol (30%, 50%, 70%, 90% and 100%) was used for serial dehydration of the samples as appropriate. The samples were then coated with carbon for viewing under a JEOL7001F SEM. Once in the microscope vacuum chamber, each sample was viewed at three different random locations (i.e., 3 images of each specimen x 3 replicate samples). Care was taken to systematically photograph the specimens at the same magnifications. A ×1000 magnification was used to explore the extent of coverage of the surface with *S. aureus*.

Statistical Analysis

The data from the cell viability assay, the lactate production assay and the ionic concentration measurements were analysed using Statgraphics Centurion XVII (StatPoint Technologies, Inc.). After descriptive statistics, data were checked for normality and for equal variances (Levene's test). When data were parametric, the data was analysed for treatment or time effects using one way ANOVA with Fisher's LSD test post-hoc. In cases of unequal variances, the data were transformed before analysis and where the data remained non-parametric, the Kruskal Wallis test was used. Data are presented as mean ± S.E.M unless otherwise stated. The default 95 % confidence level was used for all statistics.

RESULTS

Dialysis experiment and the stability of coatings

Prior to the analysis of the release of zinc from the coatings in the presence of the *S. aureus* (see below), the dissolution of apparent total Zn from the coatings were analysed in the presence of simulated body fluid to aid the interpretation of the bioassays and to inform on the stability of the coatings. The results are reported in Figure 4. The total concentration of Zn in the beakers from the samples without any added zinc was minimal, as expected. For the coatings containing Zn, there was an exponential rise in the total Zn concentration in the external compartment of the dialysis bag, reaching a maximum total Zn concentration of 8.17 ± 0.42 μg/L and 4.24 ± 0.36 μg/L from TiO_2-ZnO/350 and TiO_2-ZnO-HA/350 respectively. The maximum dissolution rates were 4.35 ± 0.17 μg/h and 2.60 ± 0.19 μg/h for Zn from ZnO/350 and TiO_2-ZnO-HA/350 respectively. The dissolution rate from TiO_2-ZnO/350 was significantly higher than that from TiO_2-ZnO-HA/350 (One-way ANOVA, $p<0.05$, $n=3$). It was also observed that the discs were not exhausted of Zn as Zn was measured inside the

dialysis bag at the end of the experiment and the resulting concentrations were 9.42 ± 1.20 µg/L and 6.78 ± 1.56 µg/L from TiO_2-ZnO/350 and TiO_2-ZnO-HA/350 respectively.

Figure 4 also showed the dissolution of calcium and phosphorus from the coated samples. Similar saturation mode in the total concentrations was observed for both Ca and P in the beakers (Figure 4B and C). The maximum concentration of Ca reached was 105.27 ± 9.40 mg/L and 93.00 ± 1.21 mg/L from TiO_2-ZnO/350 and TiO_2-ZnO-HA/350 respectively with a maximum dissolution rate of 65.89 ± 2.00 mg/h and 70.12 ± 0.98 mg/h respectively. The maximum concentration of P reached was 31.20 ± 0.35 mg/L and 30.21 ± 0.11 mg/L from TiO_2-ZnO/350 and TiO_2-ZnO-HA/350 respectively with a maximum dissolution rate of 21.79 ± 0.33 mg/h and 22.50 ± 0.17 mg/h respectively. At the end of the experiment the concentration of Ca and P in the dialysis bag were 85.09 ± 1.04 mg/L and 27.02 ± 0.53 mg/L for TiO_2-ZnO/350 and 82.85 ± 2.28 mg/L and 25.80 ± 0.71 mg/L for TiO_2-ZnO-HA/350.

Confirming zinc release in the broth during *S. aureus* exposures

The measured total Zn concentrations in the broth during the exposure of *S. aureus* to the different composite coating and relevant controls are shown in Figure 5A. For the controls and materials without added zinc, as expected they showed only a background concentration of the metal (around 1.4 µg/L). The positive controls of $ZnCl_2$ and nZnO, as expected, showed high concentration of zinc: 405 ± 11.3 mg/L and 354 ± 7.16 µg/L respectively. Where the coatings contained nZnO, total Zn (form unknown) was readily measured in the broth (Figure 5A). The broth exposed to both TiO_2-ZnO/350 and TiO_2-ZnO-HA/350 discs had 45.0 ± 7.2 and 22.6 ± 0.9 µg/L of total Zn respectively; with significantly less total Zn from the latter coating with HA (Kruskal-Wallis, $p<0.05$; $n = 6$). This suggests the HA coating is impeding the release of total Zn into the media, but nonetheless, this was still enough to be biocidal (see below).

Cell Morphology and Survival

Specimens from the controls and treatments were examined for abundance and morphology of the bacteria by electron microscopy at the end of the experiment and the resulting surface was reflected in Figure 6. The bacteria in the wells without any discs in them (i.e., a control grown directly on the plastic of the culture plate) survived and grew on the whole surface of each well, as expected (Figure 6A). The bacteria cultured on the TiO_2 NTs discs also grew over the whole surface of the discs, although slightly less dense than the plastic control (Figure 6B). In contrast, the treatments with either just the zinc salt (Figure 6C)

or ZnO alone (Figure 6D) showed very few bacteria, indicating that both treatments were very biocidal. The discs coated with both TiO_2-ZnO/350 and TiO_2-ZnO-HA/350 also had much reduced coverage of bacterial cells attached to their surfaces compared to the discs coated with just TiO_2 NTs, showing that the composites with or without the HA present retained antimicrobial properties.

Electron microscopy alone can only determine the presence of bacteria, not whether the organisms are alive or dead. The viability of *S. aureus* was therefore analysed using the L7012 LIVE/DEAD® Backlight™ Kit after 24 h of exposure to the composite materials or the appropriate controls. Bacterial cell viability was determined for the bacteria firmly attached to the substrate (Figure 7A) and those still present in the overlying broth (Figure 7B). The lactate production by the bacteria was also measured in homogenates from the biofilm (Figure 7C) and from bacteria in the overlying media (Figure 7D) to confirm that the cells had some metabolic activity. Overall, the results of the LIVE/DEAD assay and lactate production (Figure 7) reflected the morphological observations (Figure 6). In the unexposed control, as expected for plastic culture wells and in keeping with the electron microscopy observations, the attached biofilm had excellent viability (mean ± SEM, n = 6) of 100 ± 3 % and less in the cells in the overlying broth (72 ± 3 %, statistically different, Kruskal Wallis, $p < 0.05$, n = 6). Both the attached cells and those remaining suspended in the broth showed readily measurable lactate production (Figures 7C and D), with more in the attached microbes, as expected (Kruskal Wallis, $p < 0.05$, n = 6). The survival of bacteria attached to TiO_2 NTs (63 ± 3%), or in the broth overlying the TiO_2 NTs (38 ± 2 %), had slightly less viability than the plastic plate controls (statistically significant for each, Kruskal Wallis, $p < 0.05$, n = 6); but both showed similar lactate production to their respective controls. This indicted the cells observed on the TiO_2 NTs (Figure 6) were mostly alive and metabolically active. In contrast, most of the bacteria in the overlying broth, from either the zinc salt or zinc oxide nanoparticles alone, were dead (6 ± 2 % and 2 ± 0 % alive, respectively) and this was reflected in low lactate production (Figure 7D). The attached cells from the $ZnCl_2$ treatment fared better with 50 ± 2 % survival, but those from the nZnO treatment did not (only 1% survival, Figure 7A), indicating a higher biocidal activity by nZnO than $ZnCl_2$.

The discs coated with both TiO_2-ZnO/350 and TiO_2-ZnO-HA/350 were also very biocidal, although not as effective as $ZnCl_2$ or nZnO alone (Figure 7). The bacterial cells on the TiO_2-ZnO/350 and TiO_2-ZnO-HA/350 were mostly dead with only 22.0 ± 2.0 % and 30.9 ± 3.0 % of live to dead cells respectively (Figure 7A). This was significantly lower than those cells alive on the TiO_2 NTs discs (Kruskal Wallis, $p < 0.05$, n = 6), confirming that the

presence of nZnO in the coatings was killing the bacterial cells. Similar observations were made for surviving cells in the overlying broth in the TiO_2-ZnO/350 and TiO_2-ZnO-HA/350 treatments (Figure 7B). The moderately low survival was also reflected in the lactate production by the attached bacteria on the surface of TiO_2-ZnO/350 and TiO_2-ZnO-HA/350; values were 0.13 ± 0.03 mM and 0.37 ± 0.1 mM respectively which was significantly lower (Kruskal Wallis, $p < 0.05$, $n = 6$) than that produced by the bacteria on TiO_2 NTs (1.1 ± 0.3 mM lactate, Figure 7C). Indeed, in terms of lactate production by either the attached biofilm, or the overlying broth, the TiO_2-ZnO/350 discs were as effective as nZnO alone (Kruskal Wallis, $p > 0.05$, $n = 6$). The addition of HA was less effective, with the TiO_2-ZnO-HA/350 treatments showing a little more lactate production in the attached biofilm, but this was still much inhibited compared to either the unexposed controls or the TiO_2 NTs alone (Figure 7C).

DISCUSSION

Overall, this study aimed to make a biomaterial that was durable and capable of being decorated with nano HA to impart potential biocompatibility with human bone. Both these features were considered with the safety requirements of medical implants in mind. The material was also designed to offer antimicrobial properties by the addition of zinc. This was achieved by using TiO_2 NTs as a scaffold to grow nZnO, and the subsequent annealing ensured that the nZnO particles remained attached. The material, with or without HA present, showed a slow and beneficial dissolution of Zn in simulated body fluid, that was also biocidal to one of pathogens know to be a concern during implant surgery, *S. aureus*. The biocidal nature was confirmed by poor coverage of bacteria on the biomaterial and reduced bacterial survival as well as low lactate production by those microbes remaining.

Advantages and stability of ZnO nano-coatings

Although TiO_2 NTs on the surface of titanium alloy have been successfully used as a platform for the attachment of antibiotics to deliver some antimicrobial properties to bone implant material [46]. This approach is problematic because the antibiotics, as organic compounds, will inevitably be degraded, and thus offer only transient protection. There is also the concern of antibiotic resistance. The approach here, to use Zn as a biocide, therefore offers some advantages. An initial concentration of 0.075 mol/L of zinc nitrate as the source of zinc, and 0.1 mol/L of hexamethylenetetramine successfully yielded nZnO particles on the surface of TiO_2 NTs (Figure 1). The morphology of the TiO_2 NTs and the decoration with nZnO was similar to those reported by Liu *et al*. [42] using the same concentration of zinc

nitrate. In the latter study, the resulting nZnO had a thinner structure, compared to those in the present study where the annealing modified the morphology of the as-grown ZnO nano-coating to a more spherical shape and altered the size of the nZnO (Figure 1). The ability of annealing temperatures to alter the size of nZnO has been reported previously,[33] and was associated with the alteration in the crystal size and the reduced number of vacancies in zinc in the annealed zinc oxide . The annealing process is intended to improve the bonding of the nZnO (and any subsequent HA) to the relevant substrate as well as influence the size of the resulting crystals. Increasing the temperature has also been shown to improve the stoichiometry of the nZnO crystals,[33] in this study, a temperature higher than 350 ºC caused the crystals of nZnO to merge with each other reducing the porous structure of the latter coating (Figure 1). This change in turn resulted in bigger crystals of HA forming on the surface of the nZnO (Figure 2). This might be regarded as clinically beneficial for a bone implant material, as better HA coverage is known to increase biocompatability with osteoblasts.[47] There was also some loss of Ca and P from the 3x concentrated SBF in the presence of the coatings (Figure 3) and this is likely due to adsorption to the coating surface, and may in the case of HA, also contributed to crystal growth. However, the Ca and P were also labile and in the dialysis experiments with freshly prepared SBF, some Ca and P were leached into the medium (Figure 4).

Regardless of the detailed mechanisms involved in crystal formation, the resulting TiO_2-ZnO and TiO_2-ZnO-HA combinations with an annealing temperature of 350 ºC was selected for antimicrobial testing, partly on the basis of the results from the incubations in SBF (Figure 3). These experiments confirmed that µg/L concentrations of total Zn were released from the coating in SBF, and of the annealing temperatures used, 350 ^{o}C achieved the best release (Figure 3B), but with much of the original Zn remaining on the coating, as measured by EDS (Figure 3A). This indicated a slow release of zinc into a biologically relevant media, whilst maintaining the coating integrity in terms of elemental composition and surface roughness. The cause of the apparent total Zn release into the SBF, in theory, could either be due to slight erosion of the coating such that intact nZnO particles were being released into the media, or more likely, the release of dissolved Zn ions by dissolution of the nZnO particles attached to the TiO_2 NTs. The dialysis experiments (Figure 4) confirmed the latter. The dialysis tubing (measured pore size < 2 nm) enables only the apparent soluble Zn fraction to diffuse into the external compartment of the beakers, and this achieved equilibrium following a rectangular hyperbola as expected for solutes (Figure 4). The

dissolution of nZnO particles in biological fluids is well-known and nZnO is sparingly soluble such that usually a few µg/L of Zn^{2+} ions are released over 24 h, depending on the details of the material synthesis and media composition.[48] Similarly, in SBF, µg/L concentrations of apparent total dissolved Zn were released during the dialysis experiments (Figure 4). Less Zn was released by dissolution from the TiO_2-ZnO-HA/350 coating as compared to the TiO_2-ZnO/350 coating, likely because the HA top coat was limiting the accessible area of the nZnO for the media. Nonetheless, even in the presence of HA on the coating, the maximum release rate of total Zn was 2.6 µg/h and is broadly comparable to other biocidal nanomaterials such as Ag NPs.[44] This release of Zn was biocidal to *S. aureus* (see below).

Antibacterial properties

TiO_2 NTs on medical grade titanium alloy provide a nanoscale surface that may better support osseointegration of bone implant compared to titanium alloy alone, but the TiO_2 NTs do not have any inherent antibacterial properties.[42, 49] This was also the case in the present study, and although *S. aureus* grew less well on TiO_2 NTs compared to directly on the cell culture plate, there was still more than 80% viability of the bacteria and with no effects on lactate production (Figure 7). In contrast, both the positive control of 1 mmol/L $ZnCl_2$ and the nZnO particles alone were effectively killing the bacteria (Figure 7). The toxicity of $ZnCl_2$ to *S. aureus* is expected, with 2 mmol/L of Zn or much less reported to cause complete growth inhibition, depending on the strain of organism used,[50] and zinc is generally biocidal to microbes in the 1-10 mmol/L range, depending on salinity and temperature.[51] Zinc oxide nanoparticles have also been shown to effectively inhibit the growth of microbes such as *E. coli* and *S. mutans*.[23 24, 42] There are fewer reports of nZnO toxicity to *S. aureus*, although concentrations of around 100 mg/L of nZnO are reported to cause growth inhibition.[52] Nonetheless, in the experimental conditions used here, the measured 354 µg/L of Zn as nZnO in the broth was an effective biocide (Figures 6 and 7), and was more effective than the $ZnCl_2$ treatment (Figure 7). The mechanism by which nZnO causes toxicity to microbes is not fully understood, but could involve free ion toxicity derived from the dissolution of the metal ion, or by direct contact toxicity of the particle on the exterior surface of the microbe, as has been suggested for Ag NPs.[53] The dialysis experiments confirmed dissolution of Zn (Figure 4), and Zn was detected in the SBF (Figure 3).

However, the key concern was whether or not nZnO was toxic to the microbes when present in the coating. Both the TiO_2-ZnO/350 and TiO_2-ZnO-HA/350 coatings caused

growth inhibition of *S. aureus*. There were less coverage of bacteria on these coatings (Figure 6) and fewer live bacteria present (Figure 7) compared to the TiO_2 NTs coating alone. About 80% of the bacterial cells died in the presence of TiO_2-ZnO/350 (Figure 7B). This is agreement with the findings of Liu *et al* [45] and Roguska *et al* [46] in similar studies with nZnO coatings. However, in the present study the distribution of the nZnO was more uniform and more stable due to the annealing process and arguably making the coating more efficient. Regardless, the addition of nano HA on the nZnO slightly reduced the antibacterial effect of the coating. This might be expected, as the HA provides barrier that could prevent direct contact of the bacteria with the underlying nZnO. For example, the gaps in the HA coverage was of the order of 200-300 nm at most (Figure 2), and yet the bacteria are around 2 μm long (Figure 6). The addition of HA did reduce the Zn dissolution from the coating (Figure 4), perhaps because the exposed surface area of nZnO was less. The chemical reaction between HA and nZnO also have to be taken into consideration, as zinc can attach or be adsorped onto the HA particle, and hence decrease the apparent Zn leaching from the coating [54]. However, the small gaps in the HA structure did allow some Zn dissolution (Figure 4) and with a maximum dissolution rate of 4.35 μg/h. The latter would roughly equate to a release about 100 μg of Zn into a small volume (a few millilitres) around the point of surgery in a patient. Given the MIC for dissolved Zn is around 1 mmol/L (or 65 μg/mL), this would represent a desirable slow release of antibacterial Zn in the patient during and immediately after surgery, when the infection risk is greatest.

However, the coating containing nZnO did not completely kill the bacteria. The TiO_2-ZnO-HA/350 caused about 60% mortality of the bacteria present on the coating (Figure 7A). Other cations in the media, such as calcium can compete with dissolved zinc for uptake into cells,[55] and the relatively high cation concentrations in the SBF may have offered the bacteria some protection from Zn exposure. The nZnO coating was also much less effective as a biocide than Ag NPs attached to TiO_2 NTs. For example, on the same TiO_2 NTs used here, Gunputh et al. [56] showed the addition of Ag NPs killed more than 90% of the *S. aureus* present. This is not surprising, with the exceptional toxicity of Ag being associated with its ability to bind to –SH groups in proteins [53], and being a non-essential metal there is no endogenous ion transport system in microbes to regulate intracellular concentrations of silver or prevent bioaccumulation. These same features of Ag make it less desirable for use in patients compared to zinc. Clearly, the moderate biocidal properties of nZnO coatings need to be considered with the benefits of Zn as an essential metal which is inherently less hazardous than silver for humans in the long term.

CONCLUSIONS

In conclusion, a composite coating was successfully synthesised with a uniform distribution of nZnO on TiO_2 NTs. The coating appeared stable in SBF over 24 h and the dialysis experiments showed a slow, beneficial release of dissolved Zn. The addition of nano HA maintained the roughness and nanostructure of the coating, but still enable an antimicrobial Zn release from the material, which was effective at killing around 60% of *S. aureus* attached to the coating. Further work is needed to confirm the antibacterial properties of the coatings against other common causes of orthopaedic/dental implants infections such as *Streptococcus mutans and E. Coli*. Understanding the biocompatibility of the coating with human osteoblasts, and whether less HA can be used to further improve access to the antimicrobial nZnO in the coating.

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Conflict of Interest statement

The authors declare that there is no financial or non-financial conflict of interest.

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FIGURES

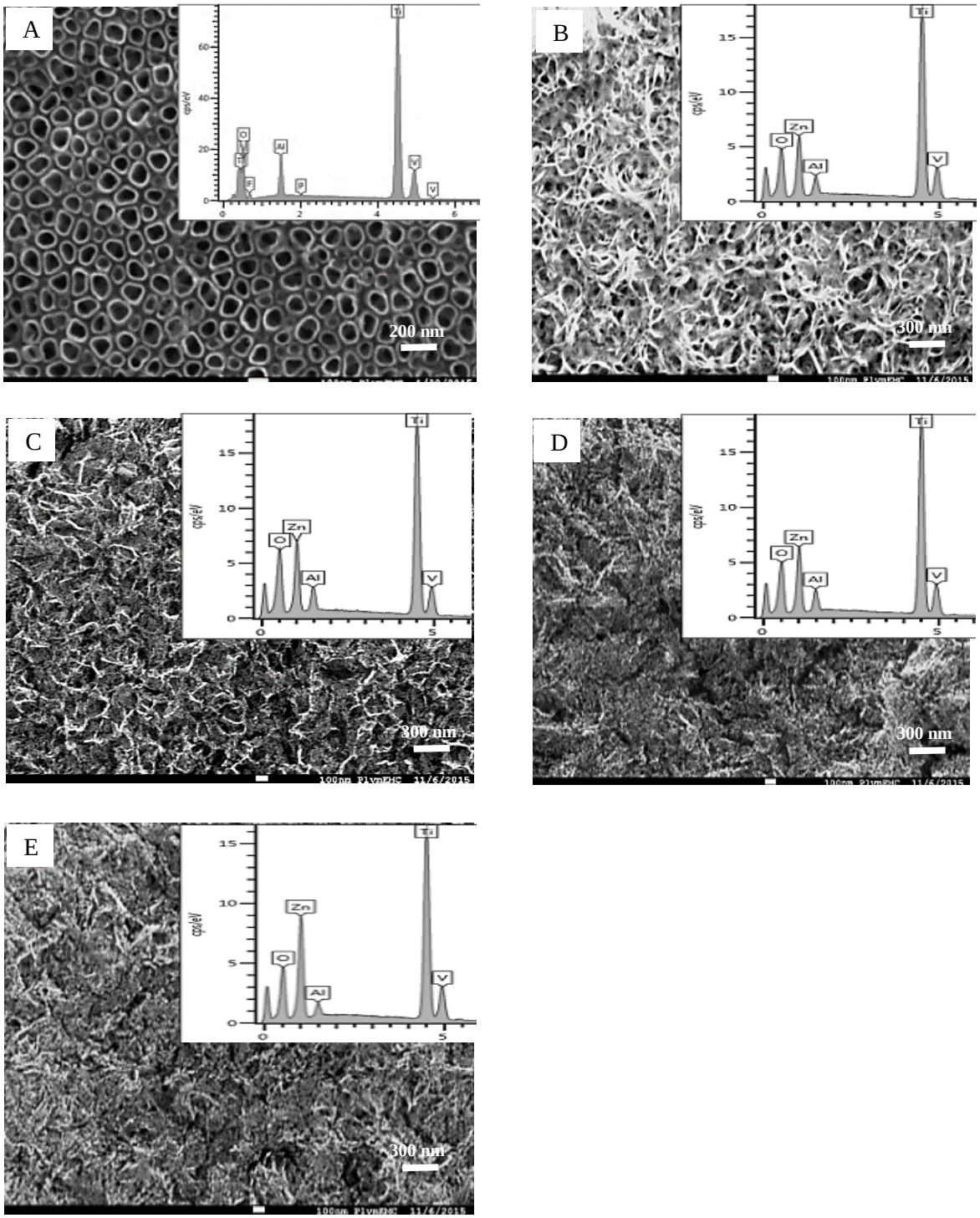

Figure 1: SEM images of Ti alloy surface with (A) the self assembled titania nanotubes (TiO_2 NTs), (B) nZnO grown on the TiO_2 NTs without any heat treatment, and nZnO grown on the TiO_2 NTs after heating to either, (C) 350 ºC, (D) 450 ºC, or (E) 550 ºC. There was no hydroxyapatite added to these samples. The insert in each panel shows the elemental composition of the coatings by EDS. Images are examples from at least three replicates.

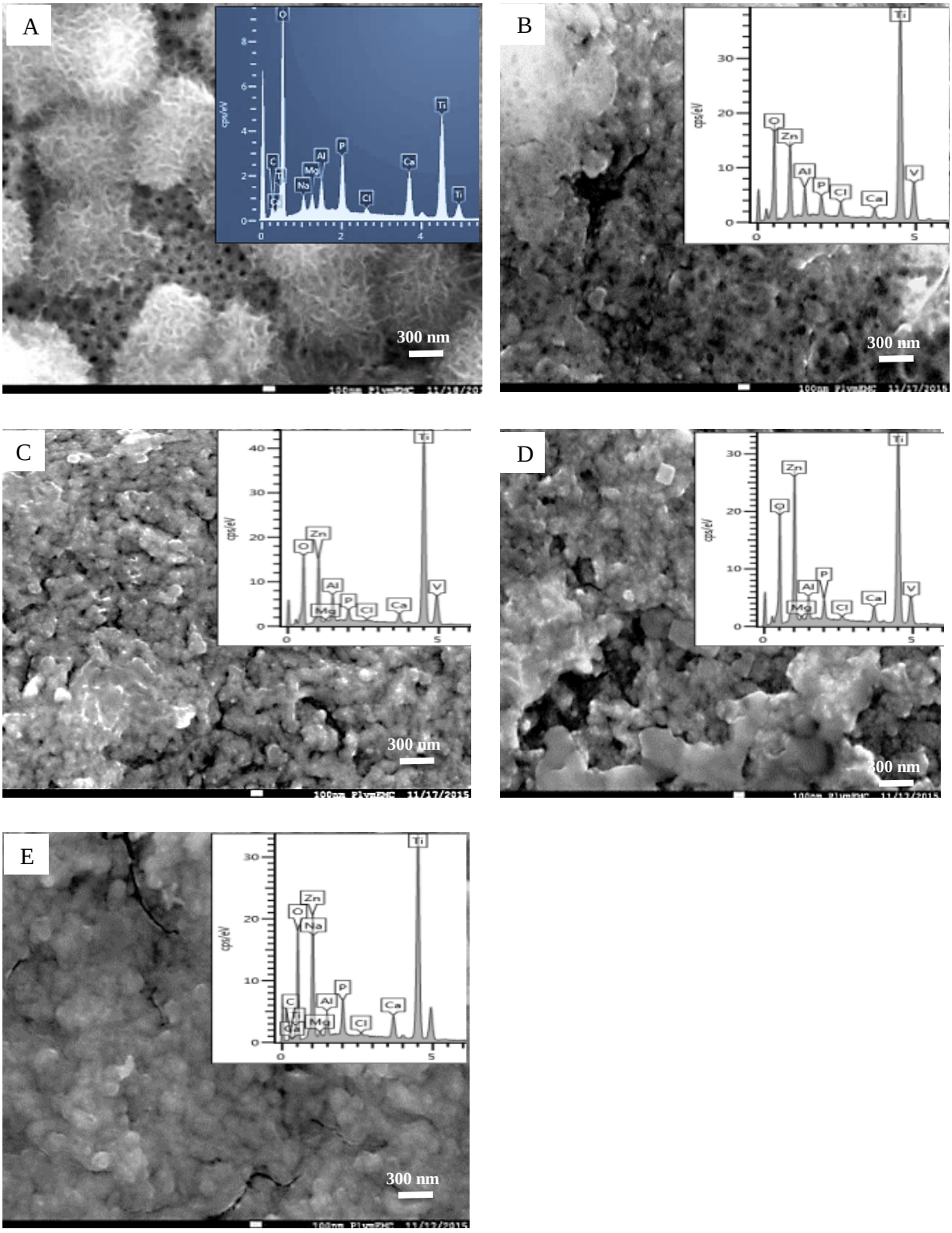

Figure 2: SEM images of HA nanoparticles on top of Ti alloy. (A) The self assembled titania nanotubes (TiO_2 NTs) with hydroxyapatite (HA), (B) nZnO grown on the TiO_2 NTs without any heat treatment, or nZnO grown on the TiO_2 NTs after heating to either, (C) 350 ºC, (D) 450 ºC, (E) 550 ºC. The insert in each panel shows the elemental composition of the coatings by EDS. Images are examples from at least three replicates.

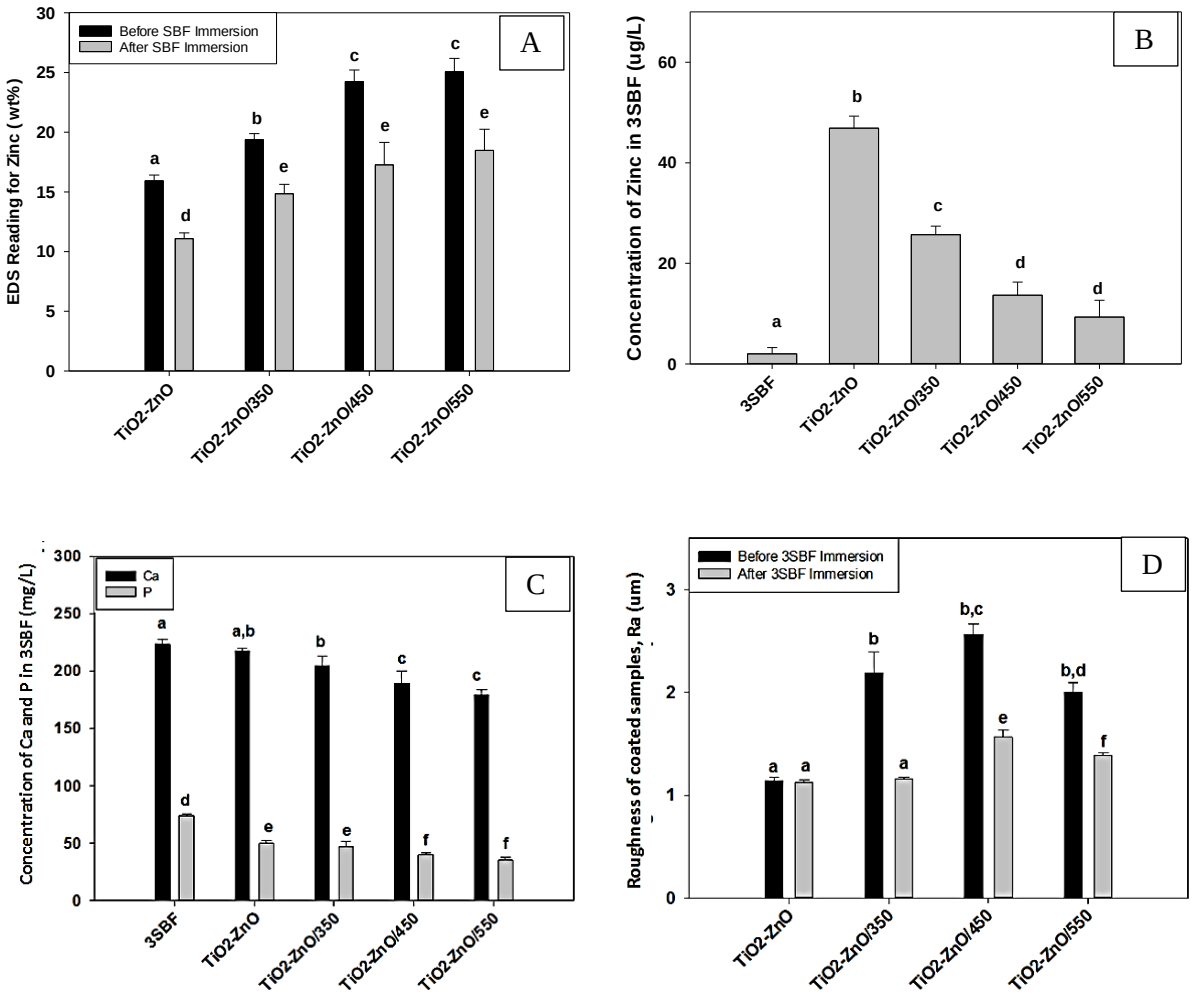

Figure 3: The effect of heat treatment on the composition and the leaching of TiO_2-ZnO coating during the synthesis of the composite coating. (A) The total concentration of zinc on the differently heat treated TiO_2-ZnO discs before and after exposure to 3SBF by EDS analysis. (B) The total concentration of zinc in the 3SBF measured by ICP-MS after 24 hour exposure. (C) The concentration of total calcium and phosphorus in the 3SBF measured by ICP-MS after 24 hour exposure. (D) Surface roughness (Ra values) for the different TiO_2-ZnO coatings before and after immersion in 3SBF, read by Olympus Laser Microscope LEXT OLS3100. Different letters indicate statistically significant differences (ANOVA or Kruskal Wallis, $P < 0.05$) between the type of coating for each measurement. Data are mean ± S.E.M, n = 3 replicate per treatment.

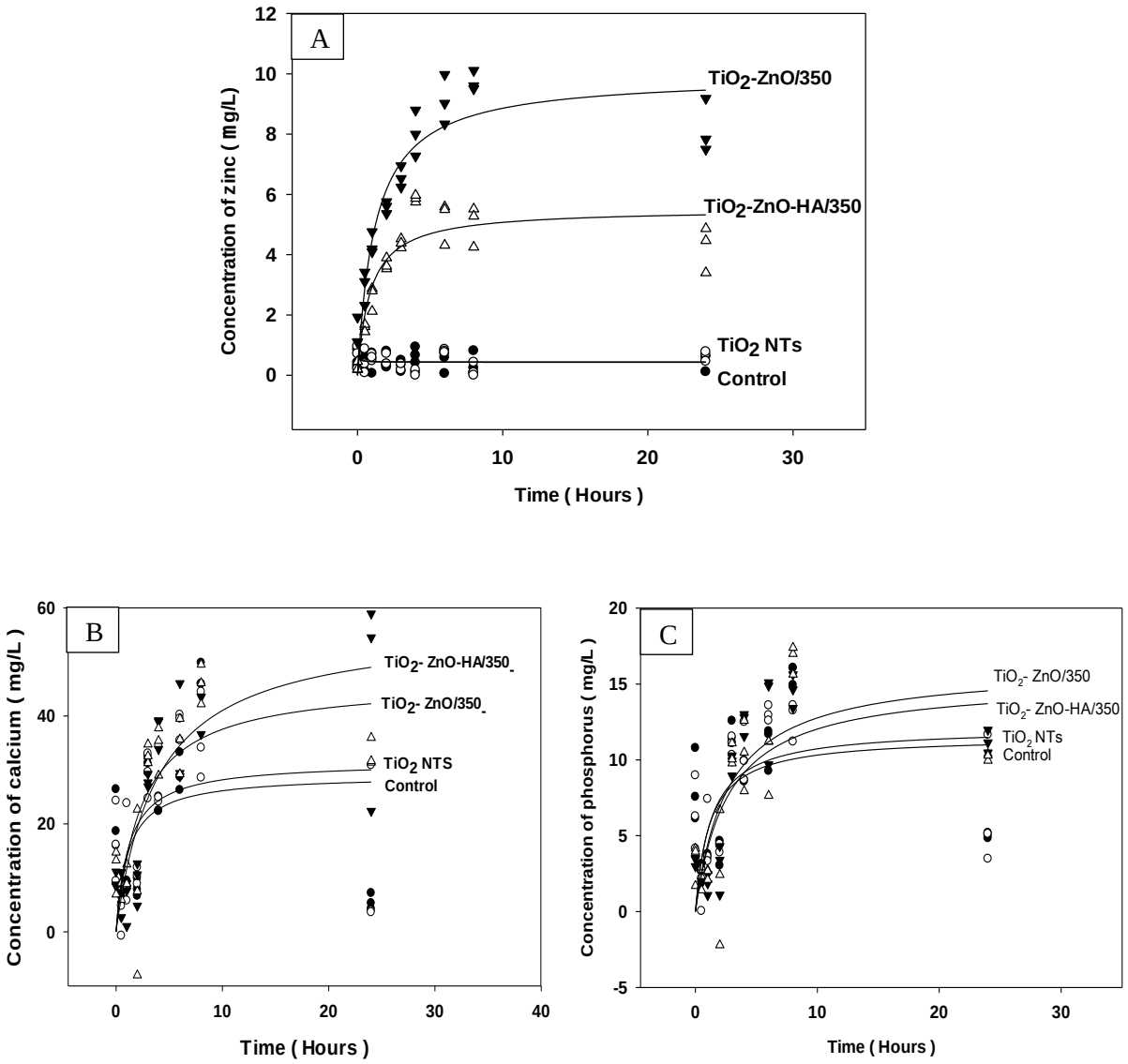

Figure 4: Dialysis of titanium alloy discs coated with either TiO_2 NTs (• Symbol), TiO_2 NTs decorated with nZnO (TiO_2-ZnO/350, ▼Symbol), or also with hydroxyapatite (TiO_2-ZnO-HA/350, Δ Symbol) in simulated body fluid (1 × SBF). Concentration of (A) total zinc measured by the ICP-MS, (B) total calcium and (C) total phosphorus measured by the ICP-OES, in SBF from the dialysis in the external compartment of the beakers. Data are single values from triplicate beakers. The curves were fitted to the raw data using a rectangular hyperbola in SigmaPlot.

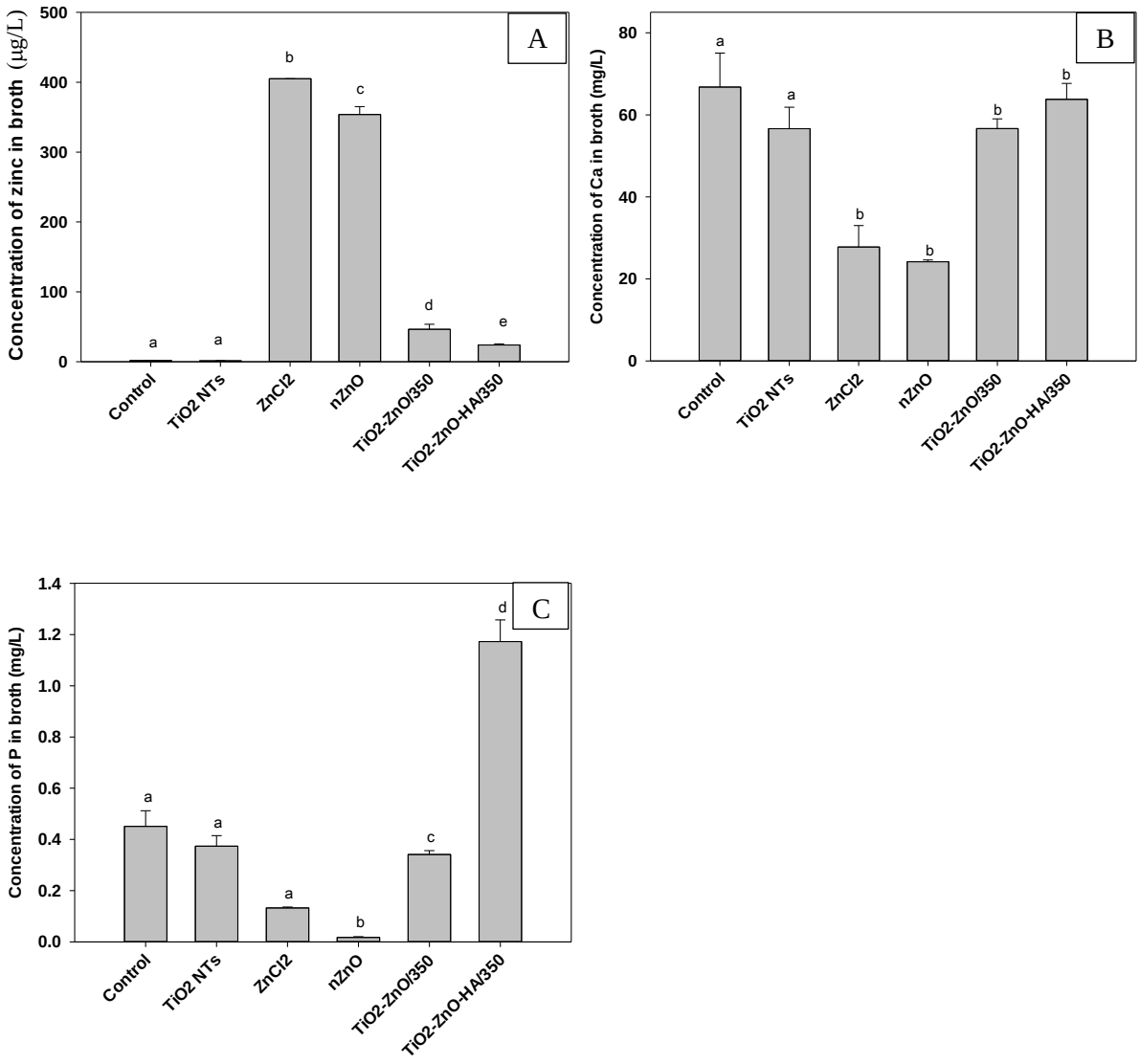

Figure 5: Total concentration of (A) zinc, (B) calcium and, (C) phosphorus, in the exposed broth after 24 hours growth of *S. aureus* in controls (cell culture plate without any titanium alloy disc), on TiO_2 NTs, in the presence of $ZnCl_2$ or a dispersion of nZnO particles alone, or TiO2 NTs decorated with nZnO (TiO_2-ZnO/350), or also with hydroxyapatite on the coating (TiO_2-ZnO-HA/350). Zinc was measured by ICP-MS and Ca and P by ICP-OES for zinc. Different letters indicate statistically significant differences (Kruskal Wallis, $P < 0.05$) between the type of coating for each measurement. Data are mean ± S.E.M, n = 6 replicate per treatment.

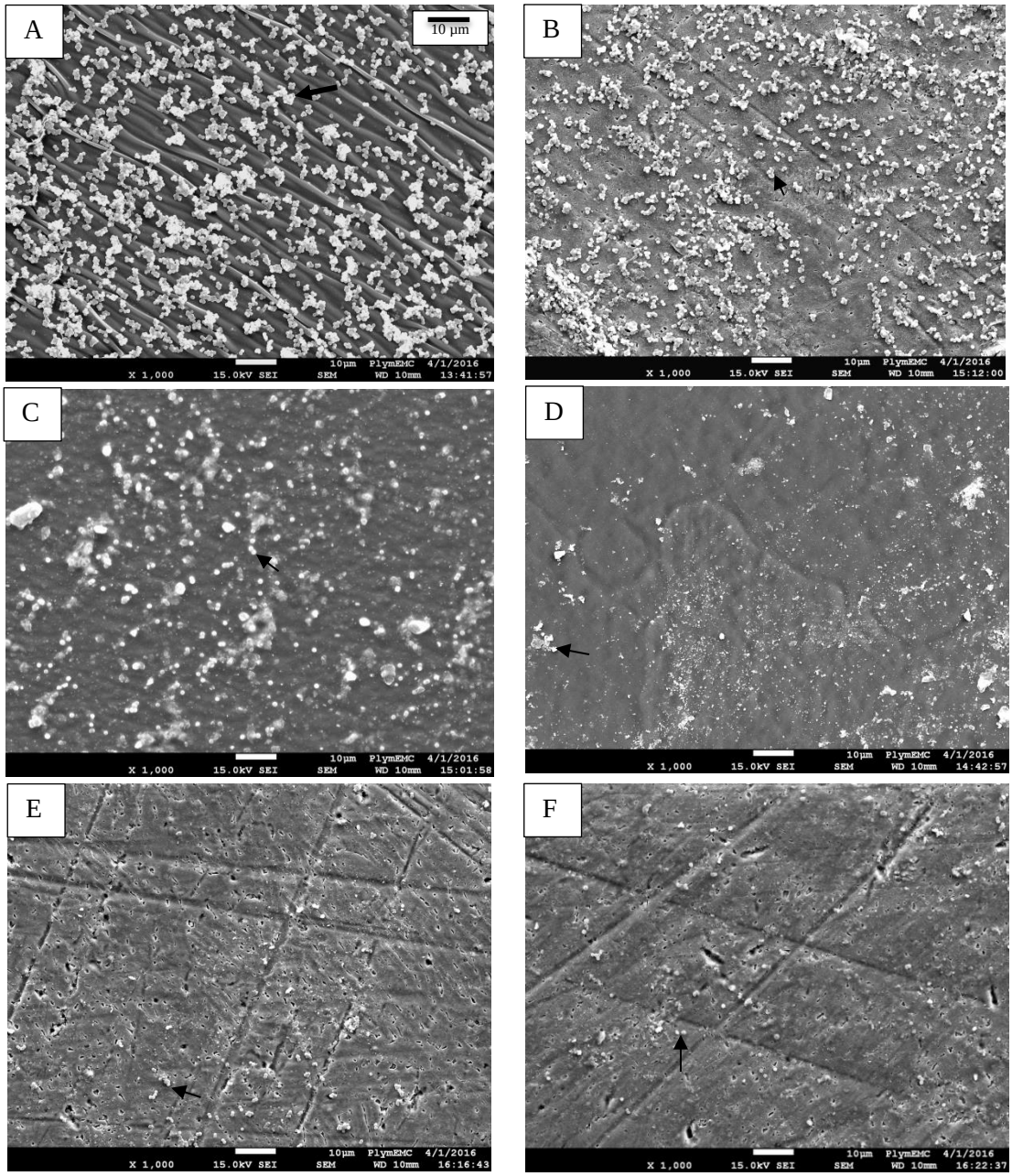

Figure 6: SEM images of attached *S. aureus* (White structures; examples showed by arrow) after 24 hours culture (A) controls (cell culture plate without any titanium alloy disc), (B) on titanium alloy with TiO_2 NTs, (C) in the presence of $ZnCl_2$, or (D) a dispersion of nZnO particles alone, (E) titanium alloy with TiO_2 NTs and decorated with nZnO (TiO_2-ZnO/350), or (F) also with hydroxyapatite on the coating (TiO_2-ZnO-HA/350). Images are representative micrographs from at n = 3 replicated samples. [Scale the same for all images]

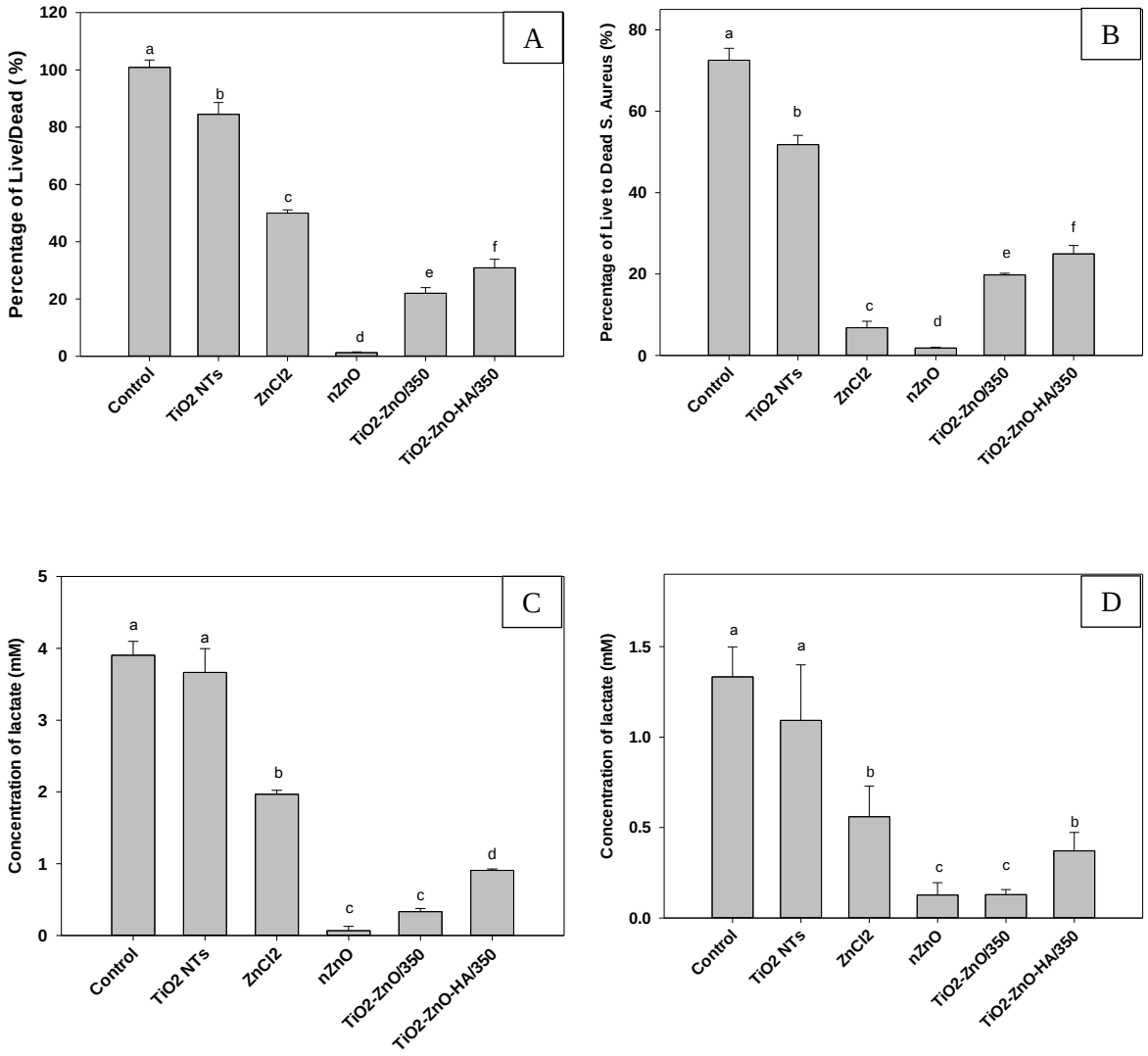

Figure 7: Percent survival (upper panels) and lactate production (lower panels) by *S. aureus* grown overnight in BHI broth at 37 ºC, on controls (cell culture plate without any titanium alloy disc), on TiO_2 NTs, in the presence of $ZnCl_2$ or a dispersion of nZnO particles alone, or TiO2 NTs decorated with nZnO (TiO_2-ZnO/350), or also with hydroxyapatite on the coating (TiO_2-ZnO-HA/350). Panels (A) and (C) are for bacteria attached to the relevant substrate, and panels (B) and (D) for microbes remaining suspended in the overlying broth. Different letters indicate statistically significant differences (Kruskal Wallis, $P < 0.05$) between treatments for each measurement. Data are mean ± S.E.M, n = 6 replicate per treatment.

Appendix

The resulting nZnO coatings synthesised in the presence of 0.05 M, 0.075 M and 0.10 M Zinc Nitrate (b, c and d respectively) as viewed under the high resolution scanning electron microscopy.