2018-10-01

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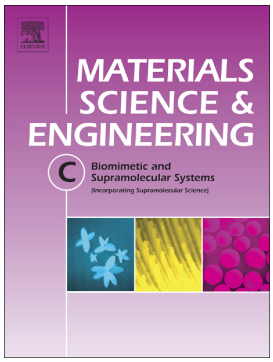
Gunputh, UF

10.1016/j.msec.2018.05.074
Materials Science and Engineering: C
Elsevier BV



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PII: S0928-4931(17)31199-2
DOI: doi:10.1016/j.msec.2018.05.074
Reference: MSC 8632

To appear in: *Materials Science & Engineering C*

Received date: 31 March 2017
Revised date: 20 February 2018
Accepted date: 26 May 2018

Please cite this article as: Urvashi Fowdar Gunputh, Huirong Le, Richard D. Handy, Christopher Tredwin , Anodised TiO2 nanotubes as a scaffold for antibacterial silver nanoparticles on titanium implant. Materials Science & Engineering C (2017), doi:10.1016/j.msec.2018.05.074

Anodised TiO_2 nanotubes as a scaffold for antibacterial silver nanoparticles on titanium implant

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Abstract

Medical grade titanium alloy is widely used for bone implants, but the material alone has no innate antimicrobial properties that would reduce infection risk following surgery. However, silver nanoparticles (Ag NPs) are known to be antibacterial. This study investigated the growth of Ag NPs on titanium dioxide nanotubes (TiO_2 NTs) on Ti-6Al-4V discs. The TiO_2 NTs were grown on the Ti alloy using an electrochemical method, and then decorated with Ag NPs. The Ag NPs were synthesised by chemical reduction using δ-gluconolactone. A silver ammonia solution (silver nitrate + liquid ammonia) was used as the source of silver. Two separate approaches were used: (1) The δ-gluconolactone was mixed with the silver ammonia and then exposed to the TiO_2 NTs (the 'mixing method'), which produced micron-sized clusters of the Ag NPs. (2) The TiO_2 NTs were exposed to the silver ammonia first and then to δ-gluconolactone (the 'sequential addition method'), which resulted in the formation of nano-sized clusters of the nanoparticles. The Ag-TiO_2 composites were confirmed by scanning electron microscopy and the elements analysed using energy dispersive X-ray spectroscopy (EDS). The composite coatings were exposed to a simulated body fluid for 24 hours in order to determine the total Ag released. The release from the micron-sized clusters from the mixing method (14.6 ± 0.67 ppm) was higher than that from the nano-sized clusters (4.05 ± 0.36 ppm) when 0.015 M of silver ammonia was used. Additionally, *Staphylococcus aureus*, was cultured on the composite materials for 24 hours. Both the micron- and nano-sized clusters of the Ag NPs were found to be antibacterial using the Live/Dead assay. Overall, δ-gluconolactone was successfully used to reduce silver to Ag NPs on the surface of TiO_2 NTs. The sequential addition method was the preferred method of synthesis because of its slower silver release, better coverage of the Ag-NPs on the TiO_2 NTs and strong antibacterial properties.

Keywords: *Titanium alloy, silver nanoparticles, TiO_2 nanotubes, anodisation, antimicrobial coating*

1. Introduction

Titanium dioxide nanotubes (TIO_2 NTs) grown on titanium alloy has gained a lot of attention since the early ninety's and has made an impact in different areas such as self-cleaning systems, solar cells, sensing, biomedical implants and drug delivery [1-3]. In the medical world, these nanotubes have been mainly been used on the surface of bone/dental implants with the aim of preventing rejection followed by life-threatening secondary surgery. Titanium dioxide NTs are regarded as being more biocompatible than plain titanium alloy due to the nano-topography of the coating, which mimics bone surface morphology [4-6]. However, on their own, the nanotubes are not anti-microbial, and their biocompatible topography inevitably also enables the growth of micro-organisms on the surface [7].

Attempts have been made to incorporate antibiotics on or into the nanotubes (e.g., vancomycin [7], with the prospect of decreasing the risk of infection after placing an implant in the human body [7-10]. However, traditional antibiotics which are organic in nature, can become ineffective due to the development of resistance by bacteria. Furthermore, traditional antibiotics are targeted to a limited number of microorganisms and cannot combat multiple infections [11-13]. Consequently, research in the development of metallic nanoparticles as antibacterial agents has gained much attention such as silver [14]. The precise mechanism by which Ag NPs are antimicrobial are still being debated, but likely involves direct contact of the particles with the cell wall of the organism and then the localised release of toxic Ag^+ ions by dissolution [15]. It is therefore important that the Ag NPs are presented on the surface of the implant where this contact toxicity with Ag can occur. This has been successfully achieved with durable Ag NP coating on human dentine that inhibited the growth of the oral pathogen, *Streptococcus mutans* [16*]*. Annodisation with silver directly on the titanium alloy can result in a coating of Ag NPs that are antimicrobial, but such a surface would also need further coating with synthetic hydroxyapatite to impart biocompatibility with human cells [17]. Titanium alloy with a covering of TiO_2 nanotubes doped with silver metal by electron beam evaporation has shown some antibacterial

properties [18]. However, the synthesis of Ag NPs onto TiO_2 nanotubes requires careful consideration, especially when a clinical application is considered. [19-21].

Silver nanoparticles can be synthesised using a variety of techniques such as electrochemical method [20], thermal decomposition [18], and laser ablation [22]. However, chemical reduction is one of the simplest method of fabricating Ag NPs on to the surface of TiO_2 nanotubes, whereby a reducing agent is used to reduce the silver ions to Ag NPs [23, 24]. In this context, several types of reducing agents have been used such as ascorbic acid [25], sodium citrate [26], sodium borohydride [27], hydrazine [28] and hydroquinone [29].

The use of hazardous reagents, such as borohydride and hydrazine, in the synthesis of an implant for the human body is not desirable for reasons of clinical safety. For some of the other less toxic substances, there are concerns that they may also alter the morphology or mechanical properties of the TiO_2 nanotubes, possibly compromising it as a bone implant material. There is a need for reducing agents that are non-hazardous to patients and preferably biologically inert to human cells, but also do not produce toxic chemicals that can damage the TiO_2 nanotubes. δ-gluconolactone is one possible candidate.

The primary aim of the present study was to explore the utility of δ-gluconolactone for reducing silver ions to Ag NPs on the surface of TiO_2 nanotubes grown on Ti-6Al-4V and to optimise the synthesis method by exploring different ways of preparing the reaction mixture. The goal was to prepare a composite material that would give a slow release of (antimicrobial) dissolved silver in the typical high ionic strength conditions of human plasma, and/or also show toxicity to microbes settled onto the surface of the implant material. For this latter goal, the release of dissolved silver was demonstrated in a simulated body fluid and toxicity to microbes shown using *Staphylococcus aureus*.

2. Materials and Methods

2.1 Growth of TiO_2 nanotubes

Titanium dioxide NTs were synthesised as previously described [30]. Briefly, Ti-6Al-4V titanium alloy discs of 1 mm thickness and 15 mm diameter were polished using #400, #800 and #1200 grit silicon carbide paper (Elektron Technology Ltd, UK) and finally polished with a diamond paste at 6 µm and 1µm. Subsequently the discs were anodised in a mixture of 1M ammonium hydrogen phosphate (NH_4HPO_4) and 0.5 weight percent (wt %) ammonium fluoride (NH_4F) at pH 4, and at 20 V with an initial sweep rate of 0.5 volts per second (V/s) using a dual output programmable power supply for 1 hour (Metrix electronics limited, UK). The resulting TiO_2 NTs were visually confirmed by electron microscopy. The synthesis typically provides TiO_2 NTs of 116.2 ± 6.4 nm (Mean ± S.E.M, n=9) diameter on the titanium alloy.

2.2 Growth of silver nanoparticles

After confirming the presence of TiO_2 nanotubes, attempts were made to coat the material with Ag NPs using a reduction reaction. A silver ammonia solution was prepared at room temperature and with continuous stirring by mixing 2.545 g of silver nitrate and 900 mL of ultrapure water, followed by 15 mL of 1 M NaOH. A precipitate of silver oxide formed, but was continuously mixed to remain in suspension. Concentrated liquid ammonia (13.4 M; density, 0.910 kg/m^3) was then added dropwise to the mixture until all the oxide had dissolved. Pure water was then added to bring the final volume to 1000 mL. The resulting solution of silver ammonia, $[Ag(NH_3)_2]^+$, (15 mM) was allowed to stir for a further 10 minutes to ensure complete reaction and mixing. Afterwards, 2 mM δ-gluconolactone solution (Sigma Aldrich, UK) was prepared in 12 mM NaOH, the volume of which was dependent on the experiment performed. Two different methods of reducing the silver ions to Ag NPs were explored. The first approach involved pre-mixing the silver ammonia solution with δ-

gluconolactone, then applying the mixture to the TiO_2 NTs (hereafter called the 'mixing method'). In order to optimise the reaction mixture, the concentration of δ-gluconolactone was maintained at 2 mM and the concentration of silver ammonia was varied from 5-15 mM (see Table 1). The second approach involved sequentially exposing the TiO_2 NTs to the reactants (hereafter called the 'sequential addition method'). First, the nanotubes were exposed to 20 mL of 15 mM silver ammonia and then the samples were washed with deionised water and air-dried. Then the Ag-dosed samples were exposed to 20 mL of 2 mM δ-gluconolactone (Table 1). In the sequential method, the reaction was optimised by varying the silver ammonia incubation from 1-10 minutes, and also by trying two durations of δ-gluconolactone treatment (5 and 10 minutes respectively). After the formation of Ag NPs by these methods, the coated samples were ultra-sonicated (12 MHz) in 25 mL of distilled water at room temperature with the aim of removing loosely attached ions.

2.3 Characterisation of the surface coating

The Ag NP-coated TiO_2 NTs prepared by each of the above methods were observed for the coverage and morphology of the Ag NPs on the surface of the composite by scanning electron microscope (JEOL7001F SEM). Three replicates for each coating were viewed and with at least three areas on each sample photographed. The SEM attempted to be systematic by examining each sample at low magnification to confirm the coverage and distribution of the Ag NPs on the composite. While high magnification was used to analyse the morphology of clusters of nanoparticles. Energy-dispersive X-ray spectroscopy (EDS) coupled with AZtec analysis software (Oxford Instruments, UK), attached to the SEM, was employed to confirm the elemental composition of the coatings; including the presence of silver.

2.4 Silver dissolution in simulated body fluid

This experiment aimed to demonstrate a local release of silver from the composites in a biologically relevant media. A simulated body fluid (SBF) of known chemical composition was used for these experiments, according to Kokubo's recipe [31] (in mmol/l): Na^+, 142; K^+,5.0; Mg^{2+},1.5; Ca^{2+}, 2.5; Cl^-, 147.8; HCO_3^-, 4.2; HPO_4^{2-}, 1.0; SO_4^{2-},0.5. The amount of the various salts used were as follows to make 1 litre of SBF: 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 M HCl, plus a few drops of 1 M HCl to further adjust to pH 7.4. Each of the composite discs from each of the synthesis methods described above (surface area exposed being 1 cm^2) were individually immersed in 25 mL of SBF; each in 100 mL plastic bottles in triplicates for each composite and placed in an incubator at 37 ºC for 24 hours. This incubation time was selected based on our experience of Ag dissolution measurements [32], and to capture the maximum release of silver from the coatings that occurs in the first few hours when the diffusion gradient into the test vessel is maximal. After the 24 hours, 5 mL of the SBF was collected into 15mL Falcon tubes, acidified with two drops of 70 % nitric acid to ensure any metal remained in solution. The samples were subsequently analysed by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) against matrix-matched standards. Sample blanks were included in the measurements runs to check for instrument drift and the detection limit for silver was 1.19 µg/l.

2.5 Antibacterial properties

This experiment aimed to give a preliminary, but clear demonstration of the anti-microbial properties of the composites. The approach involved growing *Staphylococcus aureus* on the composites for 24 h and then counting the proportions of live and dead microbes using fluorescent probes. *S. aureus* was chosen as it is considered to be one of the main causes of infection in orthopaedic and dental implants [33, 34]. Briefly, *S. aureus* was cultured in brain heart infusion (BHI) broth (Lab M Ltd, UK) at 37 ºC. A bacterial suspension having

optical density 0.018 at 595 nm absorbance (Spectrophotometer Genesys 20, Fisher Scientific, UK) was prepared in the BHI broth at a concentration of 1×10^7 cells/mL. The coated discs prepared from each of the synthesis methods described above were then exposed to *S. aureus* in a 24-well, flat-bottom sterile polystyrene plates (Thermo Fischer Scientific, UK). Two mL of the bacterial culture of *S. aureus* was pipetted in each well of the 24-well plate. After 24 hours exposure, the external media was carefully removed and the cells washed 3 times with sterile saline. The remaining adherent bacteria were detached from the surface of the discs by sonication (12 MHz for 60 s) in 2 mL of sterile saline. The resulting suspension was used in the live/dead kit to determine the proportions of viable cells [32]. In this experiment, TiO_2 NTs without any silver were used as a coating control, and wells without any disc was used as a reference for the bacterial growth. The entire experiment was conducted in triplicate.

The cell viability of the *S. aureus* detached from the surface of the discs and the relevant controls were assessed using the L7012 LIVE/DEAD® Backlight™ Kit (Invitrogen Ltd, UK) as previously described [16]. One hundred µL of the detached cells from each replicate of the different composites were transferred to a V-bottom 96-well microplates (Corning, UK). The microplate was centrifuged at 4000 rpm for 10 minutes in a 2040 Rotors microplate centrifuge (Centurion Scientific Ltd, UK), after which the pellets in each well were washed with 1 mL of sterile NaCl saline and centrifuged at 4000 rpm for another 10 minutes. The final washed pellets were re-suspended in 1 mL of saline out of which 100 µL was pipetted into another 96 well plate flat bottom microplate. Then, 100 µL of freshly mixed dyes from the LIVE/DEAD kit was added to those wells and mixed thoroughly. The microplate was then incubated in the dark at room temperature for 15 minutes after which the fluorescence of the wells were immediately measured on the Cytofluor II, fluorescence plate reader at an excitation wavelength of 485 nm and emission wavelength 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 exposed broth and the incubated cell

suspension from the different samples and controls. The kit was calibrated against bacterial standards consisting of dilutions of live and dead cultures to give a range between 0-100 % live cells [16].

2.6 Statistical Analysis

The data were analysed with Statgraphics Centurion XVII (StatPoint Technologies, Inc.) and figures drawn using SigmaPlot. Briefly, after descriptive statistics and a variance check (Levene's test), data were analysed using one way ANOVA for treatment effects within each synthesis method. The Fisher's LSD test was used post-hoc to identify the locations of any differences. Data are presented as mean ± S.E.M and the default 95 % confidence interval was used for all statistical analysis.

3 Results and Discussion

Titanium dioxide NTs were grown successfully on the titanium alloy and produced a good coverage of nanotubes that were consistent with our previous work [30]. The use of silver ammonia and gluconolactone yielded Ag NPs (Figures 1 and 2) that were spherical in shape and had a mean diameter of 102 ± 21 nm (Mean ± S.E.M, n = 9) that was consistent on all the coatings. However, the precise details of the synthesis method also mattered, and the distribution and the clustering of the Ag NPs on the composite surface varied according to the synthesis method. Regardless, some silver was released from the composites (Figure 3) and all the composites containing silver were antibacterial to *S. aureus* and demonstrated effective killing of the microbe within 24 h (Figure 4).

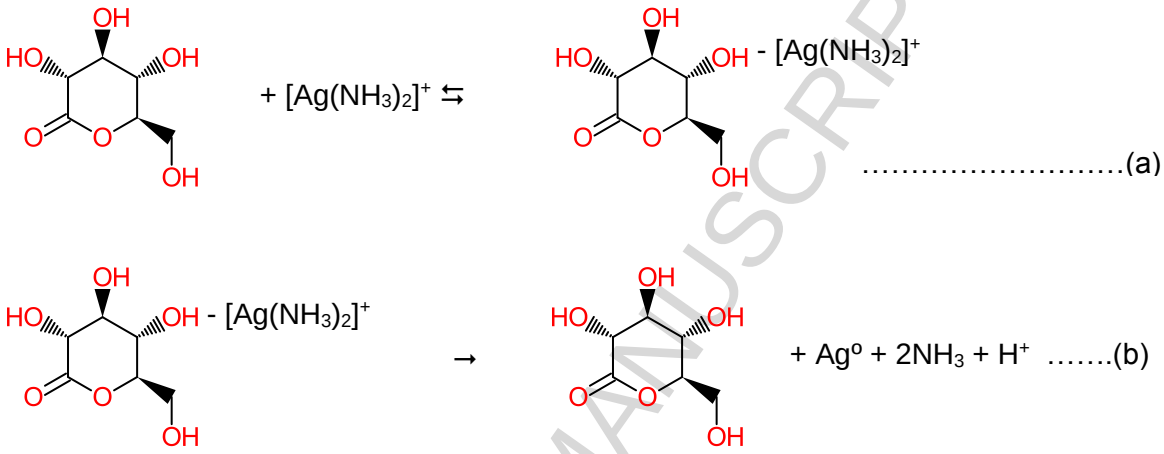
3.1 Silver nanoparticles synthesis using the mixing method

When TiO_2 nanotubes was exposed to a mixture of silver ammonia solution and δ-gluconolactone for 10 min, the resulting Ag NPs formed clusters having an overall aggregate diameter in the micrometre range; hereafter referred to as 'micro-clusters' (Figure 1). For the

mixing method of synthesis, the concentration of δ-gluconolactone was maintained at 0.002M. When the concentration of silver ammonia solution used was 5 mM, there was a uniform distribution of the clusters of Ag NPs with about 1-10 μm space between the clusters as confirmed by Figure 1A. At higher magnification (Figure 1D), the clusters were found to be of an average size of 1 by 0.5 μm. The size ranges of the clusters are reported in Table 1. When the concentration of silver ammonia was increased, the distribution and spacing of the clusters remained the same. Nonetheless, the size of the clusters increased with the concentration of the silver ammonia solution used (Figure 1B and 1E) and was confirmed by measurements of the clusters (Table 1). The EDS analysis in Figure 1G confirmed the presence of titanium, oxygen and silver on the surfaces of the composite, as expected. The presence of silver in the EDS confirmed the presence of Ag NPs. However, the individual particles did not attach to the inner walls of the TiO_2 NTs, and this is consistent with other attempts to decorate nanotubes with spherical particles using photoreduction, electrodeposition, sputtering, and silver mirror reaction [24, 35-38]. The clustering effect in the present study prevented the individual nanotubes being coated with a uniform layer of individual nanoparticles, but instead the nanoparticles were attached to a few nanotubes. The reason for the clustering is likely explained by the reaction chemistry steps in this particular synthesis method.

Mixing the silver ammonia solution with the δ-gluconolactone likely reduced the silver ion to Ag^0 nanoparticles in the mixture as per equation (a) and (b). The initial step is the formation of a cationic silver ammonia complex (equation (a)) which then reduces to Ag^0 in the acid conditions (equation (b)). The Ag NPs were then attached to the TiO_2 NTs. δ-gluconolactone is a relatively big molecule due to its cyclic molecular structure, and it had four –OH bond which were presumably all available to react with the silver ammonia complex. Hence, one δ-gluconolactone molecule, in theory, is able to simultaneously react with four silver ammonia complexes. This would result in Ag NP formation in close proximity to each other. Thus clusters were likely already formed in the mixture before adhering to the

walls of the TiO_2 nanotubes during the 10 minutes of exposure to the reaction mixture. Increasing the concentration of the silver ammonia complex would promote the reduction of silver ions. Since the concentration of δ-gluconolactone remained unchanged throughout, the number of clusters did not increase appreciably with the increase in the concentration of $[Ag(NH_3)_2]^+$. Nonetheless, the number of silver nanoparticles increased which in turn led to an increase in the size of the micro-clusters formed on TiO_2 nanotubes.



3.2 Silver nanoparticles synthesis by the sequential addition method

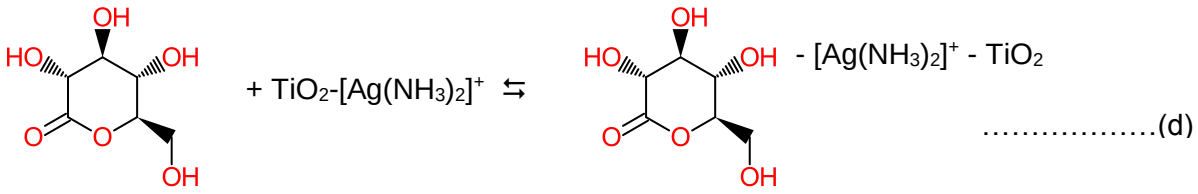
Using sequential additions of the reagents, the clusters of Ag NPs formed on the surface of the TiO_2 NTs where much smaller than those made by the mixing method; with nano clusters forming instead of micron clusters (Figure 2). The surfaces of the TiO_2 NTs from the sequential addition method for silver were uniformly covered with the clusters of Ag NPs (Figure 2). At higher magnification, EDS was also conducted. Figure 2G confirmed the presence of silver on the surface through EDS analysis of the nanoparticles clusters. The presence of Ti and O was concomitant with the presence of TiO_2 NTs.

In the sequential addition method, the TiO_2 NT surfaces were first exposed to the silver ammonia complex solution for times varying from one to ten minutes, while the exposure to the δ-gluconolactone solution was fixed at five minutes (Figure 2). Increasing the time in the presence of the silver ammonium complex from one to ten minutes resulted in a reduction in

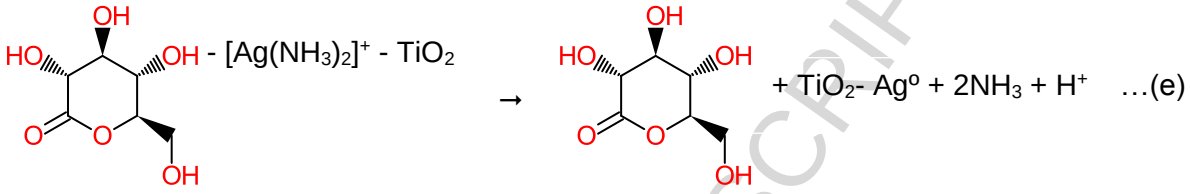
the size of the nano-clusters on the surface of the nanotubes (Figure 2). The sizes of the clusters (but not the primary diameter of the individual particles) varied from 200-500 nm, 100-200 nm and less than 100 nm for silver ammonium incubation times of 1, 5 and 10 minutes respectively (Table 1).

Exposing the TiO_2 nanotubes to the silver ammonia solution first, allowed the silver ammonia to attach to the nanotubes as one whole complex, $[Ag(NH_3)_2]^+$ as per equation (c). Subsequently, when exposed to δ-gluconolactone, the silver ammonia complex was reduced to Ag NPs as per equation (d) and (e). They still formed clusters because of the 4 –OH present on the δ-gluconolactone molecule, which brought the silver nanoparticles closer to each other. The clusters were smaller, but closer to each other, in Figure 2F compared to 2D; suggesting that a longer exposure time to the silver source yielded more nano-clusters closer to each other on TiO_2 nanotubes. The longer TiO_2 nanotubes stayed in the $[Ag(NH_3)_2]^+$ solution, the more complex molecules likely became attached to the walls of the nanotubes. Subsequently, the more reaction sites were available when the δ-gluconolactone was added, resulting in a spread of Ag NP clusters on the surface. Since the exposure time to the δ-gluconolactone solutions was fixed at five minutes, there was not enough time to build up the bigger clusters as seen in Figure 2C and 2F. A more uniform distribution was seen with nano-clusters as compared to micro-clusters, which indicates the sequential addition method gives a better coating.

$TiO_2 + [Ag(NH_3)_2]^+ \leftrightarrows TiO_2\text{-}[Ag(NH_3)_2]^+$ .....(c)



……(d)



…(e)

3.3 Silver release from the coatings

Figure 3A shows the total silver released into the SBF from the composite materials as prepared by the mixing method. The blank confirmed there was no silver contamination in the apparatus or procedure, and as expected only a trace amount of silver is found in TiO_2 nanotube coated alloy, and likely liberated from the alloy itself when nanotubes are present, rather than the nanotubes. There was a statistically significant increase in the amount of total silver released into the SBF according to the concentration of silver ammonia solution used in the original synthesis (One-way ANOVA, $p < 0.05$). The silver releases ranged from 3.35 to 14.6 ppm (Figure 3A). Figure 3B showed the total silver concentration releases after 24 hours from composites made by the sequential addition method. The silver release declined with increasing incubation time in the silver ammonium complex during the synthesis (Figure 3B); perhaps suggesting the longer incubations produced the more stable coatings. Regardless, even with the longest incubation with the silver ammonia solution, the amount of silver released from the coating was around 4 ppm. In any case, both synthesis methods resulted in a silver release that would be biocidal (see below).

3.4 Antibacterial Properties

Figure 4A and B confirmed the antibacterial properties of all the samples coated with Ag NPs. There were much fewer bacteria surviving on the Ag NP-coated surfaces than the TiO_2 nanotubes without silver or the control of bacteria grown without any discs present (One-way Anova, $p<0.05$). Indeed, there was little or almost no live cells in the presence of Ag NPs be it in micro clusters or nano clusters. Besinis et al., 2014 showed that Ag NPs coated onto dentine discs were more than 99 % biocidal to *Streptococcus mutans* [16]. Other studies have also shown Ag NPs coatings to be antimicrobial to bacteria including *Aggregatibacter actinomycetemcomitans* [20] and *S. aureus* [21] . The proportions of the observed killing of *S. aureus* (Figure 4) due to direct contact toxicity or release of dissolved silver is not certain from the measurements made here. However, it is interesting that in suspensions of *S. mutans*, silver concentrations from Ag NPs as low as 3.125 mg/l is highly biocidal [16]. It is therefore likely the silver release from observations in SBF (Figure 3), on its own, could explain the antimicrobial effect in the short term, while the presence of Ag NPs on the coating offers the potential for more persistent antimicrobial properties of the material.

4. Conclusions

This study explored two approaches to coat TiO_2 NTs coated Ag NPs that also used a less hazardous reducing agent, δ-gluconolactone. Both methods were successful at forming Ag NPs with a primary particle size around 100 nm, but there was a stark difference in the clustering of the Ag NPs according to the synthesis method. The mixing method led to micron clusters of Ag NPs, but the sequential addition method led to much smaller nano clusters of particles. Both types of clustering of Ag NPs proved antibacterial to *S. aureus*. The release of some silver from the coating in the first 24 h is also beneficial from the viewpoint of patient care, where arguably, the greatest risk of infection is in the few hours immediately after implant surgery. However, further studies are needed to inform on the

molecular mechanism of bacterial killing from the composites used here, as well as establishing the biocompatibility of the implant in a wound environment with respect to clinical safety.

Acknowledgements

The authors would like to acknowledge the funding through a joint PhD studentship for UG by the Faculty of Science and Environment and Peninsular Schools of Medicine and Dentistry. The assistance by the technical team in the School of Marine Science and Engineering, the School of Biological and Biomedical Science and the Electron Microscopy Centre (EMC) of Plymouth University is gratefully acknowledged.

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Table 1: Variables used in the synthesis of silver nanoparticles on TiO_2 nanotubes grown on medical grade titanium alloy and the size range of the clusters of particles obtained.

[silver ammonia solution] (mM)	[delta gluconolactone] (mM)	Incubation time with each reagent (minutes)	Cluster size range
Mixing method			
5	2	10 (silver ammonia + δ-gluconolactone together)	1 by 0.5 µm
10	2		1 by 3 µm
15	2		5 by 5 µm
Sequential addition method			
15	2	1 (silver ammonia) then 10 δ-gluconolactone)	100 by 100 nm
15	2	5 (silver ammonia) then 10 (δ-gluconolactone)	350 by 350 nm
15	2	10 (silver ammonia) then 10 (δ-gluconolactone)	150 by 150 nm

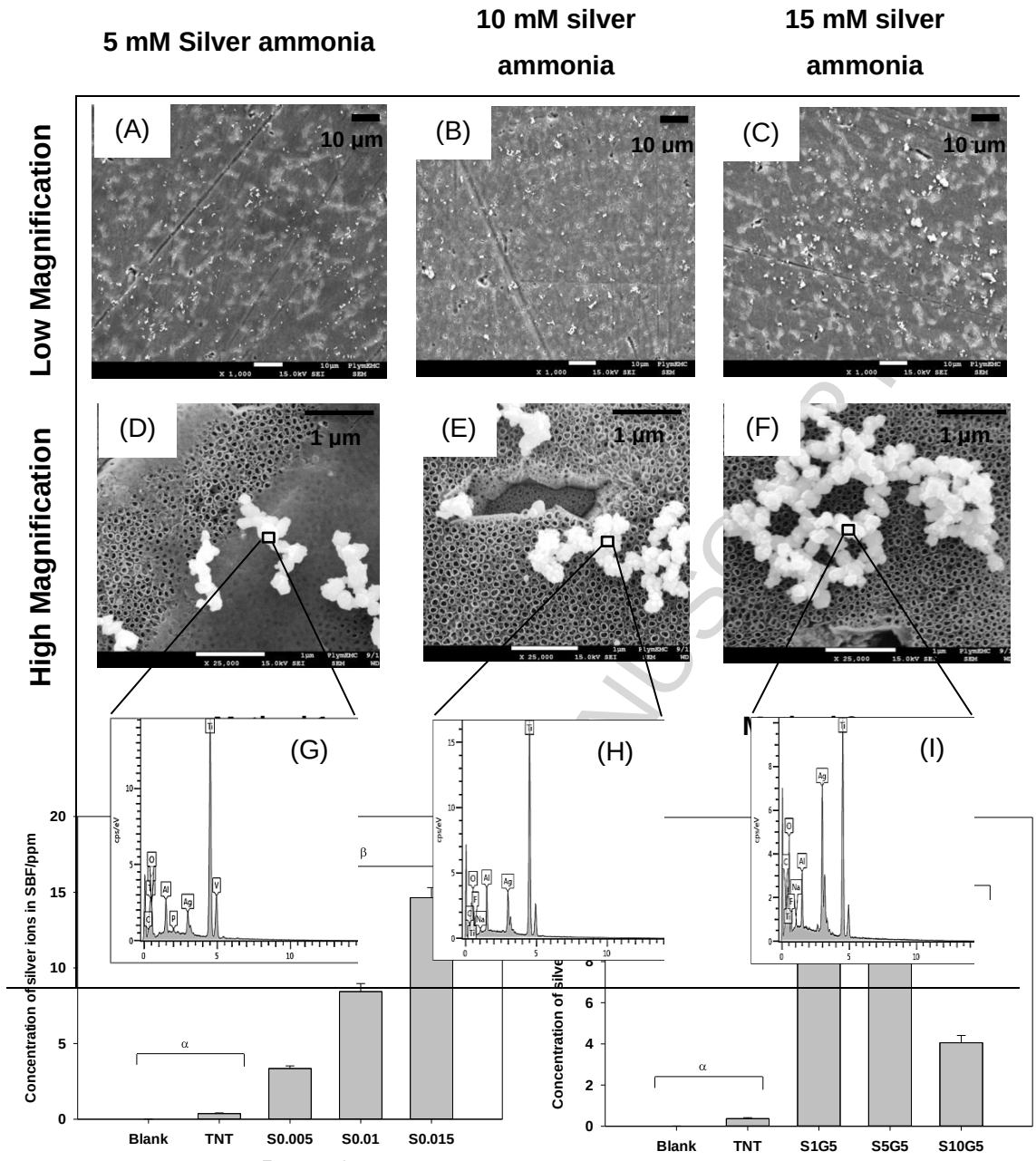

Figure 1. SEM images of silver nanoparticles forming micro-clusters on TiO_2 nanotubes grown on medical grade titanium alloy, along with the images of the composite coating prepared with different concentrations of silver ammonia solution (A) 5 , (B) 10 and (C) 15 mM respectively; all viewed at a low magnification of ×1000. Panel D-F shows the respective coatings at a higher magnification of ×25 000. Panel (G-I) shows the EDS analysis of the silver nanoparticles grown on theTiO_2 showing the elemental composition. The areas being analysed are highlighted in panels D-F.

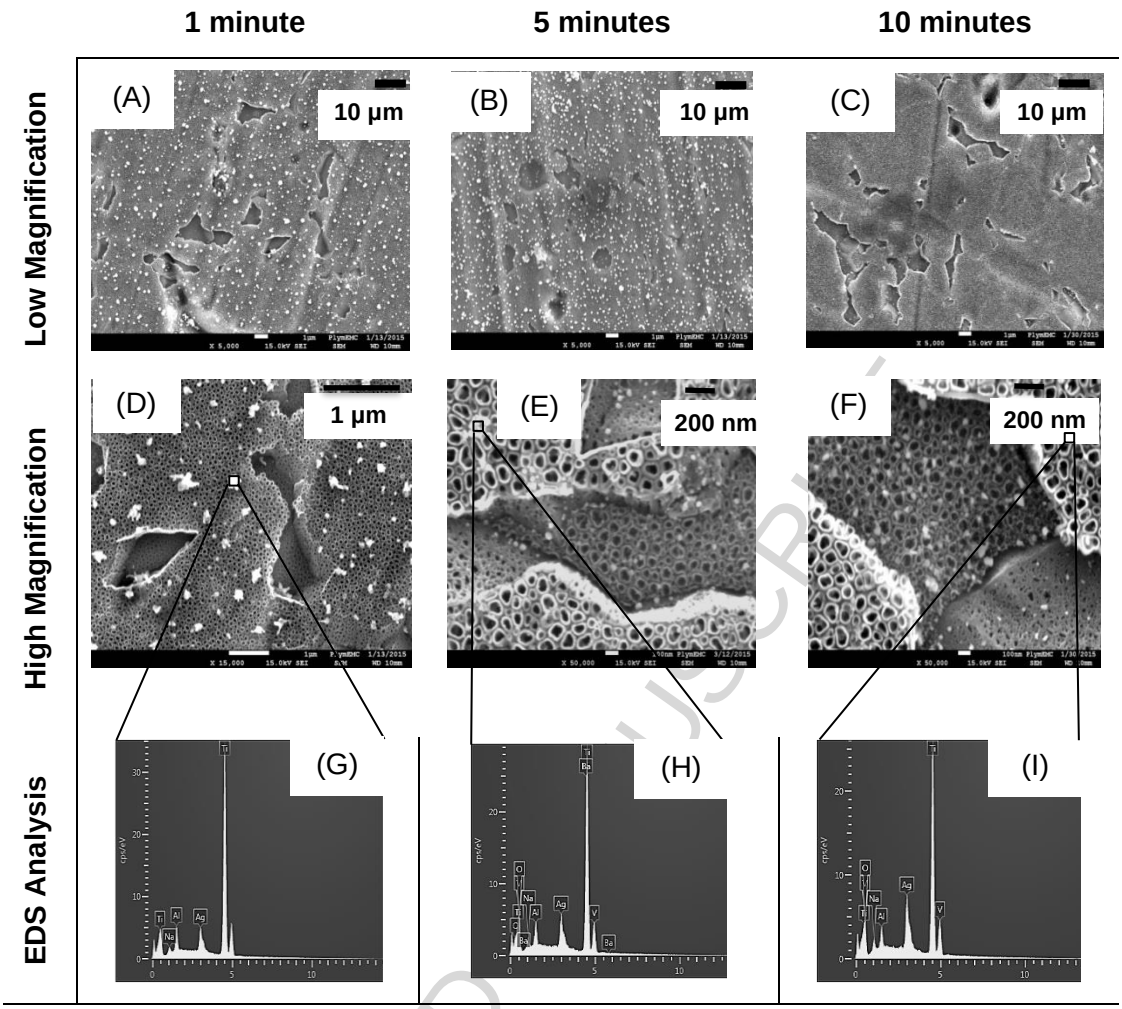

Figure 2: SEM images of nanoclusters of silver nanoparticles grown by initial exposure of TiO_2 nanotubes to 15 mM silver ammonia followed by 2 mM δ-gluconolactone. The exposure time to silver ammonia was (A) 1, (B) 5 and (C) 10 minutes respectively. The δ-gluconolactone concentration was maintained at 5 minutes. Panels (D), (E) and (F) show higher magnification SEM images of these respective treatments. Panel (G-I) shows the EDS analysis of the silver nanoparticles grown on the TiO2 showing the elemental composition. The areas being analysed are highlighted in panels D-F.

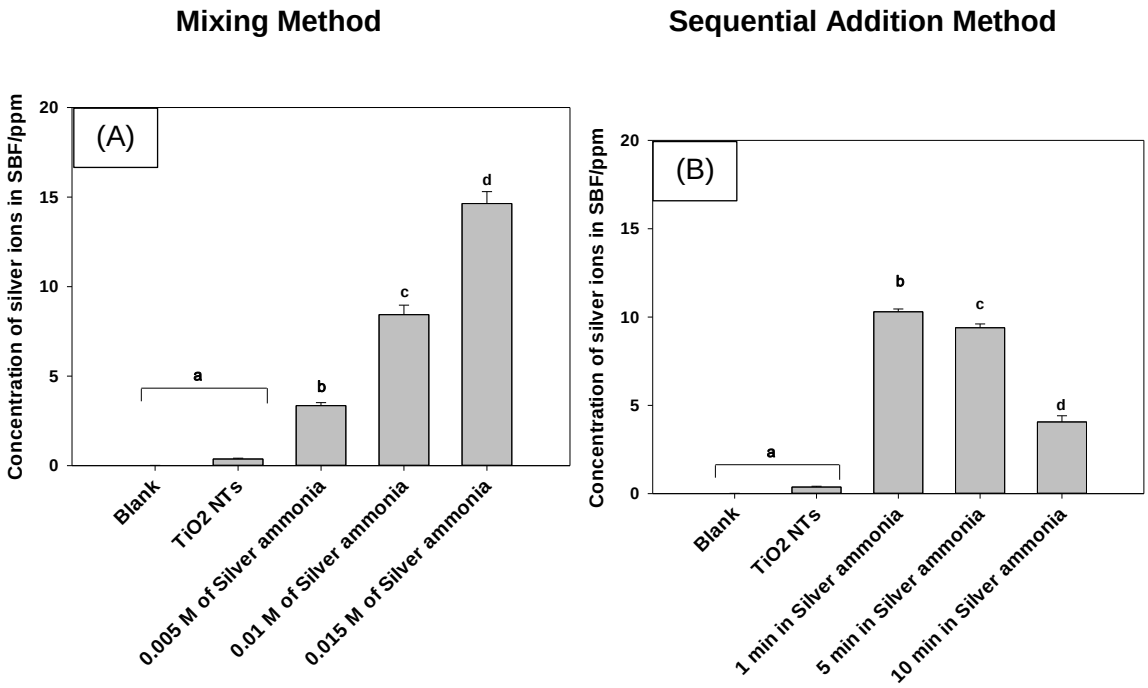

Figure 3: Concentration of total silver in acidified SBF measured by ICP-OES after 24 hours exposure of the titanium discs from: (A) the mixing method and (B) the sequential addition method of silver nanoparticles synthesis. Data are means ± S.E.M., n = 3 replicates. The different letters indicate the statistically significant differences between samples within synthesis method (one-way ANOVA, $P < 0.05$).

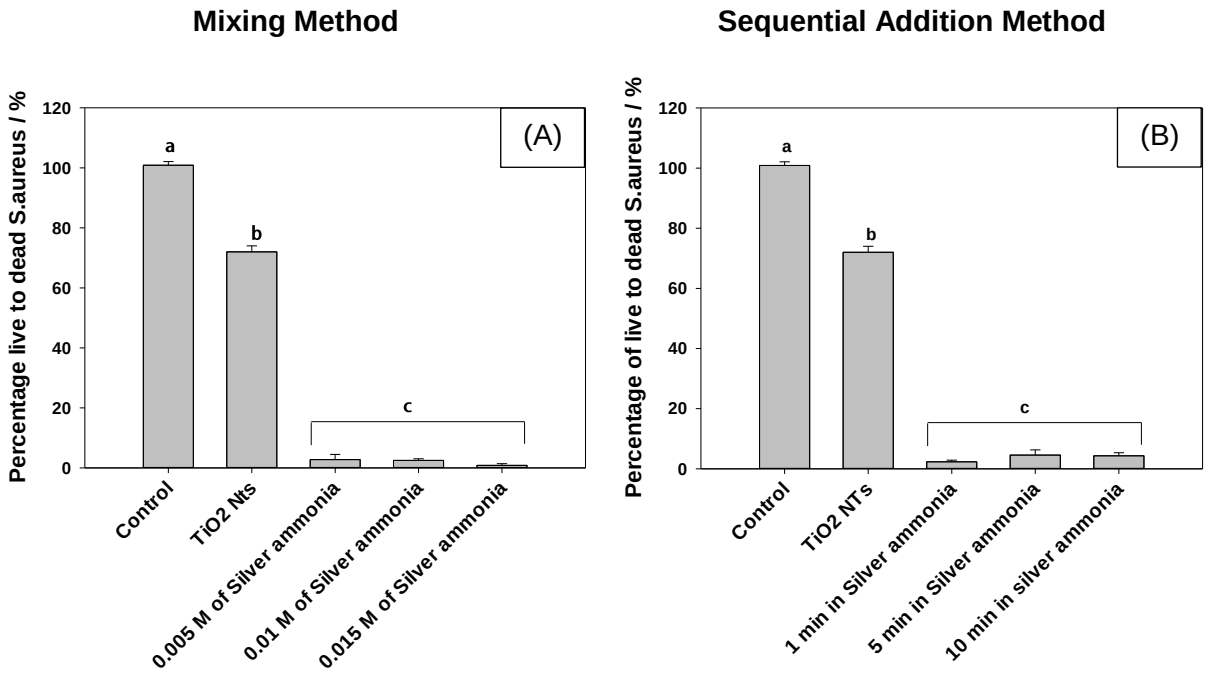

Figure 4: The percentage live to dead bacterial cells after 24 hours exposure of *S. aureus* in BHI broth to the titanium discs prepared using (A) the mixing method and (B) the sequential addition. The control is broth and bacteria only (no titanium disc). The data is presented as mean ± S.E.M., n = 3 replicates. Different letters represent statistically significant differences among the samples within synthesis method (One-way ANOVA, $P < 0.05$, n=3).

Highlights

1. Silver nanoparticles were successfully synthesised on medical grade titanium alloy surface with anodised TiO_2 nanotube honeycomb structure.
2. Exposing the TiO_2 nanotube to the mixture of the silver source and reducing agent would result in the formation of micro-clusters of silver nanoparticles. Nonetheless, allowing $[Ag(NH_3)_2]^+$ to attach to the walls of TiO_2 nanotube first and then reducing with gluconolactone provided a better distribution of nano-clusters of silver nanoparticles.
3. The latter method also resulted in the release of less silver ions in the first 24 hours of exposure to simulated body fluid as compared to the result of the former, therefore latter is more desirable for medical implant.
4. Live/Dead assay was performed on the fabricated coatings and showed that the Ag nanoparticles possess excellent antibacterial properties.