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Mechanical degradation of TiO₂ nanotubes with and without nanoparticulate silver coating

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Abstract

The primary objective of this research was to evaluate the extent of mechanical degradation on TiO₂ nanotubes on Ti with and without nano-particulate silver coating using two different lengths of TiO₂ nanotubes- 300nm and ~1µm, which were fabricated on commercially pure Titanium (cp-Ti) rods using anodization method using two different electrolytic mediums - (1) deionized (DI) water with 1% HF, and (2) ethylene glycol with 1% HF, 0.5 wt%. NH₄F and 10% DI water. Nanotubes fabricated rods were implanted into equine cadaver bone to evaluate mechanical damage at the surface. Silver was electrochemically deposited on these nanotubes and using a release study, silver ion concentrations were measured before and after implantation, followed by surface characterization using a Field Emission Scanning Electron Microscope (FESEM). *In vitro* cell-material interaction study was performed using human fetal osteoblast cells (hFOB) to understand the effect of silver coating using an MTT assay for proliferation and to determine any cytotoxic effect on the cells and to study its biocompatibility. No significant damage due to implantation was observed for nanotubes up to ~1 µm length under current experimental conditions. Cell-materials interaction showed no cytotoxic effects on the cells due to silver coating and anodization of samples.

Keywords

TiO₂ nanotubes; Silver coating; Mechanical damage; Bone implants; Cytotoxicity

1.0 Introduction

Ti and its alloys have gained significant importance in recent years in various fields of biomedical devices where it has been used as dental and orthopedic implants. Ti and its alloys are known for having good mechanical properties, excellent corrosion resistance, good biocompatibility and excellent fatigue resistance (Regoinini et al., 2013, Sepedeh et al., 2012, Das et al., 2007). Titanium being bioinert in nature, the process of osseointegration i.e., direct bonding to the bone can be slow. Thus surface properties of titanium can be

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modified further to improve *in vivo* osseointegration (Das et al., 2007, Das et al., 2009). Various surface modification techniques have been used to modify Ti surface, and growth of *in situ* titanium dioxide (TiO₂) nanotubes on Ti has gained popularity over the years not only in the biomedical field, but also towards solar energy related applications like dye synthesized solar cells (DSSC's), photovoltaic cells, lithium ion batteries and many more (Das et al., 2008a, Jaroenworarluck et al., 2010, Regonini et al., 2010, Shankar et al., 2007, Macak et al., 2008). Various methods such as hydrothermal, sol-gel process, as well as various vapor deposition techniques (Tuli et al., 2011, Li et al., 2013, Mor et al., 2006, Yoriya et al., 2012, Sreekantan et al., 2011, Ortiza et al., 2009) have been used to grow TiO₂ layer on the titanium substrate but an increasingly common approach used over the years is the electrochemical anodization process due to its low set-up cost, ease of operation and flexibility towards growing controlled dimensions of nanotubes.

Surface modification of titanium substrate in the form of growing bioactive TiO₂ nanotube arrays has been shown to facilitate the process of osseointegration and also helps in improving the apatite forming ability (Das et al., 2007, Das et al., 2009, Roy et al., 2012, Fielding et al., 2012, Das et al., 2008b). Apart from the osseointegration, another concern with Ti or other metallic implants is the problem of infection, which may lead to early revision surgery. Formation of biofilm on implant surface is one of the main reasons for implant failure, particularly for wounds related to open fracture. Proper antibiotic measures need to be taken immediately during and after the surgery to prevent infection at the wound site. Use of antimicrobial coatings on medical implants is gaining importance in research in order to mitigate this problem. Antimicrobial elements such as silver or drugs like gentamycin have gained importance for use as coatings (Roy et al., 2012, Fielding et al., 2012). Among them, silver is highly toxic to microorganisms and relatively less toxic to human tissues. Unlike silver, whose use dates back to several hundred years, other commercial antibiotics have met with less success in the commercial market for a broad variety of microorganisms. Also silver coated devices have already received approval by the US Food and Drug Administration (FDA). Apart from being antibiotic, silver is non-cytotoxic when consumed in appropriate doses, and can provide antimicrobial effects for long term applications in implants (Das et al., 2008b, Schierholz et al., 1998, DeVasConCellos et al., 2012, Shin et al., 2011, Chang et al., 2011, Bosetti et al., 2002, Brutel et al., 2009). In recent years, we have shown and patented the idea of a combination treatment of both TiO₂ nanotubes on Ti along with particulate silver deposition (Bandyopadhyay and Bose, 2013). The fundamental idea behind this dual treatment option is to simultaneously enhance osseointegration of Ti implants while improving their infection resistance *in vivo*.

When bone-implant materials are taken into consideration for long term applications, one important attribute that needs considerable attention from clinician's perspective is the surface mechanical properties. TiO₂ nanotubes are known to have lower elastic modulus than the substrate Ti (Melaiye et al., 2005, Xiao et al., 2008). Crawford *et al.* have shown the apparent elastic modulus values of TiO₂ nanotubes with a length of approximately 650nm to be around 36–43 GPa as compared to the substrate which is known to have around 105–110 GPa. Also a relation between the coating thickness and the apparent elastic modulus has been shown where an increase in TiO₂ nanotube thickness further lowers the elastic modulus

(Crawford et al., 2007). Moreover, nanotubes orientation, the extent of damage to nanotubes during surgery, and phase transformation can play an important role towards *in vivo* performance of the implant (Shivaram et al., 2014). Therefore, this study mainly focuses on the aspect of damage evolution when the TiO₂ nanotubes are subjected to implantation *ex vivo*. The primary objectives of our work are to first understand how the nanotubes perform mechanically during surgery and then measure if the nano-particulate silver can adhere to the surface of the TiO₂ nanotubes even after implantation? If the answers are no to either one of those questions, then any such surface treatments may not make sense clinically. We hypothesize that normal implantation process will not damage or degrade TiO₂ nanotubes or adhered silver particles on it. To validate our hypothesis, nano-particulate silver coatings were deposited on the anodized Ti surface, where the adherence of silver was tested via *ex vivo* implantation in an equine cadaver bone followed by silver release study before and after implantation. It should be noted that during this study, a broad range of experiments was performed to optimize various process parameters, which were not discussed unless deemed necessary. Finally, *in vitro* cell-materials interaction of the samples were performed using human fetal osteoblast cells (hFOB) to understand the biocompatibility of anodized surfaces using an MTT assay for proliferation, and SEM images for early stage adhesion to measure the cytotoxic effect of silver coating on anodized surfaces.

2.0 Materials and Methods

2.1 Fabrication and characterization of TiO₂ nanotubes on Ti

Commercially pure Ti (cp-Ti) (President Titanium, MA, USA) rods were used to fabricate TiO₂ nanotubes by anodization. Samples were prepared from the rod which was 15mm in length and 2.5mm in diameter. Samples were ground using silicon carbide paper in increasing grades from 500 to 1000 grit followed by polishing with 1 µm alumina suspension. The samples were cleaned ultrasonically using deionized water and 200 proof ethanol. After ultra-sonication the samples were dried at room temperature. The anodization setup consisted of a beaker where the cp-Ti rod was used as anode suspended from one end, and a platinum cathode was suspended on the other end using platinum wires. A detailed description of the anodization process can be found in one of our previous paper (Shivaram et al., 2014).

2.2 Nanoparticulate silver deposition on Ti rods

Nanotubes fabricated cp-Ti rods were used for electrodeposition. Electrodeposition was performed with an aqueous solution of 0.1M AgNO₃ with platinum as the anode material. A DC power supply (Hewlett Packard 0–60 V/0–50 A, 1000 W) was used to carry out the electrodeposition process. The deposition was performed by applying a DC current between 0.01 and 0.05A keeping the voltage constant at 3V for 45sec at room temperature. After deposition, the excess loose silver was wiped off using a tissue and DI water. Silver coated samples were heat treated at 500°C for 7 min in a furnace in air atmosphere and then allowed to cool naturally at atmospheric conditions. These optimized process parameters were established after several experiments performed with different electrolyte concentrations, electrodeposition time, and heat treatment conditions to achieve strongly adherent silver nano-particulate on cp-Ti rods. Also electrodeposition was performed using

0.05M and 0.2M AgNO₃ solutions which were used as low and high silver concentration solutions, respectively, to obtain an idea how the release kinetics vary with the change in the concentration of electrolyte solution. All other parameters such time, voltage and heating temperature were kept constant.

2.3 Damage and *ex vivo* silver release study in DI water

The Ti anodized rods were used to study the damage of nanotubes with and without silver deposition upon implantation into a cadaver bone. For this damage study, an equine cadaver bone was used and the implantation was done in a similar manner as that of a surgical implantation procedure. First a hole equivalent to the diameter of the rod was drilled into the cadaver bone using a mechanical drilling machine. Then the rod was implanted into the bone ensuring sufficient contact has been made with the bone by hammering it to ensure mechanical abrasion macroscopically. The rod was then removed from the bone and cleaned. For silver release study, samples were then further used for *ex vivo* silver release studies in DI water. A schematic of the implantation process is shown in Fig. 1. Then to understand the release kinetics of silver upon implantation, nano-particulate silver deposited on anodized Ti rods were placed in the DI water, and silver release profile was measured using an atomic absorption spectrophotometer (AAS, Shimadzu AA-6800, Shimadzu, Kyoto, Japan) for a period of 14 days. After the end of each time point, the solution was replaced with fresh DI water. Two sets of silver deposited anodized Ti rods were studied – 1) rods inserted into an equine cadaver bone mimicking surgical procedure related to surface wear and 2) rods without the insertion, which served as a control. Release study of silver ions was also performed by using 0.05M and 0.2M AgNO₃ solutions which were used as low and high silver concentration solutions, respectively, to get an idea how the release kinetics vary with the change in the concentration of electrolyte solution. Both sets of samples were silver deposited under the same condition using all three concentrations. After the completion of the experiments, the collected solution was then analyzed for silver ions content using AAS. Samples were tested in “Flame Mode” using air and acetylene (C₂H₂) fuel, and the data acquisition was performed using commercial Shimadzu WizAard software. The machine was calibrated using Ag⁺ standards (High-Purity Standards, Charleston, SC, USA) of known concentrations from 0 to 5µg/ml. During testing a pre-spray time of 30s and an integration time of 10s were used.

2.4 Contact angle measurements

A sessile drop method was used to measure the contact angles on the surface of the samples using a face contact angle set-up which was equipped with a microscope and a camera. A 0.5–1.0 µl droplet of distilled water was suspended from the microliter syringe tip and the surface of the disc was moved up towards the tip of the syringe to ensure the droplet makes just enough contact with the surface of the disc. Images were then collected with the help of a camera and the contact angle was measure between the drop and the substrate using the magnified image collected from the computer. For each anodized condition, two samples were used to collect the contact angle data in water. Then from each sample an average of six measurements were used to calculate the contact angle. Diiodomethane was used as the apolar liquid whereas glycerol and formamide were used as the polar liquids in the following equation (1) to calculate the surface energy.

$$\gamma_L(1+\cos\theta)=2(\gamma_s^{LW}\gamma_L^{LW})^{1/2}+2(\gamma_s^+\gamma_L^-)^{1/2}+2(\gamma_s^-\gamma_L^+)^{1/2} \quad (1)$$

In Eq. (1), θ is the contact angle of liquid L and solid S, γ^{LW} is the apolar component of the surface energy, γ^+ is the Lewis acid component (electron acceptor) and γ^- is the Lewis base component (electron donor) (Van Oss et al., 1990, Das et al., 2007).

2.5 *In vitro* cell-materials interaction study

For cell culture, circular disc samples 12.5mm in diameter and 3 mm in thickness were used to study the bone cell-material interactions using human fetal osteoblast (hFOB) cells. Only one anodization composition using the HF electrolyte was used for the *in vitro* studies. Anodization and silver deposition were performed in the same manner as mentioned in the previous sections. Sterilization was performed on all samples by means of autoclaving at 121 °C for 30 min. Human osteoblast cell line hFOB 1.19 (ATCC, Manassas, VA) was used in this study. Cells were seeded onto the disc shaped samples placed in 24 well plates. A 1:1 mixture of Ham's F12 Medium and Dulbecco's Modified Eagle's Medium (DMEM/F12, Sigma, St. Louis, MO), with 2.5 mM L-glutamine (without phenol red) was used as the base medium for this particular cell line. The medium was added with 10% fetal bovine serum (HyClone, Logan, UT) along with 0.3 mg/ml G418 (Sigma) and the cultures were kept at 34 °C under an atmospheric condition of 5% CO₂. It was ensured that during the duration of the experiment the medium for all samples was changed every 2 days.

To evaluate the cell proliferation, MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay was used. A 5 mg/ml MTT (Sigma) solution was prepared by dissolving MTT in PBS followed by filter-sterilization using a filter paper of 0.2 µm pore. Dilution of MTT was performed by adding 100 µl of MTT into 900 µl DMEM/F12 medium. Then 1 ml of diluted MTT solution was then added to each sample in 24 well plates which were kept for incubation for a period of 2hrs for the formation of formazan crystals. After incubation, 1 ml of solubilization solution which consisted of 10% Triton X-100, 0.1 N HCl, and isopropanol was added to dissolve the formazan crystals. One hundred microliters of the resulting supernatant was transferred into a 96 well plate in triplicate and read by a microplate reader at 570 nm. 4 and 7 days MTT assay was performed to evaluate the cell proliferation and see if any cytotoxic effects resulted after 7 days.

All the SEM samples were fixed using 2% paraformaldehyde/ 2% glutaraldehyde in 0.1 M cacodylate buffer overnight at 4 °C. Post-fixation was performed using 2% osmium tetroxide (OsO₄) for 24h at 4 °C. Dehydration to the fixed samples was performed in a series of ethanol (30%, 50%, 70%, 95% and 100% three times for each concentration respectively) and was followed by a drying procedure using hexamethyldisilane (HMDS). After drying, the samples were gold coated before being observed under FESEM.

3.0 Results

3.1 Surface morphology of TiO₂ nanotubes with and without silver

Fig 2a shows the scanning electron microscopy (SEM) images of first set of nanotubes anodized in DI water (with 1vol% HF) based electrolyte at 20V for 45 mins. The nanotubes

obtained using this electrolyte were $300 \pm 25\text{nm}$ in length and $100 \pm 20\text{nm}$ in diameter. Fig 2b shows the SEM images of second set of nanotubes anodized in an ethylene glycol based electrolyte (1vol. % HF, 0.5 wt. % NH_4F , 10vol. % DI water) which were anodized at 40V for 60mins to get longer length of nanotubes and the resulting nanotubes were $950 \pm 65\text{nm}$ long and $100 \pm 15\text{nm}$ in diameter. The diameter and length of nanotubes were calculated using the ImageJ software. Fig 2a shows the SEM image indicating low and high magnification (inset) images of TiO_2 nanotubes grown in HF medium and similarly Fig 2b shows the SEM images of nanotubes grown in ethylene glycol (EG) medium (Shivaram et al., 2014).

Fig 3a and 3b shows the SEM images of resulting silver deposition in nanoparticle range on both sets of nanotubes. The deposition resulted in particles from micrometer to nanometer range, but care was taken to ensure the loosely adhered particles were removed and cleaned using DI water to give the final deposition in nanometer range.

3.2 Damage and silver release study

The TiO_2 nanotubes fabricated Ti rods with and without silver were implanted into the equine cadaver bone to study the damage to the nanotubes during implantation. Implantation was performed *ex vivo* in a manner similar to the surgical implantation process. Fig 4a and 4b show the SEM images of nanotubes with 300nm length before and after implantation whereas Fig 5a and 5b show the ones with 950nm length before and after implantation. Both sets of images represent nanotubes without silver coating and show no significant damage or change in the morphology of nanotubes under current conditions before and after the implantation, which signifies the strength and damage resistance of these nanotubes which it can endure during this implantation for a length of approximately 1 micron. Also no signs of delamination of the coating or any macroscopic damage were observed due to implantation. Fig 6a and 6b show SEM images of nanotubes with 300nm length with silver coating before and after implantation. Fig 7a and 7b show the similar set of SEM images of nanotubes with 950nm length with silver coating before and after implantation.

Silver was deposited on Ti rods anodized using both conditions and were implanted into an equine cadaver bone to study the release of silver ions before and after implantation. Release study of silver ions were performed in DI water for a period of 14 days and cumulative release profiles were plotted for all concentrations as shown in Fig 8 and Fig 9. Also from Fig 10a and 10b, presence of silver can be seen even after 14 days, which signifies the importance of good adhesion of silver particles to the surface. Release profiles of silver ions for all concentrations were plotted where the ones without implantation into the cadaver bone acted as control with respect to the ones which were implanted *ex vivo*. No significant changes were seen in any release profiles and the total cumulative release was well below the potential toxic limit of 10 ppm ($\mu\text{g/ml}$) mentioned for the human cells (DeVasConCellos et al., 2012).

3.3 Contact angle and surface energy measurements

Contact angles and surface energies were measured for samples anodized under both conditions, keeping polished Ti surface as the control sample. Table 1 shows the contact

angle and surface energy data for all samples. Contact angles of anodized samples are lower compared to the control sample, which results in higher surface energy for treated samples compared to the control sample, an indication of better wettability due to anodization. Better wettability and higher surface energy also plays an important role to improve cell-materials interactions.

3.3 *In vitro* cell-materials interaction study

In our previous work, we have already shown that silver deposited metal surfaces can effectively eliminate bacterial colonies (Das et al., 2008b, DeVasConCellos et al., 2012). Therefore, our goal in this work was only limited to show that these surfaces are still biocompatible even after silver loading and do not induce any cytotoxicity. Cell proliferation was determined using the hFOB cell line via MTT assay to measure any cytotoxic effect on the cells due to surface modification. Cell proliferation was determined for the 4 and 7 days for samples anodized in HF electrolyte keeping non anodized cp-Ti surfaces as a control. It can be noticed from the MTT results from Fig 11 that there is an increase in the cell density on anodized and silver coated samples as compared to the control samples. For simplicity, control samples without nanotubes and silver coating were named as Ti-control and samples anodized in HF medium and with silver coating were named as NT-HF and NT-HF-Ag, respectively. Results from Fig 11 indicate no cytotoxic effect due to the surface modifications. Cell spreading for all anodized samples with respect to Ti-control surface was similar ($p < 0.001$ -extremely significant) except for NT-HF anodized sample with respect to Ti-control sample for 7 days where significant difference ($p^{**} < 0.01$) was observed. Fig 12 shows the SEM images of cell morphologies for anodized, silver coated and control samples after 4 and 7 days of cell culture. The images show better cell attachment for anodized samples and silver coated samples as compared to the control samples. Also the images show good cell adhesion with filopodia extensions coming out of cells to grasp the surface and presence of good amount of extracellular matrix (ECM) can also be seen. The NT-HF samples showed presence of abundant cells as compared to the control samples on day 7 along with good cell proliferation, and cell adhesion. The NT-HF-Ag samples as compared to NT-HF samples showed an increase in cell density from day 4 to day 7. Also, all cells showed an elongated morphology with presence of small calcified nodules mostly on Ti-control samples (Das et al., 2007, Roy et al., 2012, Fielding et al., 2012).

4.0 Discussions

4.1 Nanotube formation

The formation of nanotubes in a fluoride based electrolyte takes place as a result off three simultaneously processes: (1) field assisted oxidation of Ti metal to form titanium dioxide, (2) field assisted dissolution of Ti metal ions in the electrolyte, and (3) chemical dissolution of Ti and TiO₂ due to etching by fluoride ions (Jaroenworarluck et al., 2010, Regonini et al., 2010, Shankar et al., 2007, Mor et al., 2006). The above three processes can be described by the following reactions:

Based on the formation mechanisms in fluoride based electrolytes, two different electrolytes were used for obtaining two different lengths of nanotubes. Lower water content in the electrolyte yields in longer length nanotubes (Li et al., 2013, Mor et al., 2006). Keeping this in mind the second electrolyte in an ethylene glycol based medium for used with a view to obtain nanotubes with longer length nanotubes keeping the diameter fairly constant. Several electrolytes were experimented during this study. Fig 2a and 2b shows the nanotube dimensions using respective electrolytes with two different lengths.

4.2 Degradation study of nanotubes

TiO₂ fabricated rods were implanted into the equine cadaver bone in the manner mentioned above thereby mimicking the surgical implantation procedure. As seen from Fig 4 and Fig 5, no noticeable signs of degradation or damage were seen on the nanotubes under the current experimental conditions, i.e., no signs of nanotube collapsing or change in nanotube diameter or delamination of nanotubes along with no signs of smudging or smearing of nanotubes. Previous studies have shown certain mechanical properties of TiO₂ with quantitative analysis focusing on elastic modulus and hardness using Nano indentation or atomic force microscopy which was not the objective of this study (Crawford et al., 2007, Shokuhfar et al., 2009). Emphasis has not been given on quantitative analysis of mechanical stability of nanotubes but to show the response of nanotubes and the extent of damage it undergoes upon implantation as the mechanical stability of nanotubes is one of the most important aspects for clinical application of these coatings.

4.3 Adherence of nano-particulate silver coating upon implantation

Similar implantation study was performed with nano-particulate silver coatings to show the importance of adherence of the coating on the nanotubes upon implantation. Fig 6 and Fig 7 convey that message where no damage or complete loss of silver particles can be seen under the current experimental conditions. Another purpose of silver based antimicrobial coating is the ability for long term release of silver ions from the implant surface for infection control to minimize the possibility of revision surgery due to infection. Various methods have been used by researchers to show particulate coating or doping with antimicrobial elements. Similar measures were sought in previous studies by our group as well (Das et al., 2007, Das et al., 2009, Roy et al., 2012, Fielding et al., 2012, das et al., 2008b). A possible disadvantage of particulate coating could be the release of loosely adhered silver particles into the body in one time during surgery itself, which could potentially lead to cytotoxic effects. In this study, we have focused on mechanical degradation of nano-particulate silver coating during surgical procedure. We hypothesize that silver will strongly adhere to the implant surface during surgical procedure and provide antimicrobial treatment *in vivo*. To test the adherence of the coating, an *ex vivo* release study was performed where anodized Ti surface was coated with silver particles and then implanted into an equine cadaver bone. A similar sample without implantation served as a control. The release study was performed in DI water and as seen from Fig 8 and Fig 9. The cumulative release profiles suggest the total release of silver was well below the toxic limit. To study the effect of varying concentration of electrolyte, the samples were deposited with a higher and lower concentration of Silver, followed by similar release study. In all cases, the total release was always below the toxic limit (DeVasConCellos et al., 2012, Shin et al., 2011, Chang et al., 2011, Bosetti et al., 2002,

Brutel et al., 2000). Also no change in the release profile was observed due to implantation. A no observable adverse effect of silver up to 10 gm for a normal human being has been recommended by World Health Organization (WHO) (Brutel et al., 2000). From Fig 10a and b confirm the presence of silver on the surface of nanotubes even after 14 days. These results show good adhesion of the silver particles to the TiO₂ nanotube surface.

4.4 Biocompatibility and cytotoxicity study of nano-particulate silver coating

Once the proper adherence of nanotubes and coating was tested, focus was shifted towards the biocompatibility and cytotoxic effects of the coating and nanotubes. Although the silver release was well below the human toxic limit, biocompatibility and cell-material interaction were also important aspects. The biocompatibility and cell-material interaction were tested using an osteoblast cell line which is specific to surface attachment i.e., they initially attach to the surface and then proliferate. This attachment is governed by various surface factors like roughness, surface morphology and wettability. MTT results, as seen from Fig 11 show no cytotoxic effects due the formation of nanotubes and silver coating as cell densities of all samples are higher than the control sample. Cell proliferation was also confirmed from day 7 data compared to day 4 cell numbers. Surface properties of the material play an important role in cell proliferation, cell adhesion and its morphology (Das et al., 2007, Das et al., 2009 Das et al., 2008a). Smoother surfaces will result in lower surface attachment as compared to surfaces that are modified to induce roughness. Contact angles were measured for all samples in distilled water and shown in table 1. Anodized surfaces result in lower contact angle and in turn show better cellular attachment as compared to control samples. The changes in the contact angle between the anodized surfaces can be attributed to the orientation of nanotubes with a compact orientation as seen in 300nm nanotubes resulted in even lower contact angle (Shin et al., 2011). Keeping this in mind, contact angle and surface energy were measured only for nanotubes with 300nm length with silver coating and it can be seen, from Table 1, that the coating of anodized surfaces resulted in further decrease in the contact angle and thereby increasing the surface energy i.e., the wettability of the surface. It has been learnt that the hydrophilic nature of the surface can be influenced by annealing due to a change in morphology and phase stability (Shin et al., 2011). These hydrophilic natures of anodized surfaces play a crucial role to cell-material interactions. Hydrophilicity and hydrophobicity nature of surface influence the cellular adhesion. SEM images also reveal a good cellular attachment and proliferation of cells in the hydrophilic anodized surface as compared to hydrophobic Ti-control surface. Increase in surface wettability and surface energy also help in spreading of cells which also results in better proliferation as seen in Fig 12 (Fielding et al., 2012, Das et al., 2008b). The cells were nicely attached to surface with the filopodia extensions hold on to the surface in both anodized and silver coated samples as compared to the Ti-control sample. Increase in cell density and better attachment of cells to the surface can be observed for all samples on day 7 than on day 4. Also no cell death as such has been observed on any sample during both the time points. Concerns have been expressed with the use of silver as antimicrobial agent and this study helps in addressing this concern by showing no cytotoxic effect as on the cells due to the use of silver (Fielding et al., 2012, Das et al., 2008b, Schierholz et al., 1998, DeVasConCellos et al., 2012, Chang et al., 2011, Bosetti et al., 2002, Brutel et al., 2000, Xiao et al., 2008). The cell morphology, cell adhesion and cellular attachment, everything concerning cell-material

interaction has been addressed in this study with a positive effect over the use of silver as a nano-particulate coating on anodized surface of Ti (Xiuyong et al., 2013, Agata et al., 2012, Necula et al., 2009, Lingzhou et al., 2011, Li et al., 2014).

5.0 Conclusions

This work reports the damage study of TiO₂ nanotubes with two different lengths grown using two different electrolytes along with the cell-materials interaction at the surface with and without silver coating. TiO₂ nanotubes were grown on Ti substrate by anodization method and nano-particulate silver coating was electrodeposited using 0.1M AgNO₃ solution. Damage study on both sets of TiO₂ nanotubes with and without silver coating was performed by implanting it into an equine cadaver bone. No significant damage due to implantation was observed for nanotubes up to ~1 µm length under current experimental conditions and the presence of silver was seen even after implantation, which signifies the adherence of both nanotubes and the coating. Also an *ex vivo* silver release study was performed to measure the release of silver ions before and after implantation. Silver release data confirmed the presence of silver ions before and after implantation, which was also below the toxic limit. Cell-materials interaction showed no cytotoxic effects on the hFOB cells due to silver particulate coating. Also all anodized samples resulted in having a lower contact angle and in turn a higher surface energy as compared to the control samples, which played a role to enhance cellular attachment and proliferation *in vitro*. Our results show that anodized TiO₂ nanotubes on Ti are mechanically stable for clinical applications. Our results further confirm that addition of silver particles on TiO₂ nanotubes is also mechanically stable without any cytotoxic effects *in vitro*.

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Highlights

- Understanding mechanical degradation on TiO₂ nanotubes.
- Influence of mechanical degradation on silver release in vitro.
- Influence of silver particles on bone cell-materials interactions.

Figure 1.
Schematic representation of the implantation procedure using nanotubes fabricated rods.

Figure 2.

(a) SEM image showing the low and high magnification image of TiO_2 nanotubes anodized using DI water based electrolyte (~300nm long). (b) SEM image showing the low and high magnification image of TiO_2 nanotubes anodized using ethylene glycol medium (~950nm long) [Reproduced with permission from Reference 29].

Figure 3.
SEM Images showing the nanoparticulate silver coating on TiO_2 nanotubes with two different lengths (a) For 300nm length and (b) for 950 nm length nanotubes.

Figure 4. SEM Images of (a) Before Insertion and (b) After Insertion of TiO_2 nanotubes with 300nm length into the cadaver bone showing no significant damages of the nanotubes due to implantation.

Figure 5. SEM Images of (a) before and (b) after implantation of TiO_2 nanotubes with 950nm length into the cadaver bone showing no significant damages of the nanotubes due to implantation.

Figure 6. SEM images of nanoparticulate silver coating on TiO₂ nanotubes of 300nm length (a) before and (b) after implantation into the cadaver bone showing the presence of silver even after implantation.

Figure 7. SEM images of nanoparticulate silver coating on TiO₂ nanotubes of 950nm length (a) before and (b) after implantation into the cadaver bone showing the presence of silver even after implantation and also no significant damage to the nanotubes.

Figure 8. Cumulative silver ions release profile with deposition of silver on 300 nm length nanotubes performed using varying concentration of electrolytes and showing the cumulative release being well below the toxic limit.

Figure 9. Cumulative silver ions release profile with deposition of silver on 950 nm length nanotubes performed using varying concentration of electrolytes and showing the cumulative release being well below the toxic limit.

Figure 10.
SEM Images of both set of nanotubes (a) 300nm length and (b) 950 nm length showing presence of silver particles after 14 days release study.\

Figure 11.

Optical density values measured using MTT Assay after 4 and 7 days of culture at a wavelength of 570nm by the reader. The cell density is significantly different for anodized samples when compared to Ti-control samples denoting negligible cytotoxicity. $p^* < 0.001$ and $p^{**} < 0.01$.

Day 4

Day 7

Figure 12.

SEM images showing a comparison of cell morphology for anodized and control samples at time point's day 4 (a, b, c) & 7 (c, d, e) respectively.

Contact angle and surface energy measurements for samples with 300nm and 950nm length nanotubes keeping polished Ti surface as control.

Table 1

Sample	Contact Angle (Degrees)				Surface Energy (mJ/m ²)
	DI Water	Diiodomethane	Formamide	Glycerol	
TiO ₂ nanotubes with 300 nm length	18.1±1.8	33.7±1.7	23.9±1.4	39±1.1	78±1.09
TiO ₂ nanotubes with silver (300 nm)	14.7±2.3	27.9±1.1	17.6±1.5	35.8±0.3	92.43±1.5
TiO ₂ nanotubes with 950 nm length	22.2±1.5	36±0.8	24.5±1.3	40.1±0.9	75.76±2.81
Ti-control	59.3±3.7	40.5±1.7	29.1±1.5	41.3±2.1	69.53 ±2.12