Effects of titanium nanotubes on the osseointegration, cell differentiation, mineralisation and antibacterial properties of orthopaedic implant surfaces

The development and pre-clinical evaluation of nano-texturised, biomimetic, surfaces of titanium (Ti) implants treated with titanium dioxide (TiO2) nanotube arrays is reviewed. In vitro and in vivo evaluations show that TiO2 nanotubes on Ti surfaces positively affect the osseointegration, cell differentiation, mineralisation, and anti-microbial properties. This surface treatment can be superimposed onto existing macro and micro porous Ti implants creating a surface texture that also interacts with cells at the nano level. Histology and mechanical pull-out testing of specimens in rabbits indicate that TiO2 nanotubes improves bone bonding nine-fold (p = 0.008). The rate of mineralisation associated with TiO2 nanotube surfaces is about three times that of non-treated Ti surfaces. In addition to improved osseointegration properties, TiO2 nanotubes reduce the initial adhesion and colonisation of Staphylococcus epidermidis. Collectively, the properties of Ti implant surfaces enhanced with TiO2 nanotubes show great promise.

Over the past six decades, the surface technologies of implants have progressed from bioinert surfaces such as porous Ti and tantalum, to bioactive surfaces such as plasma-sprayed hydroxyapatite and other ceramics, to the most recent and probable future generation of biomimetic engineered, nano-texturised surfaces such as Ti treated with Ti oxide (TiO2) nanotube arrays. These surfaces mimic the nano-morphology of the external cellular surfaces of the osteoblasts that surround the implant. A substantially increased surface area has unique chemical characteristics provided by the nanotubes, allowing increased interaction between the surface of the implant and adjacent cells. Nano-surface mechanisms increase the rate of initial osseointegration between Ti alloys and the surrounding tissue, greatly increasing the strength of the bond between implant and bone.

Nano-technology is the control of matter at a scale of approximately 1 to 100 nanometres (nm), where novel properties and function occur because of size. Thus, for the surface of an implant truly to possess nano-technology, aspects of shape and structure must be formed at the nano-scale which specifically enhance the properties of the implant. The impact that nano-texturising has on the interactions of the surface of the implant can be illustrated by imagining the macro, micro, and nano-surface area of a 1 cm × 1 cm × 1 cm cubic implant, which has 6 cm² of macro surface area (about the size of a sugar cube), as shown in Figure 1. If such an implant is divided into 1 mm cubes, the total surface area increases to 60 cm². If it is further divided into 1 nm cubes, the surface area increases to 60 000 000 cm², equivalent to approximately 1.5 acres of surface area. Likewise, nano-texturing an implant with features such as tubes that have both internal and external surface areas greatly increases the
area available for osseointegration between the nano-scale features of the tubes and those of the cells.

TiO₂ nanotube arrays are formed on Ti surfaces through a specialised efficient anodisation process that is followed by heat treatment. The arrays consist of rows of vertically aligned nanotubes with engineered and reproducible internal diameters, outer diameters, and lengths, resulting in a nano-textured surface of nanotubes that has defined, non-random physical and chemical properties. In order to treat a Ti implant, it is first immersed in a fluoride-rich electrolyte. As a voltage is applied, a thicker compact layer of TiO₂ develops on the outer surface. Then, during an extended anodisation process of approximately ten minutes to 45 minutes, depending on the volume and surface area of the implant, billions of vertically aligned nanotubes are integrated into the outer TiO₂ layer, creating a surface of nano-texturised TiO₂ nanotubes. After anodisation, the surface is heat treated to 500°C for two hours to convert the TiO₂ nanotubes from an amorphous phase to an anatase phase. This post-anodisation heat treatment improves both the toughness of the nanotubes and their osseointegration potential. The resultant surface is not a coating but a transformed TiO₂ surface, which not only greatly increases the available surface area for processes such as cell adhesion, but also provides a nano-texture that interacts with outer cell membrane surfaces. Such TiO₂ nano-texturised surfaces exhibit tensile and shear adhesion strengths that exceed the typical loads experienced during the introduction of implants, their use and their removal.

The anodisation process can be adjusted to create nano-texturised surfaces of vertically aligned nanotubes with accurately controlled pore diameters. For example, specimens can be made with a mean outside diameter of the nanotube of 30 nm (SD 10 nm), 70 nm (SD 10 nm), or 100 nm (SD 10 nm), and with specific thicknesses and heights of the walls. A nano-texturised surface with a mean diameter of the nanotube of 100 nm has a high hydrophilicity when compared with a non-nano-texturised Ti surface or a nano-texturised surface with a mean diameter of 30 nm. The hydrophilicity of the surface of a nanotube array is inversely related to the diameter of the tube. The higher the hydrophilicity, the more absorbent the surface and the smaller the contact angle surrounding liquid on the surface.

The formation of vertically aligned TiO₂ nanotubes on the surface of a Ti implant can positively affect the osseointegration, cell differentiation, mineralisation, and antimicrobial properties. Figure 2 shows an example of a tibial tray that incorporates macro features, micro porosity, and nano-texturing to enhance fixation.

We present a review of recent research to summarise the outcomes that can be achieved by the creation of vertically aligned TiO₂ nanotubes on a Ti implant surface.

**Osseointegration and cell differentiation**

Oh et al. studied the in vitro behaviour of osteoblasts cultured on vertically aligned TiO₂ nanotubes and investigated the effect of such a nano-structure on the morphology of osteoblasts and the kinetics of cell proliferation. A layer of vertically aligned TiO₂ nanotubes on a Ti surface was created by anodisation, and MC3T3-E1 mouse osteoblast cells were seeded on the experimental substrate and on a control substrate of pure non-texturised Ti. The presence of nanotubes positively affected the adhesion and propagation of the osteoblasts, with the
filopodia of the growing cells spreading across the pores of the nanotube arrays, producing an interlocked cell structure. With the passage of time, the number of adhered cells on the TiO₂ nanotubes increased significantly by approximately 300% to 400% compared with the number of cells adhering to the Ti metal surface. This effect is most likely to be due to the increased surface area, the increased hydrophilicity, and the unique topography of nanotubes that increases the negative charge on the outer rim of the tubes.¹⁹

Gongadze et al.²⁰ have suggested that the attraction between the negatively charged Ti surface and a negatively charged osteoblast is mediated by charged proteins with a distinctive distribution of quadrupolar internal charge. Similarly, cation-mediated attraction between fibronectin molecules and the Ti surface is expected to be more efficient for a high surface charge density, resulting in integrin mediated osteoblast adhesion. The osteoblasts are most strongly bound along the sharp convex edges of the surfaces of the TiO₂ nanotube where the magnitude of the density of the negative surface charge is the highest. A vertically aligned nanotube configuration may be a useful route for accelerating the proliferation of various other types of cells in addition to osteoblasts.¹⁷

As described above, the pore diameter of TiO₂ nanotubes can be controlled by adjusting the potential during anodisation. In an in vitro examination of human mesenchymal stem cells (hMSC), Oh et al.¹⁵ created TiO₂ nanotubes with pore diameters ranging from 30 nm to 100 nm. Varying the pore diameters of nano-tubular-shaped TiO₂ surface structures independently allowed either augmented hMSC adhesion or a specific differentiation of hMSCs into osteoblasts by using only the geometric cues, without the introduction of osteogenic-inducing media. The behaviour of hMSC in response to the varied sizes of nanotubes revealed a significant change in a relatively narrow range of sizes. As shown in Figure 3, small (~30 nm diameter) nanotubes promoted adhesion without noticeable differentiation, whereas larger (~70 nm to 100 nm diameter) nanotubes elicited a dramatic elongation of
stem cells (~10-fold increase), which induced cytoskeletal stress and selective differentiation into osteoblast-like cells.

Similarly, osteoblasts can exhibit increased elongation when cultured on TiO₂ nanotubes of increasing diameter. Brammer¹⁸ prepared TiO₂ nanotubes of 30 nm, 50 nm, 70 nm and 100 nm pore diameter on Ti substrates by anodisation, and seeded the substrates with MC3T3-E1 mouse osteoblasts to investigate cellular behaviour in response to the different sizes of nanotube. They observed that a change in osteoblast behaviour was obtained in a relatively narrow range of nanotube dimensions; those with a small diameter (~30 nm) stimulated the highest degree of osteoblast adhesion, while those with a larger diameter (70 nm to 100 nm) stimulated a lower population of cells with extremely elongated cellular morphology and much higher levels of alkaline phosphatase, as shown in Figure 4. Increased elongation of nuclei is also seen with larger diameter nanotubes.¹⁸

The rate of cell adhesion may be significantly increased on nano-textured surfaces. Peng et al²¹ found, in an in vitro study comparing the cellular response with different textured surfaces, that the adhesion of C3H10T1/2 mouse cells on the surface of Ti specimens treated with TiO₂ nanotube arrays, 30 nm or 80 nm in diameter, was significantly enhanced when compared with control samples cultured on polished Ti and acid-etched Ti. A surface analysis of the four groups (group one, polished Ti; group two, acid etched Ti; group three, nanotubes with a mean diameter of 80 nm; and group four, nanotubes with a mean diameter of 30 nm) identified increased surface roughness, decreased water contact angles and an enhanced concentration of oxygen and fluorine atoms on the surfaces of TiO₂ nanotubes. The density on the 30 nm and 80 nm TiO₂ nanotube arrays was found to be higher than that on the mechanically polished and acid-etched Ti sheets (p < 0.01), and the cell density on the 80 nm TiO₂ nanotube arrays was markedly higher than that of the other groups at two, eight, 12, and 24 hours. The authors concluded that the surface of a TiO₂ nanotube can reduce bacterial colonisation (as described below) and enhance C3H10T1/2 cell adhesion. Many physical and chemical properties of the surface of a TiO₂ nanotube may contribute to these effects.

Adhesion strength, or implant-bone bonding, was measured in an in vivo study comparing the surface of a TiO₂ nanotube implant with the surface of a Ti grit-blasted implant. Bjursten et al⁷ implanted discs with TiO₂ nanotube surfaces and control discs with Ti grit-blasted surfaces in compression on the flat cortical bone non-load-bearing surface of the proximal anterior tibias of rabbits. Weight-bearing was allowed immediately following surgery. The grit blasted surfaces had a micro-surface roughness with features approximately 6 μm long by approximately 2 μm deep and an outer layer of approximately 5 nm of amorphous TiO₂. The TiO₂ nanotube treated implants had an approximately 250 nm to 300 nm thick layer of anatase TiO₂ nanotubes that were 100 nm outer diameter by 80 nm inner diameter formed by anodisation. After removing the tibial periosteum in the area of attachment of the implant, and reaming the exposed bone surface flat with a circular reamer, the implants were held in compression by Teflon (DuPont Co., Wilmington, Delaware) coverings that were installed over the disc-shaped implants to hold them securely.

After four weeks, the coverings were removed, and screws were attached to the discs in harvested necropsy samples and pulled slowly at 0.1 mm/sec to measure adhesion strength in pure tension. Pull-out testing indicated that the strength of bonding of the TiO₂ nanotubes improved by nine-fold (p = 0.008) compared with the blasted micro-textured surface (mean 10.8 N (SD 3.1 N) vs mean 1.2 N (SD 2.7 N)), as shown in Figure 5. Furthermore, the TiO₂ nanotube implants showed significantly higher bone-implant contact area (mean 78.3% (SD 33.3)) compared with the micro-textured implants (mean 21.7% (SD 24.7)). Histological analysis, illustrated in Figure 6, confirmed greater bone-implant contact area, new bone formation and increased levels of calcium and phosphorus on the surfaces of the nanotubes compared with the micro-textured implants. Energy dispersive radiograph mapping of the interface after tensile testing indicated a higher surface area and increased calcium and phosphorus on the TiO₂ nanotube surfaces compared with the blasted implant surfaces. The percentage of surface area covered by calcium and phosphorous, which is indicative of strong

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**Fig. 4**

Scanning electron microscope micrographs of osteoblasts (which appear dark) on (left to right) flat titanium (Ti) and 30 nm, 50 nm, 70 nm, 100 nm diameter Ti dioxide (TiO₂) nanotube surfaces after 24 hours of incubation. The arrows indicate strikingly long cellular extensions across the substrate on the 100 nm nanotubes. Red brackets show increased cellular elongation on the larger ~70 nm to 100 nm diameter nanotubes. Flat and more rounded cells are shown on Ti and 30 nm to 50 nm TiO₂ nanotube surfaces.
osseointegration, was approximately 41.7% on TiO₂ nanotube surfaces versus only 8.3% for the micro-textured surfaces. The bond between newly formed bone and the surfaces of the TiO₂ nanotubes was so strong that fracture occurred within the growing bone rather than at the implant-bone interface.

A nano-surfaced material in the range below 100 nm more closely mimics the natural hydroxyapatite and collagen constituents of bone than a micro-scale textured surface alone. The nano-topography of the TiO₂ nanotubes more closely resembles the porous structure of native bone, allowing for more optimal interactions for osteogenesis. As protein adsorption on the surface occurs first on implantation, the nanotubes provide a more favourable structure to attract proteins, such as vitronectin and fibronectin, which promote the adhesion of osteoblasts onto the surface of the implant. Nanotubes improve adhesion and proliferation for osteoblasts through their improved focal adhesion. Thus, one of the major benefits of nano-surfacing seems to be the initial enhancement of osteoblast attachment and accelerated osseointegration.

When sufficient time is allowed without micro-movement between the implant and bone, an implant surface without nano-structure eventually seems to catch up, although at a slower rate, and produces a comparable degree of bone formation with a nano-textured surface. However, the quality of osseointegration and bone bonding may still not match that of a nano-textured surface.

Ti nanotubes may also be formed on Ti coatings applied to cobalt chrome (CoCr) implants. Figure 7 shows in vitro osteoblast formation on a CoCr implant with a Ti coating and TiO₂ nanotubes after 24 hours. Similar to TiO₂ nanotubes on solid Ti implants, the osteoblasts are elongated, with prominent filopodia. These results are like the results seen in Figure 4 (osteoblast cells on TiO₂ nanotubes on a Ti implant). In addition, the cells may show large focal adhesions, which are vital for proper cell function.

**Mineralisation**

Bone mineralisation occurs when an inorganic substance, such as calcium or phosphorus, precipitates in an organic matrix such as an osteoblast. As reported by Frandsen et al, TiO₂ surfaces with Ti nanotubes show an increased rate of mineralisation compared with a pure Ti surface. In this *in vitro* study, the behaviour of human osteoblast cells on TiO₂ nanotubes and tantalum (Ta) coated TiO₂ nanotube surfaces of nearly identical nano-topography were compared to assess the effect of changes in surface characteristics due to a Ta coating alone. Although the rate of mineralisation of the surface of a TiO₂ nanotube array is about three times faster than that of Ti micro-textured by blasting, and about twice as fast as micro-textured Ta, the rate can be further enhanced by adding nano-particles of Ta to the nanotubes. The ‘osteofunctionality’ was enhanced on the Ta surface as measured by alkaline phosphatase activity, bone nodule formation, and the deposition of matrix minerals. The Ta surface promoted an approximately 30% faster rate of mineralisation and bone-nodule formation compared with the results on bare TiO₂ nanotubes. Table I
and Figure 8 compare the rates of mineralisation for phosphorus and calcium on Ti and Ta substrates, on TiO\textsubscript{2} surfaces with Ti nanotubes and on Ti nanotubes further enhanced with a Ta nano-particle coating. The Ta enhanced nanotubes provide an almost four-fold increase in the rate compared with the micro-texturised Ti surfaces used in most implants today. These findings enhance our understanding of cell behaviour in response to subtle
alterations in nanostructure and surface chemistry and offer further insights into the potential for compelling manipulation of biomaterial surfaces.

Antimicrobial properties

TiO2 nanotube arrays on Ti implants have shown the additional benefit of antimicrobial capabilities. Peng et al,21 in combination with the increased cell adhesion reported above, found in an in vitro evaluation that the inclusion of TiO2 nanotubes on Ti surfaces reduced the initial adhesion and colonisation of Staphylococcus epidermidis compared with polished or acid-etched Ti surfaces. Bacterial colonisation on nanotubes decreased significantly from the colonisation present on polished Ti or acid-etched Ti. The nanotubes with the larger mean diameter (80 nm) had the highest antimicrobial effect. This effect, along with the increased cell adhesion reported above, may be due to many factors, including both physical characteristics and chemical composition. It is hypothesised that the negative charge of the hydrophilic nanotube surface attaches to positively charged osteoblasts while the same surface repels the negatively charged microbes, reducing the build-up of biofilm and resisting infection. These findings show promise for clinical and commercial application, since Staphylococcus epidermidis is the most common pathogen associated with orthopaedic infections.23

Ta and silver have been shown to reduce bacterial adherence to implants.24,25 However, the entire implant or its whole surface need not be coated with these materials to have a measureable effect. Nano-particles of silver on the surface of an implant have been shown to decrease the formation of biofilms on implants.26-28 Apart from being bacterial resistant, nano-particles of silver are non-cytotoxic in appropriate doses and can provide long-term antimicrobial effects.28 The fundamental idea behind this dual treatment is to enhance the osseointegration of Ti implants at the same time as improving their resistance to infection in vivo.

Yavari et al26 prepared scaffolds of porous Ti with TiO2 nanotubes by anodisation and then soaked the nanotubes in silver nitrate having low (0.02 M), medium (0.1 M), and high (0.5 M) concentrations. The antimicrobial behaviour and viability of the cells of the treated nanotube scaffolds were assessed. At up to 24 hours after treatment, the biomaterials were found to be extremely effective in preventing the formation of biofilms on the scaffolds and decreasing the number of planktonic bacteria, especially for the medium and high concentrations of silver ions. The antimicrobial effects of the biomaterials, particularly the ones with greater concentrations of antimicrobial agents, continued until two weeks however, for the groups with the highest concentrations of silver, the viability of the cells was adversely affected. The potency of the biomaterials in decreasing the number of planktonic bacteria and deterring the formation of biofilms make them promising candidates for fighting implant-associated infections.

Take home message:
- The texture of an implant surface at the nano level impacts osseointegration and anti-microbial properties.
- TiO2 nanotube arrays are superimposed nano-texturised features on macro and micro porous tissue ingrowth surfaces.
- Macro, micro and nano texturisation is being considered for the next generation of orthopaedic joint implants.
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