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Review: The Potential Impact of Surface Crystalline States of Titanium for Biomedical applications

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Abstract:
In many biomedical applications, titanium forms an interface with tissues which is crucial to ensure its long-term stability and safety. In order to exert control over this process titanium implants have been treated with various methods that induce physicochemical changes at nano and microscales. These past 20 years, most of the studies have been conducted to see the effect of topographical and physicochemical changes of titanium surface after surface treatments on cells behaviour and bacteria adhesion. In this review, we will first present briefly some of these surface treatments either chemical or physical and we will explain the biological responses to titanium with a specific focus on adverse immune reactions. More recently, a new trend has emerged in titanium surface science with a focus on the crystalline phase of titanium dioxide and the associated biological responses. In these recent studies, rutile and anatase are the only two polymorphs used for biomedical applications. In the second part of this review, we will cover this emerging topic of the control of the crystalline phase of titanium and discuss its potential biological impacts. More in-depth analysis of treatment related surface crystalline changes can significantly improve the control over titanium/ host tissue interface and can result in considerable decreases in implant related complications which is currently a big burden on healthcare system.

Keywords: Titanium, Surface treatment, Implants, Biofilm, Cell Adhesion, Cell/Implant Interface

1. Titanium as a gold standard in implantology: Room for Improvement?

In the last 20 years, the use of titanium and its alloys for medical devices has been well explored. From the earlier use of this metal after the second world war in dental implants to nowadays many improvements have been achieved (Liu et al., 2004a). Different surface treatments can lead to attainment of required properties for each anatomic situation (Geetha et al., 2009).

Most common applications of titanium implants are for hip and knee joint and dental replacements. In cardiac and cardiovascular field, titanium is utilized for artificial heart valves, endovascular stents, occlusion coils and as housing for pacemakers. Ear ossicle replacement can be achieved through partial or total ossicle prostheses. The titanium ossicles are more used than hydroxyapatite based prosthesis because of their lower toxicity for middle ear mucosa (Schwager, 1998, Ho et al., 2003). One of newest titanium use is the artificial larynx replacement executed with a trachea connecting part developed with porous titanium microbeads (Debry et al., 2014). As long as regenerative medicine does not become common for load bearing applications, titanium implants will continue to be integral part of daily clinical practice.

The improvement of titanium implant technologies is on a plateau phase where a number of unresolved problems persist. By the mid-90s the geometry of large joint and dental implants has been consolidated and the interest has shifted towards surface modifications with the view to improve the incorporation rate of the titanium implants. Various surface treatment methods have been developed in order to accelerate the osseointegration of titanium implants, however there is no unity concerning the clinical utility of such enhancements. In this review we attempt

to highlight the difficulties that are associated with the improvement of the biological enhancement of TiO_2 surfaces. We described the potential biological relevance of the crystalline features of TiO_2 that may influence the biological performance implants. In this endeavor titanium implant-associated infections will also be addressed as one of the most urgent challenges.

The underlying principle of our approach is that the biological and mechanical characteristic of a TiO_2 surface can only be construed in relation to the synthesis method. According to this approach the synthesis method determines the physical, chemical and metallurgical characteristics of a TiO_2 surface, which, in turn, influence its biological behavior. This system approach could be useful to work out the root causes of disagreements in the art. Such disagreements may originate from the seemingly conflicting results of several studies that have investigated the response of various cells on some particular surface features (e.g. adherence of stem or bacterial cells to hydrophobic vs. hydrophilic, or to nanostructured vs. micro-rough titanium-oxide surfaces). This *mechanical characteristics → biological characteristics* oriented perspective is the most common association plane through which the biomaterials are investigated (Figure 1). However, the appraisal of the biological results through this single association plane can be accomplished only within certain limitations.

The major limitation is that the biological characteristics of a TiO_2 surface is influenced by a wide-range of mechanical factors, implying physical (e.g. surface charge), physicochemical (e.g. wettability), chemical (e.g. impurities) and metallurgical (e.g. surface roughness) properties, of which total effect will determine the biological behavior. Depending on the applied synthesis method the mechanical characteristics of the various TiO_2 surfaces may be significantly different in some particular features (e.g. sandblasted/acid etched micro-rough vs. anodized nanotubular surface), while they may remain similar in others (e.g. wettability). This causal relationship between the synthesis method and the mechanical

characteristics of TiO_2 supports the relevance of an accordant association plane (Figure 1). The mechanical and, after all, the biological characteristics can only be optimized through the synthesis method and its process parameters. In this aspect, first assumption is that the synthesis method determines the crystalline morphology and phase of the growing TiO_2 layer. The second assumption is that the synthesis method influences exposed face and surface defect of crystalline phases of TiO_2. The third assumption that we will discuss in this paper is that the reactivity of crystalline phases depends on surface energy and surface defects, which influence the chemical reactivity of the TiO_2. The fourth and last assumption is that the chemical reactivity of the exposed facets influences the biological characteristics of TiO_2 surfaces.

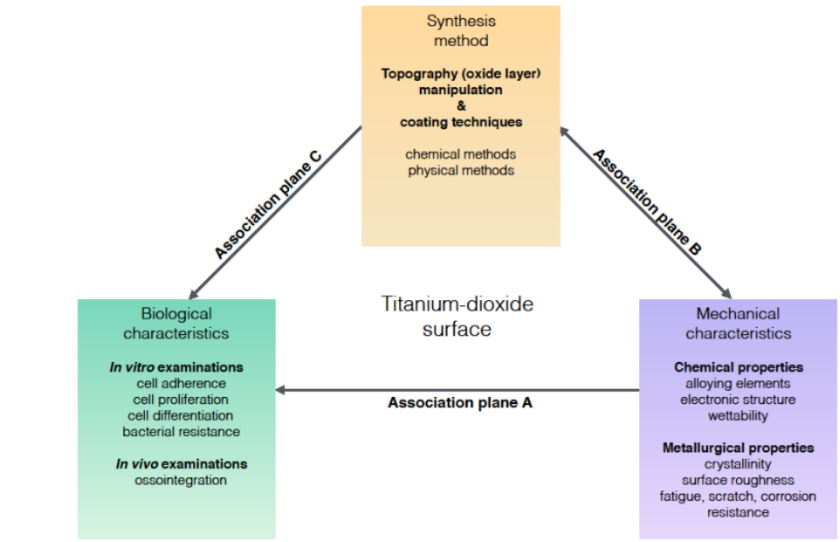


Figure 1 shows the triangle of the interrelations of biomedical aspects concerning the enhancement of titanium implant surfaces.

2. Current approaches on the surface treatment of titanium implants

Dating back from its industrial use, there are well established surface treatment methods for titanium. These can classified in three categories i) physical methods, ii) chemical methods and iii) biochemical methods.

Physical methods for Titanium treatment generally require a pre- treatment. These methods are machining, grinding, polishing, blasting and EDM (Electric Discharge Machining). With these methods, clean, rough or smooth surfaces can be formed (Karthega et al., 2010, Park et al., 2012, Celen and Ozden, 2012, Yeung et al., 2013, Seddiki et al., 2014, Ye et al., 2015, El-Hossary et al., 2015, Degatica et al., 1993). The main surface parameter affected by these treatment is the roughness and depending on whether a smooth or rough surface is required different treatments can be selected. After this pre-treatment (if required), finishing surface treatment can be performed such as Plasma immersion ion implantation (PIII), glow discharge plasma treatment, laser micro-machining, high-energy electropulsing treatment or surface mechanical attrition treatment) (Nouri and Wen, 2015, Brunette et al., 2012). A brief description of these finishing surface treatments is given in Figure 2.

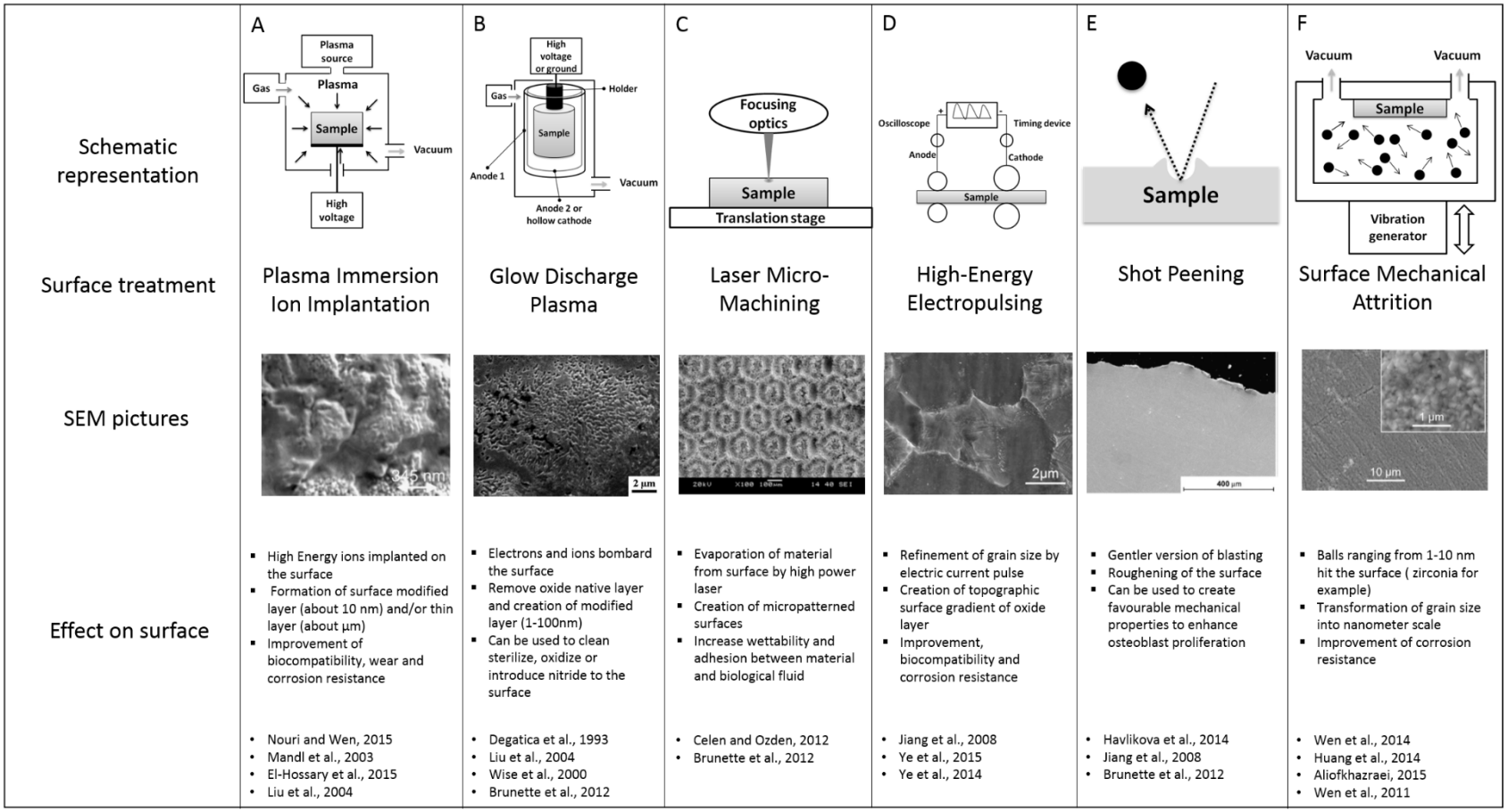


Figure 2. Effect of different physical treatments on Titanium surface topography. Schematic description of the physical treatments, effect associated and SEM pictures of the resulting Titanium surface topography after treatment. A) SEM picture from Ti surface after O-Plasma Immersion Ion implantation (Mandl et al., 2003). B) SEM picture of Ti-6Al-4V surface after Glow Discharge Plasma treatment in air at 1173 K for 2 h (Borgioli et al., 2004). C) SEM picture SEM image from honeycomb pattern obtained on Ti surface after Laser Micro- Machining treatment (Celen and Ozden, 2012). D) SEM picture of Ti-6Al-4V surface after High-Energy Electropulsing treatment (Ye et al., 2014). E) SEM picture of Ti surface after EDM and Shot Peening (Havlikova et al., 2014). F) SEM picture after Surface Mechanical Attrition treatment and thermal oxidation (Wen et al., 2014). SEM pictures reprinted with permission.

Biochemical methods will be not covered in this paper since most biochemical methods are based on the immobilization or deposition of biomolecules like peptides, proteins, growth factors where the crystalline nature of titanium will have less direct effect on cell behaviour (although it would affect the mode of deposition of the target biomolecules).

Chemical methods can be used as pre-treatment or finishing treatment. These chemical methods can be summarized mainly in four categories i) acidic such acid etching, ii) alkaline, iii) hydrothermal and iv) hydrogen peroxide treatments such as piranha solution (Liu et al., 2004b, Variola et al., 2008). In this classification we can also add electrochemical treatment and especially anodization (Das et al., 2007). A brief description of most of these treatments is given in figure 3.

As the first line of interaction is with the immune cells upon implantation; we start with the general effects of titanium on immune cell behavior.

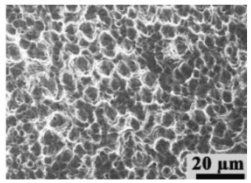
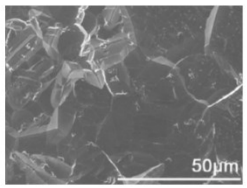
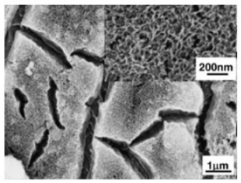
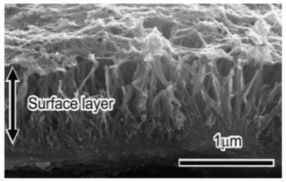
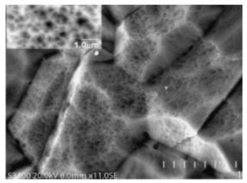
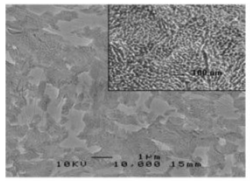
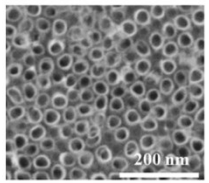
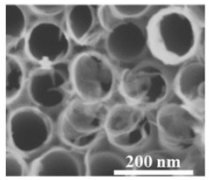
Surface treatment	A Acidic treatment	B Alkaline treatment	C Hydrogen peroxyde treatment	D Anodization treatment
SEM pictures	1  2 	1  2 	1  2 	1  2 
Effect on surface	▪ Removal of contaminations and native oxide layer ▪ Clean and uniform with micro and/or nanostructure	▪ Can lead to a structure of about 1 µm of thickness of sodium titanate gel, TiO_2 layer and micropores ▪ Improvement of biocompatibility, bioactivity or bone conductivity	▪ Lead to a two-layer oxide film; with a dense inner layer (about 5 nm thickness) and a nano-porous outer layer ▪ Oxidation, hydroxylation or roughening of the surface ▪ Improvement of biocompatibility, bioactivity and bone conductivity	▪ Modification of the structure and composition of native oxide layer ▪ Formation of porous TiO_2 layer of about 10 nm to 40 µm thickness (micron size) and/or nanotubes (between 15-100 nm) ▪ Improvement of corrosion resistance, the biocompatibility, bioactivity
	• Liu et al., 2004 • Kulkarni et al., 2014 • Jiang et al., 2013 • Lin et al., 2013	• Zheng et al., 2007 • Wei et al., 2008 • Kulkarni et al., 2014 • Liu et al., 2004)	• Kulkarni et al.,2014 • Pisarek et al., 2011 • Seddiki et al., 2014 • Liu et al., 2004	• Nouri and Wen,2015, • Zhang et al., 2013 • Jiang et al., 2013 • Zhang et al., 2014

Figure 3. Effect of different chemical or electrochemical treatments on Titanium surface topography. A) Acidic treatment : 1) SEM pictures of micropores on Ti surface caused by H_2SO_4 + HCl treatment (Jiang et al., 2013a), 2) SEM picture of micropitted surface caused by $H_2C_2O_4$ on pickled Ti surface (Li et al., 2014). B) Alkaline treatment: 1) SEM pictures of Ti surface after 3M KOH treatment (Zheng et al., 2007), 2) SEM pictures of Ti surface after 3M NaOH treatment (Pisarek et al., 2011). C) Hydrogen peroxide treatment: 1) SEM pictures of Ti surface after 15% H_2O_2 + HCl treatment (Karthega et al., 2010), 2) SEM pictures of Ti surface after H_2O_2 + H_2SO_4 treatment (Piranha solution) (Seddiki et al., 2014). D) Anodization treatment: SEM pictures of TiO_2 nanotubes with 1) 15 nm and 2) 100 nm made by ethylene glycol electrolyte (Kulkarni et al., 2014a). SEM pictures reprinted with permission.

3. Biological responses to Titanium

3.1. Immune response to Titanium

High biocompatibility and mechanical properties of titanium has made it the material of choice for a variety of implants. Still a portion of implants fails due to the development of chronic inflammation at the implant-tissue interface (Landgraeber et al., 2014). Prolonged friction between articulating surfaces as well as high mechanical stress result in the release of microscopic wear particles. The implant material and released wear particles are recognized by the immune system as foreign bodies and thus induce an inflammatory reaction (Kzhyshkowska et al., 2015).

The key immune cells involved in the initiation of the foreign body response are macrophages. Upon implantation, a protein layer is adsorbed on the surface of the implant which attracts and activates macrophages (Anderson et al., 2008). Macrophages can initiate the foreign body response either by direct recognition of the implant material and the adsorbed protein layer or by phagocytosis of wear particles (Abu-Amer et al., 2007). Several cell surface receptors, like integrins (CD11B/CD18, αvβ3, αvβ5, and α5β1) or Toll-like Receptors (TLRs) take part in this recognition and consequently activating inflammatory signaling pathways (Kzhyshkowska et al., 2015, Pearl et al., 2011, Zaveri et al., 2014). An essential pathway involved in the FBR is mediated by NF-κB (Lin et al., 2014). As a result of NF-κB activation, macrophages start to secrete a panel of pro-inflammatory cytokines (TNFα, IL-1β, IL-6, IL-8, etc.) and chemokines (CCL2, CCL4, CCL8, CCL13, etc.) which recruit additional macrophages and other immune cells to the peri-prostetic tissue and induce acute inflammation. An ineffective resolution of acute inflammation results in the development of chronic inflammation, generation of foreign body giant cells and formation of a fibrous capsule around the implanted material (Anderson et al., 2008).

Since titanium is commonly used as an implant material, numerous studies have focused on macrophage responses to it. For in vitro testing the most common cellular models have been RAW264.7 cells (mouse leukemic monocyte-macrophage cell line), THP-1 cells (human monocytic leukaemia cell line) and mouse bone-marrow derived macrophages. It has been shown that phagocytosis of titanium nano- and microparticles up-regulates the expression of TNFα, IL-1β and IL-6 on both mRNA and protein levels (Beidelschies et al., 2008, Luo et al., 2016, Minematsu et al., 2007, Naganuma et al., 2016, Pajarinen et al., 2013, Rakshit et al., 2006, Ruiz et al., 2016, Valles et al., 2006, Yang et al., 2016, Yazdi et al., 2010). Additionally, the inflammasome protein NLPR3 which is responsible for IL-1β and IL-18 activation (Martinon et al., 2002), is also up-regulated in macrophages by titanium (Naganuma et al., 2016, Ruiz et al., 2016, Yazdi et al., 2010).

Titanium can also trigger the production of pro-inflammatory chemokines in macrophages. CCL2 and CCL3 expression was up-regulated in macrophages stimulated with titanium particles (Mao et al., 2012), thus enabling macrophages to recruit additional monocytes and neutrophils to the site of inflammation. Furthermore, titanium particles induce oxidative stress by promoting the production of reactive oxygen species (ROS), NO and expression of iNOS in macrophages (Hamilton et al., 2009, Liu et al., 2010, Luo et al., 2016, Mao et al., 2012), with both ROS and RNS (reactive nitrogen species) being linked to osteolysis (Lee et al., 2005, Puskas et al., 2003).

Consistently with in vitro studies, experiments in mice also showed that titanium induces increased expression of pro-inflammatory cytokines (TNFα, IL-1β and IL-6), chemokines (CCL2 and CCL3), NLPR3, ROS and RNS (Huang et al., 2015, Jovanovic, 2015, Sang et al., 2012, Wang et al., 2011, Warme et al., 2004, Yang et al., 2016, Yazdi et al., 2010). Common in vivo implantation models consist of insertion of titanium particles, pins, rods and screws in tibia, femur, maxilla and calvaria. These models can be used not only for the analysis

of oxidative stress and pro-inflammatory cytokine expression, but also for the study of osteoclast formation and osteolysis.

The main cells involved in osteolysis are osteoclasts. While it is widely accepted that macrophages play a pivotal role in osteoclastogenesis (Takayanagi, 2010), the formation of osteoclasts in response to wear particles is highly reliant on RANKL (Receptor activator of nuclear factor kappa-B ligand) (Clohisy et al., 2003, Liu et al., 2009). RANKL expression was up-regulated in both primary human osteoblasts and in hFOB1.19 cells (human fetal osteoblastic cell line) upon stimulation with titanium particles (Cadosch et al., 2010, Pioletti and Kottelat, 2004). Moreover, *in vivo* osteolysis models revealed that the osteoclast number is increased in response to titanium particles, with a higher expression of TRAP (tartrate-resistant acid phosphatase; osteoclast marker) and RANKL in the tissue surrounding the implantation site (Jiang et al., 2013b, Shin et al., 2012, Yang et al., 2016). The current model of macrophage-osteoblast-osteoclast interaction in response to titanium is presented in Figure 4.

Macrophages can initiate an inflammatory reaction either by direct recognition or by phagocytosis of the implant material. Comparison of THP-1 reaction to titanium particles and discs demonstrated that the discs had a more stimulatory effect on cytokine production (TNFα, CCL2 CCL3, IL-1Ra) possible due to the frustrated phagocytosis phenomena (Kim et al., 2007). In order to reduce inflammatory reactions to titanium, implant's surface can be modified to improve its biocompatibility. Thus it was reported that hydrophilic surfaces on titanium discs reduced the expression of pro-inflammatory cytokines (TNFα, IL-1α, IL-1β, IL-6 and CCL2) while increasing the expression of anti-inflammatory cytokines (IL-4 and IL-10) in both macrophages (Alfarsi et al., 2014, Hamlet et al., 2012, Hotchkiss et al., 2016) and osteoblasts (Hyzy et al., 2013) when compared to hydrophobic surfaces. These results as well as findings on other materials have shown that surface modification can be adopted to minimize implant-induced inflammatory reaction (Rostam et al., 2015). However, surface modification has its

limitations and new combined techniques with the use of surface modifications and application of biodegradable, immunomodulatory coatings are a promising approach to design highly biocompatible implants (Kzhyshkowska et al., 2015).

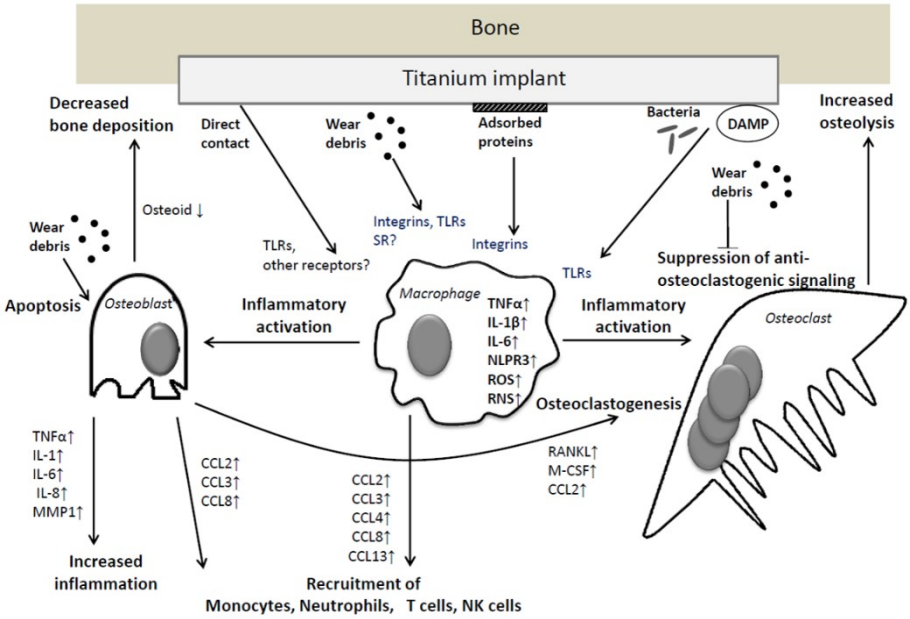


Figure 4. Macrophage-mediated immune reactions at the implant-tissue interface. Modified from Kzhyshkowska et al (Kzhyshkowska et al., 2015).

Macrophages can also trigger FBR around titanium by contact with bacteria or damage-associated molecular patterns (DAMP). These events initiate secretion of pro-inflammatory cytokines and chemokines in macrophages, which in turn activate osteoblasts. Activated osteoblasts start to produce inflammatory cytokines and chemokines, thus enhancing the inflammation. Additionally, wear particles induce apoptosis in osteoblast and as a result decrease the bone deposition. Chemokines released by osteoblasts and macrophages recruit additional immune cells to the site of inflammation. Synergistic effect of wear particle-induced anti-osteoclastogenic signaling and osteoblast expression of RANKL, MCSF and CCL2 increases the number of osteoclasts at the implant-tissue interface. Macrophage inflammatory

cytokines activate osteoclasts leading to the increased osteolysis. In order to prevent this cascade, development of anti-inflammatory titanium surfaces is necessary.

From this perspective we also intend to emphasize that, even if titanium implants are broadly considered biocompatible, unwanted events may be associated with their clinical use, such as immune response that may end up in implant loosening. The etiology of titanium implant-associated immune response is sometimes obscure owing to various unknown factors that play role in the initiation of the loosening process. These unknown factors are supposed to be delivered by some specific surface features of the TiO_2 layer, such as crystalline phase, exposed facet, electron structure and surface defect, which together could create a chemically active solid surface that may catalyze some heterogeneous redox reactions or facilitate the adsorption and desorption of macromolecules in the human body. It must be noted that the effect of such surface features have yet been investigated extensively in conjunction with TiO_2 photocatalysts and semiconductors but they have been disregarded in relation to indwelling implants

3.2.Surface modification of Titanium to control cell response

Cell attachment and spreading on titanium is a well-established phenomenon (Le Guéhennec et al., 2007, Sjöström et al., 2013, Koenig et al., 2016, Schultz et al., 2007, Vrana et al., 2012, Regis et al., 2015). Nevertheless, lack of osseointegration on untreated titanium implants is often associated with implant failure mainly due to the difficulty of the material to establish a strong bond with bone. To overcome this problem, efforts have been made on the modification of titanium surface to improve the early bone to implant interaction. This can explained why most of the studies have been conducted with osteogenic lineages (osteoblast or mesenchymal stem cells) (Fujino et al., 2014) (Gittens et al., 2013, Sverzut et al., 2012, Gittens et al., 2014).

Most of these works focuses more specifically on the nanostructuration of the interface between the implant and the tissue. Osseointegration is firstly regulated by osteoblast adhesion on the implants and this phenomenon is dependent on protein adsorption on the surface. The serum proteins such as fibronectin and vitronectin will first be adsorbed on the surface. Then the surface will interact with the cells first through nonspecific interactions (ionic, Van Der Walls). Finally cells with the engagement of their integrins will recognize cell binding domain on these proteins (Rivera-Chacon et al., 2013). The nanostructuration will increase the surface hydrophilicity, the serum protein adsorption and so improve the interaction between the implant and biological fluids. As a consequence, the initial attachment and the proliferation of osteoblasts or mesenchymal stem cells will be enhanced (Rieger et al., 2015). The structuration of the interface at a nano level can be achieved with different treatments such as acid etching, alkaline treatment or anodization. Acid etching treatment with HCl, H_2SO_4, HNO_3 or HF or their mixtures is widely used for orthopedic or dental implants. It produces pits on the surface in the range of 200 nm to 5 µm and its beneficial effect for rapid osseointegration has been shown (Le Guéhennec et al., 2007, Bächle and Kohal, 2004, Cho and Park, 2003). Some studies have shown that blasted surfaces treated with acid treatment (hydrofluoric acid) improve osteoblast adhesion and bone specific gene expression (Guo et al., 2007). In a similar study, Lamolle et al have demonstrated that fluoride modification of Titanium through HF treatment that affect both the topography (micro/nano features) and the chemical composition (presence of fluoride, hydride, oxide) had a positive effect on osteoblast attachment, proliferation (Lamolle et al., 2009). Anodization is also widely used to obtain nanostructuration by producing TiO_2 nanotubes on the surface (Kulkarni et al., 2014b). The positive effect of this treatment on osteoblast attachment, proliferation, colonization has already been proven (Yao et al., 2008, Yao et al., 2005). It has been reported that this kind of treatment have positive effects both *in vivo* and *in vitro* on the growth human MSCs and also it enables the differentiation of these

cells into osteogenic lineage. Some other works have studied the effect of the combination of multiple treatments on osteoblast or mesenchymal stem cells responses (Chiang et al., 2009). Oliveira et al. showed the effect of surface anodization with subsequent Calcium Phosphate incorporation on human osteoblastic response. They quantified factors that can influence osteoblast differentiation such as TGF-β1, BMP-2 or major osteoblast matrix proteins such as alkaline phosphatase (ALP). They were able to conclude that anodization combined with CaP incorporation enhance cell viability and ALP expression but the incorporation of CaP did not have a significant effect of osteoblast differentiation suggesting that the nanotopography was more relevant than the chemical composition (Oliveira et al., 2013). Another treatment mainly used for orthopedic or dental implants is the alkaline treatment. It has been demonstrated that immersion of titanium implants in alkaline solution such as NaOH creates nanotubes on the surface. This treatment through the nanostructuration of the interface enables the formation of hydroxyapatite layer when brought in contact with simulated body fluid and as a consequence bone-titanium bonding was improved. This treatment has shown beneficial effects for osteogenic differentiation of stem cells and for enhancing mineralization (Fujino et al., 2014). Some studies have shown that this apatite layer created with alkaline treatment was unstable and subject to delamination for long term implantation and so this treatment can be combined with heat treatment in order to stabilize this apatite layer (Nishiguchi et al., 1999). These findings indicate that Titanium topography induced by surface treatments especially at a nanoscale level is important to modulate cell responses.

3.3.Rendering Titanium surface antibacterial

Biofilm formation starts with the attachment of free-floating bacteria on a surface. The biofilm renders the bacterial colony resistant to antibiotics and immune cells (Kargupta et al., 2014). Bacterial infections can lead to the failure of orthopedic and dental implants due to bone

resorption (Ramalingam et al., 2012, Gao et al., 2011). The bacterial adhesion can be defined firstly as an initial and reversible physical phase, and secondly as a time-dependent and irreversible molecular phase (An and Friedman, 1998). It is related to chemical composition and physical characteristics of the surface (Yoshinari et al., 2000, Taborelli et al., 1997). In this context, many studies have been performed to limit bacterial adhesion to address early infection (Glinel et al., 2012, Emmerson, 1998, Harris et al., 2004, Klok and Genzer, 2015, Eckhardt et al., 2013, Cao et al., 2014, Qiao et al., 2015). In addition, another solution is the bactericidal surfaces that can kill bacteria once in the contact with the implant surface (Popat et al., 2007, Özçelik et al., 2015, Pornpattananangkul et al., 2011, Jin et al., 2014).

Surface modification of the titanium implants is also proposed to inhibit bacterial contamination without changing its bulk mechanical properties (Shibata and Tanimoto, 2015). One example is the titanium dioxide surface (TiO_2) conversion into anatase via anodization of Ti followed by a heat treatment. Anatase titanium substrates possess antibacterial properties due to the bactericidal effects of reactive oxygen species (ROS) generated by the surface anatase, (Figure 5 A) (Hu et al., 2014). Shibata and colleagues presented that titanium surfaces anodically oxidized in a solution with chloride (Ti-Cl) achieved antimicrobial activity against an oral microorganism due to the amount of ROS. Ti-Cl is shown as an efficient surface against a time-dependent degradation of biological ability due to the peroxidation effects by hypo-chloride (Fig 5B, C).

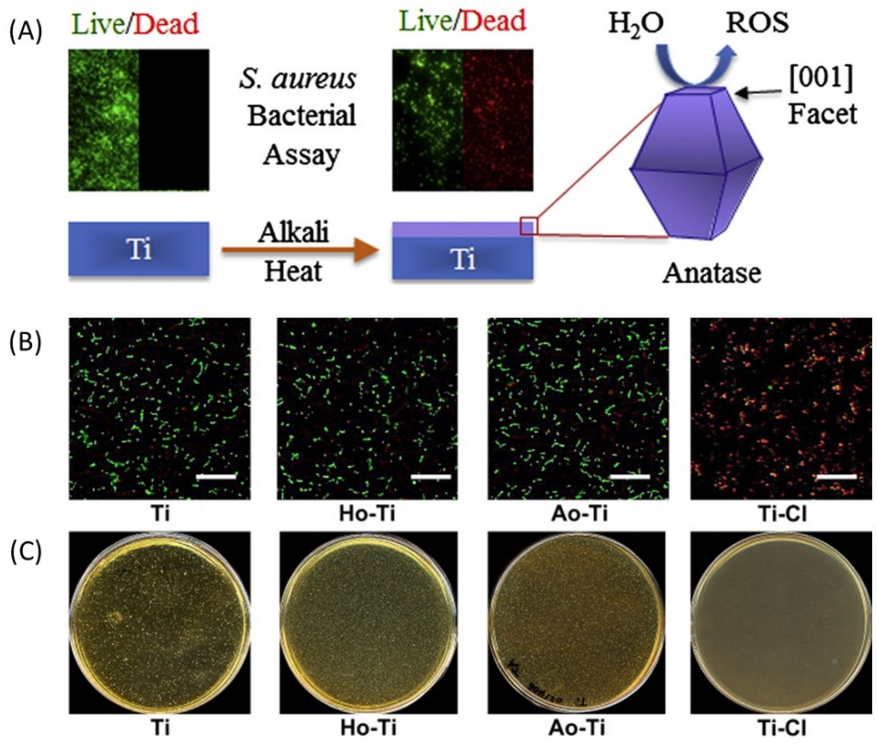


Figure 5. Bactericidal effects of reactive oxygen species (ROS) generated by the surface anatase (A). Antimicrobial efficacy on titanium samples in different conditions: control (Ti), oxidized by heating at 600°C/30min, anodically oxidized in 1M Na_2HPO_4 (Ao-Ti) and in 1M NaCl (Ti-Cl): the viable S. mutans are green while the non-viable S. mutans are red (Bar=50µm) (B); A. actinomycetemcomitans on the samples after 1h of cultivation (C).

The success of the titanium implants depends of several factors, and as mentioned before the infection around it leads to implant failure and revision surgeries. Thus for commercial uses of titanium antimicrobial activity is a necessary improvement. However, currently available implants do not contain this property as of yet, thus new systems to render titanium antimicrobial is still an active research area.

4. General overview on the crystalline phases of titanium-dioxide

The TiO_2 exists in various natural and high-pressure polymorphs, for instance anatase (tetragonal), rutile (tetragonal), brookite (orthotrombic), akaogiite (monoclinic), columbite (orthotrombic), fluorite-type polymorph (cubic), pyrite-type polymorph (cubic), baddeleyite-type polymorph (monoclinic) and cotunnite (orthotrombic), which is one of the hardest polycrystalline materials known (Diebold, 2003, Greenwood and Earnshaw, 2012, Sharpe and Pousse, 2010). However, on the current level of technology only rutile and anatase play any role in biomedical applications, hence in this paper we focus on these two polymorphs.

The recent scientific achievements in the field of the photocatalysis have markedly increased our understanding in the electronic structure of TiO_2, and so in its electrochemical and chemical properties (Schneider et al., 2014); however, the translation of such results for the improvement of medical technologies occurs after some delay. The phase transformation of anatase to rutile entails the changes in the electron structure that is responsible for the chemical properties of the TiO_2 surface that affect the biological behavior (Ning et al., 2016, Hanaor and Sorrell, 2011). Concerning photocatalytic applications the mixed-phase samples of anatase and rutile outperform the individual polymorphs (Luo et al., 2014).

4.1. Influence of the synthesis method on the formation of the crystalline phase of TiO_2

In the synthesis of TiO_2 films the formation of the initial crystalline phase significantly depends on the applied conditions and process parameters. The control of conditions that affect the kinetics of anatase to rutile phase transformation is of considerable interest, as well. Even minor changes in the process parameters may yield changes in the crystalline phase. For instance, low anodizing potentials (1-130V) facilitate the growth of smooth amorphous oxide film on the surface of titanium. The composition of the anodizing electrolyte has also a

significant effect on the growing TiO_2 film, e.g. pure anatase can be grown in H_2SO_4 electrolyte applying low potential in the range of 70 – 120V (Diamanti and Pedeferri, 2007). The effect of other process parameters can be as significant as the electrolyte is, for example, higher anodizing potential results in the formation of rutile phase beside anatase in the same H_2SO_4 electrolyte (Uttiya et al., 2014).

The crystalline phase of a TiO_2 film on the surface of titanium implants is regarded stable under physiologic conditions, which is also an essential requirement that must be fulfilled by all implantable devices in order to assure their constant biological performance and safety in the body (Brunette et al., 2012). Albeit, there is no reported data on what crystalline phase (anatase vs. rutile vs. multiphase) of TiO_2 performs the best in vivo. Concerning medical device applications, the recent surface treatment technologies have primarily been optimized for the manipulation of the topography (nano-, and micro-roughness) of titanium implants, but they can also have an impact on the crystalline phase of TiO_2.

4.2.Molecular absorption and surface chemistry of TiO_2

The absorption of molecules and atoms on TiO_2 surfaces has been extensively investigated (Brunette et al., 2012). In this review we focus on specific reactions of rutile and anatase that may have relevance concerning biological activity. Concerning rutile, most of the available data are related to {100} and {110} facets as these have the highest thermodynamic stability and so it has been easier to produce them for experimental purposes. The reaction of TiO_2 (rutile) {100} and {110} with water is characterized mainly by molecular and dissociative adsorption at point defects, especially at O vacancies. As an example, we can refer to the UV illumination of TiO_2 (110) single crystal that becomes hydrophilic owing to the formation of O vacancies (Wang et al., 1997).

In practice, anatase crystals expose a variety of facets simultaneously to varying extent, including {101}, {111}, {112}, {100} and {001}, whereas rutile expose mainly {110} facet with more than 90% extent. Österlund demonstrated by FTIR spectroscopy that formic acid and formate absorbate structure is very different on the various anatase and rutile nanoparticles (Österlund, 2010).

4.3.Engineering of high-energy facet exposing TiO_2 crystals

High-energy facets are apt to vanish in the bulk of crystals because the thermodynamically stable facets preferentially predominate on the surface in order to minimize the total surface energy of crystals. Hence, it is challenging to expose specific facets, especially high-energy facets on the surface of the TiO_2 crystals. The critical factor for controlling exposed facets of crystals is the tuning of relative stability of different facets during the growth process, which is intrinsically determined by the average surface energy of the facets (Chen et al., 2015).

4.4.Implication of the chemical reactivity of TiO_2 facets on the biological characteristics of implants

The abovementioned results can be implied on ligand-Ti bonding because the particle morphology, hence the distribution of exposed surface facets, which strongly affect their reactivity and may also have influence on the biological behavior of TiO_2 surfaces (Diebold, 2003). Formic acid and other carboxylic acids have become the most investigated organic molecules on single-crystalline TiO_2 surfaces because many of the higher carboxylic acids follow the behavior of formic acid. A wide range of intracellular (e.g. immune reaction, toxicity, etc.) and extracellular (cell and bacterial adhesion) is mediated through the chemical reaction of carboxyl groups, thus the cell response to TiO_2 surfaces may be controlled through deliberate

selection of TiO_2 nanocrystals with appropriate size that exhibit proper fractions of various facets. This has implications in immune reaction to titanium.

4.5.Current State of the art on the effects of crystalline phase on cell behavior and inflammatory properties of Titanium

The primary biocompatibility concern regarding titanium dioxide is in the form of nanoparticles as currently more than 10.000 tons of titanium nanoparticles are used annually. Thus, the effect of the crystalline phase on cell behavior has been more widely studied using NPs. For a more comprehensive review of titanium nanoparticles, please see (Rajh et al., 2014). Beyond direct toxicity, NPs might also have secondary effects such as increased rate of infection as demonstrated by *S. Aureus* infection of HeLa cells in the presence of titanium dioxide particles (Xu et al., 2016). However, in most of the toxicology studies the crystalline phase was not used as a parameter. A recent toxicogenomics study by Rahman et al. has demonstrated that rutile NPs induced significantly more neutrophil influx and changes in inflammation related gene expression compared to anatase NPs in vivo in mice (Rahman et al., 2016). For a dose of 162 μg only 8 genes were up or down regulated for anatase NPs whereas expression of 104 genes were altered for rutile NPs. Most of the upregulation was related to Acute Inflammation related canonical pathways. These results are consistent with previously obtained in vitro results with the cells from same mouse strain where rutile NPs induced cytoxicity , genotoxicity and neoplasticity while anatase NPs did not (Uboldi et al., 2016). It has been demonstrated that lung epithelial cells respond very different to anatase and rutile TiO_2 nanoparticles. Interestingly, pure anatase and rutile particles provoked differential IL-8 response in (A549) epithelial carcinoma cells (Xu et al., 2016).

As described before, anodization is one of the most common ways of obtaining nanostructured titanium surfaces with improved cell interactions. After anodization the formed

oxide layer is amorphous and it can be rendered crystalline with heat treatment; thus anodized surfaces are a possible area of investigation for determination of biological effects of crystalline structures. Moravec et al demonstrated that a treatment at 500 °C resulted in an anatase rich surface (98%) and subsequent increase in alkaline phosphatase activity of seeded pre-osteoblasts (Moravec et al., 2016). Effect of increasing the crystalline structure by thermal treatment on osteogenic differentiation was further shown by Wang et al (Wang et al., 2016); in which study they also demonstrated a significant increase in implant bone contact surfaces for the thermally treated samples (Figure 6A). However, as the anodization process results in a relatively week oxide layer than can detach from the bulk titanium surface, Mohamed et al. studied the cytotoxic effect of detached nanotubes on human dermal fibroblasts. Although, in most concentration there was not a toxic effect, at low concentration (from 0.05 to 0.25 mg/ml) a cytotoxic effect was seen with a significant increase in ROS release and indications of DNA damage (Figure 6B). Also, in the presence of the nanotubes more cells are in resting (G_0) phase (Mohamed et al., 2017). The underlying mechanism of the cytotoxicity was genotoxic effects as demonstrated by H2AX staining for DNA breaks, which was significantly increased in the case of nanotubes.

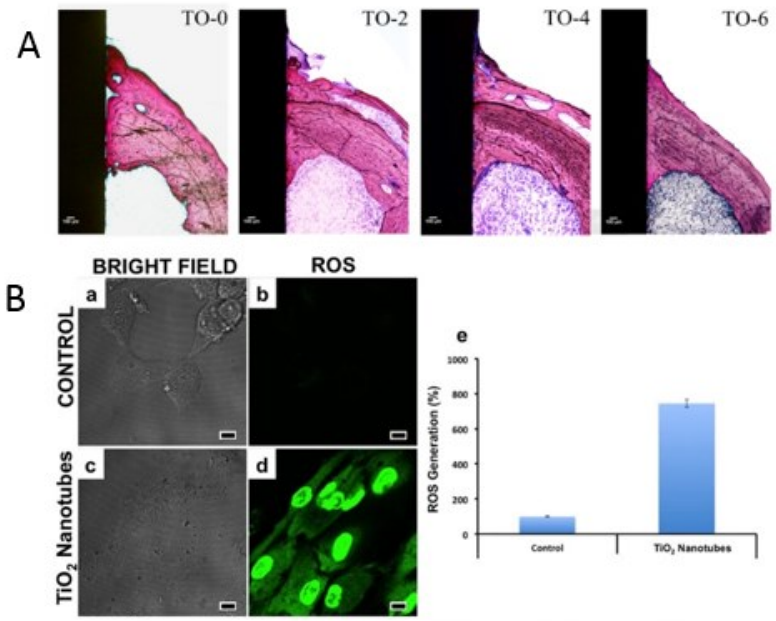


Figure 6. A) Increased bone volume and implant bone contact with crystalline titanium surfaces obtained by thermal treatment (Adapted from Wang et al.) B) Effect of detached titanium dioxide tubes on reactive oxygen species (ROS) release by fibroblasts. A more than 6 fold increase in ROS release was observed(Mohamed et al., 2017). Reprinted with permission

Although, the theoretical basis of the differential cell behavior as a function of crystalline structure of surface or particulate titanium dioxide has not been established yet; the reason can be closely linked with the interaction of the crystalline surface with the aqueous environment. Here, the effect is even subtler than the nature of the crystalline phase and the behavior of excess surface electrons even depend on which facet of the crystal is exposed to the aqueous interface (Selcuk and Selloni, 2016). As in vivo, the titanium surface will interact with complex colloids which will determine the surface cells will interact with, more in depth analysis of Titanium dioxide crystalline structure with biological activity can provide the rules of design for nanoscale surface modifications of titanium implant surfaces. For example, Ribeiro et al, recently demonstrated that anatase Titanium dioxide NPs enter the cells after

binding to calcium, phosphate and proteins available in the cell culture medium (Ribeiro et al., 2016).

Future Outlook and Conclusions:

Titanium implants will continue to play an important role in implantology for the foreseeable future; as the efforts in regenerative medicine cannot completely replace large scale systems such as femoral implants at the current stage of technology. Although titanium has proven to be an excellent material for implants, there are still significant problems such as short and long term microbial infection/biofilm formation related peri-implantitis, allergy or chronic inflammation related problems and poor tissue integration. This review aimed to provide an overview and critical analysis of available surface modification techniques to overcome those problems and recommend a future emphasis on the use of Titanium dioxide surface crystalline phases as a means of controlling biological behaviour. The future advances might provide personalized treatments of titanium surfaces taking into account the patient specific immune response, implant microenvironment and patient microbiota that will ensure long term functionality and safety *in vivo*.

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