Hindawi
Journal of Nanomaterials
Volume 2017, Article ID 6092895, 18 pages
https://doi.org/10.1155/2017/6092895

Review Article

Nanofeatured Titanium Surfaces for Dental Implantology: Biological Effects, Biocompatibility, and Safety

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Received 15 December 2016; Revised 14 April 2017; Accepted 9 May 2017; Published 4 June 2017

Academic Editor: Martin Andersson

Nanotechnology enables the control and modification of the chemical and topographical characteristics of materials of size less than 100 nm, down to 10 nm. The goal of this review is to discuss the role of titanium substrates as nanoscale surface modification tools for improving various aspects of implantology, including osseointegration and antibacterial properties. Techniques that can impart nanoscale topographical features to endosseous implants are described. Since the advent of nanotechnology, cellular specific functions, such as adhesion, proliferation, and differentiation, have been better understood. By applying these technologies, it is possible to direct cellular responses and improve osseointegration. Conversely, modulating surface features by nanotechnology could have the effect of decreased bacterial colonization.

1. Introduction

The modern implantology was developed in the late 1960 based on the studies of Brånemark, which led to the concept of osseointegration. Osseointegration is defined as the direct contact between the vital bone and the implant at the microscopic level [1–3]. Initially, titanium implants with machined surfaces were used and the healing time before prosthetic charge was empirically established within 3–6 months. Although these results represented a relevant advancement, some issues remained regarding primary osseointegration rates, especially in some categories of patients, such as those considered at risk (e.g., diabetics and smokers) [4–8].

Apart from the cases considered at risk for the general health status of the patient, implants placed in sites treated with bone regeneration procedures [9, 10] may benefit from the surface treatments that create nanometric osteoconductive features. Titanium, in particular, has initially been identified as the gold standard for dental implants due to its favorable physiochemical and biological characteristics [11–13].

Osseointegration, similar to bone fracture healing, involves many complex physiological mechanisms, such as the development through hemostasis, the organization of the clot and fibrin polymerization, neoangiogenesis, the deposition of an extracellular matrix, the colonization of bone-forming cells, and the formation of the osteoid tissue. These occur until the mineralized bone matrix and bone-implant are in direct contact with each other [14]. Osseointegration is most affected by the implant surface, because it encompasses the surface area, mineralization, and the mechanical relationship of direct bone-to-implant contact (BIC) [15–19].

In the 1990s, much focus was placed on topographical micrometric features, noting an increase of osseointegration, BIC, and resistance to applied loads [20] on sandblasted and etched surfaces. This leads to the understanding that osseointegration could indeed be influenced by various surface features [21].

The interaction between a microcharacterized implant surface and the bone has been explained according to three different theories: biomechanical, contact osteogenesis, and

osteodifferentiation. The first, expressed as a mathematical model described by Hansson and Norton [22], considers the relationship between the bone and the implant from a purely mechanical point of view. It takes into account the shape and density of pits on the surface, and according to this theory, the optimal pit values were calculated to be approximately 1.5 μm in depth and 3–5 μm in diameter. This theory, supported by subsequent findings [23–26], emphasizes the importance of mechanical retention and the forces distributed to obtain maximal osseointegration.

According to the theory of contact osteogenesis as proposed by Davies [27], a microrough surface allows for the optimal activation of the platelet factors, followed by the stabilization of the fibrin cloth and, finally, the direct growth of osteoblasts onto the implant surfaces [28]. Afterwards, numerous studies confirmed that an increase in the surface topography corresponded to an increase in extracellular matrix production and, finally, that of osseointegration [29–41].

According to the second theory, that of osseodifferentiation, the interaction between the implant surface with specific features and the adherent cells induces a preferential differentiation towards the osteoblastic line, for example, through adhesion or shape transformation [21, 29, 42–44].

Current research efforts are aimed at providing osteogenic features to implant surfaces for guiding the differentiation of adherent progenitor cells and enhancing osseointegration. For this purpose, surface modifications including microtopographical and chemical ones, or characterization with bioactive molecules, such as bone morphogenetic proteins (BMPs), have been proposed, and interest has focused primarily on modifications providing nanometer-level resolution [45–47].

Nanoscale surfaces are regarded as those with structures smaller than 100 nm in at least one dimension, and nanofeatures have been characterized on at least four commercially available implants [48].

Controlling surface features at a nanometric level allows for product surfaces that more naturally emulate biologic environment, which is regulated by nanometric scale components. Nanoscopic modifications to dental implants could affect the physicochemical and topographical features, as well as surface characteristics secondary to the deposition or adsorption of nanoscale complexes. In this review aspects regarding technology production and capability to stimulate a correct osteogenetic response by host tissue and bacterial response to nanofeatured implant surfaces are discussed.

2. Discussion

2.1. Nanotechnology and Surface Science. Nanotechnology spans the creation of functional materials, devices, and systems by controlling matter on the nanometer scale (<100 nm) and utilizing the novel phenomena and properties (physical, chemical, and biological) which occur on this scale [49]. It involves one-dimensional (nanodots), two-dimensional (nanowires), or three-dimensional (nanotubes) spatial structures, and accordingly, these materials can be nanostructures, nanocrystals, nanocoatings, nanoparticles, or nanofibers [50]. The application of nanotechnology to implants can confer new physicochemical or biochemical characteristics (e.g., binding to bacterial plaque, adsorption of proteins) that are not observed on larger scales [51].

Several techniques can be used for nanofeaturing titanium implant surfaces [52].

2.2. Physical Techniques

2.2.1. Compaction of Nanoparticles. Nanoparticles of TiO_2 can be packed onto titanium surfaces to obtain nanoscale granules. This technique was reported to increase the osteoblast adhesion with no reported changes being incurred on the physiochemical characteristics of the material [37].

2.2.2. Grit Blasting. Bioceramic grit blasting and acid etching technologies have been used in combination to produce nanometric textures on titanium implants. These surfaces have a favorable effect on initial bone healing processes [54, 55]. The materials used for blasting include aluminium oxide (Al_2O_3) or silicon carbide (SiC). The final morphology of the surfaces can be modified by particles of different compositions and sizes [56]. Smaller particles can adhere to the implant even after ultrasonic cleaning, acidic passivation, and sterilization because of the high impact velocity between small particles and implant surfaces [57, 58]. Blasting with TiO_2 has been proposed as a procedure to prevent the contamination of implant surfaces and increase both osteoblast adhesion and osteogenic differentiation [59, 60].

2.3. Chemical Techniques

2.3.1. Anodic Oxidation. Anodic oxidation or anodization is a method commonly used in the industry to create surfaces with nanometer topography, such as holes, pits, or pipes [61, 62] (Figure 1). In this treatment, direct current flows through an electrolytic solution from a titanium anode to a cathode, typically of platinum, allowing for the generation of regular and repeatable structures on the titanium surface. The diameter of the structures and the distances between them can be adjusted by controlling the intensity of the current. This technique has been used to produce nanotubes with diameters between 15 and 100 nm [63]. Small nanotubes (30 nm in diameter) can promote osteoblastic adhesion without noticeable differentiation, whereas larger nanotubes (70 to 100 nm in diameter) elicit a dramatic stem cell elongation (10-fold increase), causing cytoskeletal stress, and selective differentiation into osteoblast-like cells [62, 64]. Studies have also shown that nanotubes with a diameter between 50 and 100 nm enhance osseointegration, BIC, and bone morphogenetic protein 2 (BMP-2) expression more efficiently compared to smaller nanotubes [65]. In vivo studies showed more pronounced osseointegration when surfaces contained nanotubes compared to smooth surfaces [66], but no attempts were made, in these experiments, to compare nanosurfaces with microfeatures. It is postulated that titanium-based nanotubes allow a greater deposition of hydroxyapatite in comparison to nanoparticles [61].

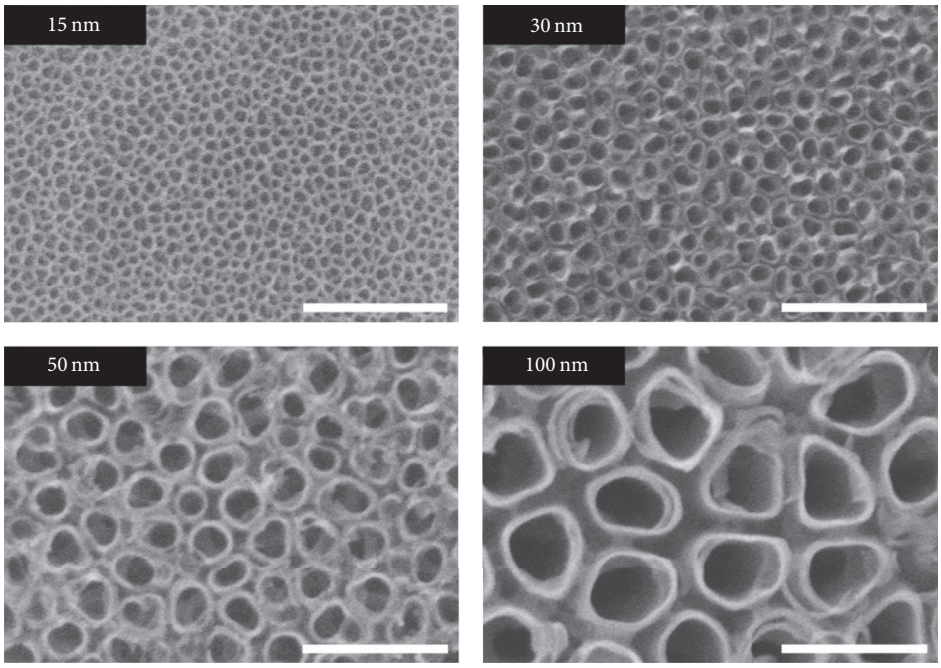

Figure 1: SEM images of vertically oriented TiO_2 nanotubes of different diameters. Scale bars: 200 nm. Reproduced from [53] with permission from The Royal Society of Chemistry.

Through anodization highly ordered porous TiO2 nanotubes with great uniformity, controllable pore size can be obtained by controlling of some parameters, like electrolyte composition, anodization time, anodization voltage, electrolyte temperature during anodization, interelectrode spacing, metal substrate, pre- and posttreatments, and annealing. For a detailed analysis of these parameters see the review by Kulkarni and Coll. [63]. An advantage of the anodic oxidation process is that entities from the electrolytic solution, such as calcium, can be incorporated in the oxide layer [62]. Moreover, through the anodization process, titanium surfaces can be covered by a thin layer of another material, like silver, which possesses antibacterial properties [67]. An in vivo study compared three surfaces: a machined surface, an HCl-treated surface, and an HCl-treated, anodized surface. The HCl-treated/anodized surface showed superior results regarding bone density, number of trabeculae, pull-out forces, and BIC [68]. There is a growing body of data that demonstrates the benefits of using TiO_2 nanotubes for enhanced orthopedic implant surfaces [69].

2.3.2. Acid Oxidation or Peroxidation. The rationale for using acid oxidation and peroxidation to nanoscopically texture metals is to permit simultaneous etching and oxidation of the surface in a controlled manner [17]. For orthopedic implantology, titanium is generally used in these processes, but other metals such as tantalum (Ta) or chromium-cobalt-molybdenum alloys (CrCoMo) have also been used [50]. For dental implantology, it is possible to obtain nanopits, sized 20–100 nm, on the titanium surface with a treatment of strong acids, such as H_2SO_4/H_2O_2 or HCL/H_2O_2 or HF/H_2O_2 [53] (Figure 2). Moreover, varying the acidic (or basic) component in mixtures containing H_2O_2 appreciably changes the nanopatterns on Ti surfaces [70]. H_2O_2/HCl processing adjoined to adequate thermal cycles produces a titanium gel coating that favors apatite deposition in vitro [71]. Oxidation in H_2O_2/0.1 M HCl at 80°C resulted in a good adhesion of arginine-glycine-aspartic acid peptides as well as the calcified matrix formation of rat bone marrow stromal cells [72]. Several studies demonstrated the ability of HF acid, when treated on TiO_2 grit-blasted Ti implants, to enhance the osteoinductive function, differentiation from mesenchymal stem cells into osteoblasts, and early osseointegration [30, 35, 73, 74]. H_2SO_4/H_2O_2 creates a nanopattern which has been demonstrated in vivo to be linked with an enhanced proliferation of essential osteoblastic cells and the simultaneous inhibition of unwanted fibroblastic cells [75]. It has also been observed that H_2SO_4/H_2O_2 oxidative nanopatterning promoted the differentiation of stem cells. Unlike the etching of Ti with H_2SO_4/H_2O_2, treatment with NH_4OH/H_2O_2 created nanoporosity without increasing the thickness of the original oxide layer and did not increase the osteoinductive properties [70].

2.3.3. Alkali Treatment. Implant surfaces treated with NaOH produce a nanoscale topography which exposes the surface active groups through the formation of a sodium titanate gel [76]. This gel can turn into anatase by simple hydrothermal treatment, giving rise in the authors opinion to highly bioactive surfaces [77].

Surfaces obtained by various technologies can be treated with NaOH to increase their reactivity. For example, nanotubes treated with NaOH obtained by anodization, or macroporous Ti surface layer formation by plasma spraying,

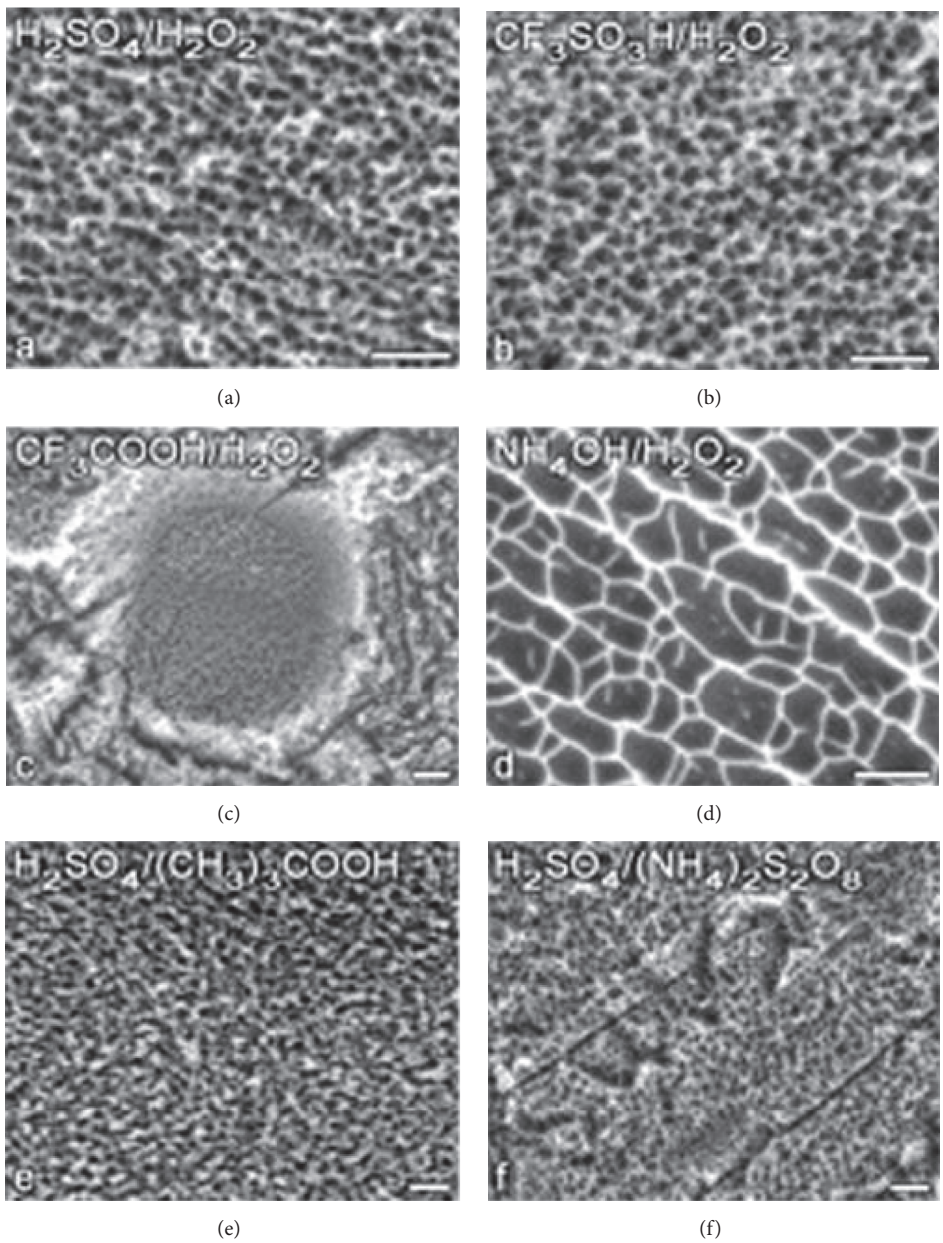

Figure 2: Characteristic SEM images of Ti surfaces nanostructured by oxidative etching with different solutions (scale bar 100 nm). Reproduced from [53] with permission from The Royal Society of Chemistry.

are converted into a bioactive sodium titanate layer that can induce the formation of a bone-like apatite in simulated body fluid [37, 78]. NaOH-treated surfaces can subsequently be silanized to achieve antibacterial features [67]. NaOH treatment in combination with heat treatment produces nanotrabecular and nanotuft-like structures and enhances osteoconductivity [79].

2.3.4. Alkali-Acid Treatment. Titanium surfaces can be altered by sequential treatment with NaOH, HCl, and thermal cycles leading to a titanium oxide layer of anatase and rutile phases that was shown, in vitro, to possess high apatite-forming abilities [80, 81].

2.4. Coating Techniques

2.4.1. Sol-Gel Coating. A solution from alkoxides, metal salts, or other suitable precursor is deposited on the substrate by either dip, spin, or spray coating. Exposed to water, hydroxides or hydrated oxides form and gelation occurs, and further heating produces an oxide layer without residual organic material. The sol-gel coating method enables the coating of an implant surface by a layer of sol-gel containing hydroxyapatite, which is stabilized through thermal cycles [82–85]. The coating can also contain fluorohydroxyapatite or fluorapatite to increase the biological activity and reduce resorption rate of the coated layer [86–88]. Sol-gel derived nanoporous TiO_2 coatings have been proven to enhance soft

tissue attachment in rat and dog models [89–91]. Similarly, in a human experimental study, results indicated that a significantly greater proportion of oral mucosa came into contact with the nanoporous TiO_2 surface than that with the unmodified surfaces [92]. This technique is also used to coat bone substitutes with a nanolayer of osteoconductive material [93].

2.4.2. Self-Assembly of Monolayers (SAMs). Molecular self-assembly allows for the deposition of a self-assembled monolayer onto a substrate and subsequent exposure of an active terminal group that promotes specific biological functions such as the adhesion of protein and cells [94] or bone deposition [95]. This technique sounds promising because it allows obtaining a selective adhesion to the surface. For example, osteoblasts can be favored and bacteria disadvantaged. In vivo results, however, showed contradictory data. In fact, although BIC was significantly higher in SAMs than in standard titanium surfaces at two weeks, at four weeks the traditional surface showed a greater BIC. Therefore, further studies should be conducted before this technology can be used in humans [95].

2.4.3. Plasma Spray. Plasma spray coatings are used in a wide range of industries, such as the automotive, aerospace, and petroleum industries, to name a few [96]. With this technology, the coating material, generally a powder, is heated to high temperatures for a very short period of time and is then accelerated against the substrate, where the coating is then formed [97]. Plasma spray technology allows a substrate to be coated with various types and layers of different materials and to obtain an array of nanostructures with features smaller than 100 nm. Hydroxyapatite is a frequently used coating material in implant dentistry [98] due to its ability to stimulate rapid biological responses [99–103]. Problems with this technology are related to the production process, where high temperatures are required. This leads to the modification of hydroxyapatite to generate the more unstable, water-soluble products of CaP, α-tricalcium phosphate, and β-tricalcium phosphate [98, 99, 104–107]. The coating is not very adherent to the substrate and can easily degrade, losing its biological stability [98]. Moreover, problems related to strength and resistance to fatigue may arise. Similar to the plasma spray technique, the high velocity of oxy-fuel combustion spraying allows for the formation of a coating layer of hydroxyapatite powders. This technique also has limitations arising from aqueous solubility and coating detachment [108].

2.4.4. Ion Beam Deposition. Ion beam deposition can be applied to overcome the limitations of the plasma spray technique by depositing of a very thin layer of osteoconductive bioceramic material onto the implant surface. The very thin layer could allow for a more gradual exposure of the implant surface, which is more aligned with the rate of bone growth [98, 109].

2.4.5. Pulsed Laser Deposition. Coating with hydroxyapatite layers can also be obtained through laser treatments, which increases the bond strength between the coating and the substrate [110]. This method allows coating a substrate with hydroxyapatite vapor obtained by heating an hydroxyapatite target with laser. Quality of the coat depends greatly on processing parameters and the whole procedure is not plain.

2.4.6. Electron Beam Evaporation. High energy electron beam bombardment on the source material to be deposited produces large amounts of heat that vaporize atoms and molecules of the coating material. The generated vapor is directed to the top of the vacuum chamber and coats the substrate, generating a layer that masks the original surface. A study reports for this technique a rate of deposition of 3.0 Å/s and at a thickness of 500 nm, obtaining a coating that contains nanosize grains [111].

2.4.7. Deposition of a Supersonic Beam of TiO_x Clusters. This technique is based on the ablation of a titanium rod by a helium plasma jet, ignited by a pulsed electric discharge. TiO_x ions condensed to form clusters form a seeded supersonic beam, which is collected on a substrate located in the beam trajectory [112]. This surface demonstrated the capability to promote human bone marrow mesenchymal stem cells osteoblast in an in vitro study [113, 114].

2.4.8. Discrete Crystalline Deposition. This method allows for the deposition of discrete calcium phosphate nanoparticles (20–100 nm) on a titanium implant with predisposing substrate microtopography produced by acid etching. This is done to obtain a bioactive surface and an increased mechanical interlocking with the surrounding bone. The deposition is achieved by immersing the metallic implants in a suspension of calcium phosphate nanoparticles followed by thermal cycles to result in approximately 50% of the metallic surface being covered by the nanoparticles, with the remaining surface being metal oxide [115].

2.4.9. Electron Beam Lithography. Electron beam lithography has been used to fabricate ultra-precise nanotopographies of ordered arrays less than 10 nm in size [116]. The advantages of this method are illustrated by recent studies comparing the effects of periodic arrays of nanopores created by electron beam lithography with those of disorganized arrays of the same nanopores. The results indicated that the haphazard arrangement was better for stimulating the differentiation of human mesenchymal stem cells into osteogenic cells [117].

2.4.10. Lithography and Contact Printing. Colloidal lithography has been used to fabricate controlled, well-defined nanoscale surface topographies [118–120]. Titanium-coated hemisphere-like topographical nanostructures sized 50–200 nm are able to influence the morphology, proliferation, and osteogenic differentiation of human mesenchymal stem cells [121]. Recently, a significant enhancement in bone formation was detected on Ti implant surfaces modified by 60 nm hemispheres, suggesting that bone formation is in fact dependent on the size of the nanofeatures [122]. Moreover, an implant with hemispheres of 80 nm was found to attenuate

the inflammatory response while enhancing mineralization during osseointegration [123].

2.5. Biochemistry. Surface characterization at nanoscale level can alter also the chemical organization of matter. Some study with X-ray diffraction identified altered crystal structures in nanophase compared to conventional titania compacts. Conventional titania possessed an exclusively rutile phase, while nanophase titania was a mixture of anatase and rutile [124]. Since ceramic crystal structure as well as surface roughness promotes osteoblast function [125], nanophase titania can improve bone formation.

2.6. Biomimetics. The ability to control 3D features at nanolevel allows better replication of natural cellular environment, which is regulated by molecular behavior at nanoscale. For example, the major inorganic component of bone, hydroxyapatite, is between 2 and 5 nm in width and 50 nm in length while the major organic component of bone, collagen I, is a triple helix, 300 nm in length, 0.5 nm in width, and has a periodicity of 67 nm [125]. Also the surface roughness of natural bone possesses nanometric features and nanophase titania can better replicate them. A study evaluated the roughness of bone, conventional titania, and nanophase titania [124]. The root-mean-square surface roughness values of bone are in the range of 25–32 nm, while those of conventional titania and nanotitania are in the range of 11-12 nm and 22–29 nm, respectively.

2.7. Nanotopography Altered Cellular Responses. Nanostructured surfaces affect the mechanical properties of cells and may provide new methods for altering the response of cells to external signals [120, 124, 126–128]. Surface nanotopographies and chemistries confer to the material unique properties that modify cell adhesion processes by direct (cell–surface interactions) and indirect (affecting protein–surface interactions) mechanisms [47].

2.8. Protein/Surface Interactions: Surface Wettability. Protein adsorption has been found to be a key factor in determining the peculiar behavior of cells on nanostructured surfaces [129, 130]. By modifying the surface energy or wettability of the surface, the adsorption of proteins is influenced, and the interactions of cells with the surface can therefore be altered.

The adsorption of fibrinogen and platelet adhesion, for example, is inversely related to the hydrophilicity of the surface [94, 131]. In a study, the authors found that cells were unable to adhere to hydrophobic surfaces previously coated with albumin but could easily adhere to hydrophilic surfaces covered with albumin [132].

The initial protein–surface interaction may explain the greater adhesion of osteoblasts on nanoscale substrates as a critical aspect of the osseointegration process. Surface effects are often mediated through integrins, with the vitronectin receptor being the predominant form of integrin receptor involved in substrate adhesion [133]. Vitronectin is strongly adsorbed onto nanoscale substrates [134], contributing to the enhanced adhesion of osteoblasts. Proteins that contain the Arg-Gly-Asp (RGD) attachment site, together with the integrins that serve as receptors for them, constitute a major recognition system for cell adhesion [135]. The distribution and density of RGD regulate effective cell spreading, formation of focal adhesions [136], and cell motility [137]. Surface changes that modify the RGD binding have been suggested to be a key factor in altering osteoblastic differentiation [138]. However, the relevance of this mechanism is still controversial. For example, Cai et al. reported that the absorption of albumin and fibrinogen and the proliferation and viability of osteoblasts did not differ in titanium surfaces with surface roughness values of 2 to 21 nm. This indicated that albumin and fibrinogen adsorption processes were unaffected by changes in roughness within that range [139].

2.9. Cell Adhesion, Spreading, and Motility. Nanoscale features play a critical role in both cell adhesion and cell motility. Reactions of cells to surface topographies include changes in cell orientation, cell motility, cell adhesion, and cell shape [140]. As previously described, integrin receptors modulate the adhesion between cells and extracellular matrices. In addition, integrin activation can affect cell functions such as motility, proliferation, and differentiation by initiating multiple intracellular signaling pathways [141]. Cell motility may be regulated by varying the ligand spatial presentation at the nanoscale level, and integrin ligand clustering is required to support cell locomotion [137]. An increased surface charge density at highly curved surfaces and sharp edges can explain the mechanism of adhesion between the negatively charged titanium surface and a negatively charged osteoblast, mediated by charged proteins with a distinctive quadrupolar internal charge distribution, fibronectin, and vitronectin [142, 143]. Nanofeatures of an alloplastic surface may have unique attributes affecting cell interactions. Both the dimension and the density of nanofeatures can affect the cell behavior [140]. Currently, the smallest dimension recognized by a eukaryotic cell is approximately 10 nm [144].

On flat titanium surfaces containing submicrometer-scale roughness produced by chemical etching or porous anodization, cells exhibit a greater thickness and delayed appearances of focal contacts compared to smooth, polished, and electropolished surfaces. Submicrometer-scale roughness favors the formation of long, numerous filopods. In an in vitro study on titanium substrata, the addition of nanotopography onto microtopography led to the presence of more focal contacts distributed throughout the entire cell surface. Microscale and nanoscale roughness exert synergistic effects on cell proliferation [145]. The growth and spatial orientation of cells were controlled in vitro by substrates with nanoscale features, showing that substrates with specific nanofeatures can in fact direct cell growth [146–148].

In this respect, there are, however, dissenting opinions. In studies examining various substrates with 24, 50, 200, and 1500 nm grain size, Dulgar-Tulloch et al. observed that human mesenchymal stem cells (HMSCs) adhesion on alumina and hydroxyapatite were significantly reduced in the materials with 50 and 24 nm surfaces, as compared with the 1500 and 200 nm surfaces. Conversely, the adhesion on titanium substrates was found to be independent of grain size [149].

In another study [117], highly ordered nanotopographies produced low to negligibly low cellular adhesion and osteoblastic differentiation, whereas nanodisplaced topographies significantly increased the osteospecific differentiation. This suggests that the use of disorder may be an effective strategy in the development of materials for regenerative medicine and tissue engineering.

2.10. Proliferation. Nanoscale features can affect cell proliferation, although experimental evidence is not univocal. Several studies indicated that nanostructures on titanium oxide surfaces could enhance cell adhesion and proliferation [112, 144, 150].

In a study [148], titanium surfaces with microdefects produced by photolithography and nanodefects produced by acid etching or anodizing and combinations of these surfaces were compared to assess the response of osteoblasts to nano-, micro-, or combined surfaces. Acid etching produced a peaky and pointy morphology, whereas anodizing produced a sponge-like surface with belt-like rings around the pore openings. Smooth surfaces showed the highest cell proliferation, while nanofeatured surfaces showed the highest osteoblast differentiation and osteogenic activities [151].

Webster et al. evaluated the activity of osteoblasts and osteoclasts cultured on nanophase ceramics and compared them to conventional ceramics [39, 152]. They observed a lower surface occupancy of osteoblast colonies, as well as a greater cell proliferation, osteogenic activity of osteoblasts, and activity of osteoclasts on nanophase ceramics.

In a previous study done [149], HMSC proliferation was minimal on 50 and 24 nm substrates of any chemistry tested and thus significantly lower than the densities observed on either the 1500 or 200 nm surfaces after three or more consecutive days of culture. Furthermore, HMSC proliferation was enhanced on the 200 nm substrates, compared with results obtained on the 1500 nm substrates after seven or more days of culture. Moreover, rat osteoblast and fibroblast adhesion and proliferation exhibited similar trends to those of HMSCs on all substrates tested.

Realistically, nanofeatured surfaces allow for more effective cell adhesion to the substrate, reducing expansion and cell flattening and therefore reducing the surface coverage. However, cells grown on the nanosurfaces have a greater differentiation and osteogenic activity.

2.11. Selectivity of Adhesion. An important observation is that nanosurfaces can selectively promote cell adhesion. An example of this selective response was described by Dulgar-Tulloch et al. in studies examining a nanophase ceramic/polymer composite that was effective in promoting osteoblast adhesion but prevented and/or minimized fibroblast adhesion [153]. In another study, this difference was not observed [149]. Moreover, nano- and submicron surface features reduced the adhesion of and proinflammatory cytokine release from macrophages and immune cell responses after implantation [154].

Notably, nanophase ZnO and TiO_2 may reduce *Staphylococcus epidermidis* adhesion and increase osteoblast functions necessary to promote the efficacy of orthopedic implants [155]. The importance of selective adhesion will further be discussed in the bacterial accession section.

2.12. Differentiation. Some studies have shown that increased cell differentiation takes place on the nanoscale surface [30, 156]. Huang et al. evaluated the response of osteoblast-like cells on microcrater, nanoplate, and nanoleaf surfaces obtained by microarc oxidation [157]. The adherent cells were polygonal-shaped on the microcrater surface, roundish on the nanoplate surface, and elongated on the nanoleaf surface. The distortion of cell nuclei was noticed only on nanoleaf surfaces. Compared with microcrater surfaces, nanoplate surfaces slowed down cell proliferation, while nanoleaf surfaces supported it. Cell differentiation was enhanced on nanoleaf surfaces compared with those on the other two surfaces.

Aniket et al. [158] investigated the effects of nanoscale roughness and the chemistry of bioactive silica-calcium-phosphate nanocomposites (SCPC50) coated on Ti-6Al-4V implants and proved that nanoscale surfaces lead to early osteoblast differentiation. Cell attachment was higher on SCPC50-coated substrates compared to the uncoated controls; however, cells on the uncoated substrate exhibited greater spreading and superior quality of F-actin filaments than cells on the SCPC50-coated substrates.

According to another study [159], differentiation is not the only variable to take into account, but the quality of mineralized tissue being developed is also important. Enhanced osteogenic cell differentiation on modified titanium is not a sufficient indicator of enhanced in vitro mineralization.

Some techniques allow for the correction of bone defects by grafting adult mesenchymal cells [160]. The use of nanofeatured surfaces, in association with these bone regeneration techniques, could allow for better new-bone formation and ultimately improve osseointegration and implant success.

2.13. Bacterial Adhesion. Unlike failure in reaching osseointegration, which involves very limited implants and arises within a short time [161, 162], peri-implant infections can occur a long time after implant placement. In this circumstance, in addition to implant failure there is also the prosthetic failure to be taken in consideration. Notwithstanding that some studies reported extremely high peri-implant infection rates, exceeding 80% of the implant population [163–178], recently those studies were questioned [179] and the estimate of real prevalence of the pathology was lowered. Those studies were biased indeed by several defects such as variety of definition of the pathology, discrepancy between the extremely high prevalence of the pathology and the still extremely high rate of success at long time, difficulty to obtain an exact diagnosis with clinical examination and X-rays, and inability to compare different protocols [179]. Furthermore, it is not clear if the peri-implant infection is the primary cause of bone resorption or the result of different pathologic processes, like residual cement in peri-implant sulcus, mistakes in surgical protocol or prosthetic restoration, metabolic disease, and smoke habit. Thus, a realistic estimate of long-term prevalence of peri-implant infections is around 1-2% [179]. Currently, a frequent, professional plaque removal

protocol is considered as an appropriate strategy for the prevention of peri-implant infections [180].

Consequently, research in antibacterial properties is not less promising than in enhancing osseointegration. Nanofeatured surfaces modified to reduce plaque build-up and the promotion of plaque removal may be an effective long-term strategy for reducing true peri-implant infections.

The relationship between these diseases and bacterial plaque accumulation is well established [181–195]. Bacterial adhesion occurs in four phases: transport of the bacterium to the surface, initial adhesion with a reversible and irreversible stage, attachment by specific interactions, and, finally, colonization to form a biofilm [196]. Following the primary attachment of single cells, bacteria start to colonize and grow on the implant surface developing multilayered cellular biofilms. These microbial communities are highly organized and structured. They are embedded in extracellular polymeric matrices that develop into maturation, achieving virulent and pathogenic features [197, 198].

Bacterial communities are important to note because they emphasize the ability of bacteria to self-organize and resist environmental perturbations since they are more effective cooperatively than in isolation and respond to environmental changes as a unit rather than as single entities [199].

It is postulated that biofilms contribute to antibiotic resistance by at least three mechanisms: reduced penetration of antibacterial drugs across the extracellular polymeric substance, a favorable environment within the inner layers, and bacterial cell differentiation and role specialization providing increased protection. These mechanisms permit the microbial community to resist antibiotic concentrations 1000-fold higher than those necessary to eradicate planktonic populations [200, 201].

Differences in sulcus microbiota composition were observed between successful and compromised implants. From a high proportion of coccoid bacteria, with a low proportion of anaerobic and aerobic species, a small number of Gram-negative species, and low detection of periodontal pathogenic bacteria in healthy sites, the microbiota shifts to mainly Gram-negative anaerobic rods and spirochetes in diseased site [202–204]. Successful implants are colonized principally by oral streptococci, capnocytophagae, *Veillonella parvula*, *Peptostreptococcus micros*, and *Fusobacterium nucleatum* [197].

Communication among different species within biofilms appears to be the key process in explaining how plaque can act as a single unit, and how specific types of bacteria emerge and impair the balance within the host [205]. Bacteria tend to agglomerate in clusters according to their nutritional requirements [197, 206]. The species within complexes are closely associated with each other and precise connections are also established between certain complexes. Bacteria belonging to green and yellow clusters are associated with healthy tissues, while the red and orange clusters are detected at pathological sites [205].

The bacterial plaque composition is affected by periodontal conditions: in partially edentulous patients, more motile rods and spirochetes were found [197, 207]. In a clinical study comparing de novo plaque formation around implants and teeth, colonization by periodontal pathogens was observed two weeks after abutment connection in the peri-implant sulcus, and after three months, the microbiota was strikingly similar for the implants and teeth [207].

Ideally, to enhance the success of implants, their surfaces should inhibit bacterial colonization and concomitantly promote osteoblast function. Bacterial adhesion to biomaterials has previously been linked to factors such as the surface free energy [196, 208, 209], chemical composition, and physical characteristics of the surface including material surface irregularities and roughness [209–212]. Studies of bacterial colonization on nanostructured titanium surfaces demonstrated improved colonization efficiency when the surface roughness was increased [209, 213].

Since the size and rigidity of osteoblasts and bacteria are different, the idea of using surface topography to selectively control the response from osteoblasts and bacteria seems attractive. In principle, the surface topography could alter the response of bacteria and osteoblasts, since their rigidity and sizes are different [144]. Rough surfaces allow for increased biofilm formation compared to smoother implants [144, 196, 212, 214–216].

Puckett et al. have recently studied the correlation between bacterial adhesion and the spatial organization of nanofeatures of different shapes and sizes on TiO_2 surfaces [209]. The study showed that nanorough titanium surface produced by electron beam evaporation allows the lower bacterial adhesion and nanotubular and nanotextured surfaces produced by two different anodization processes, on the contrary, enhance bacterial adhesion more than a conventional smooth surface. Therefore, it is reasonable to think that very rough surfaces, such as nanotubular ones, which allow a large osseointegration, also enable an enhanced bacterial growth. On the contrary, it was proven that reducing the roughness below to 0.2 μm did not significantly influence the microbial composition (not adhesion) [212, 217, 218].

Smooth, turned (TU) titanium, nanoporous TiO_2 coated (SG), and anodized Ca^{2+} modified (OC) surfaces have all been shown to be suitable not only for osseointegration but also for soft tissue healing [92, 219]. Titanium surfaces modified with ion implantation (Ca+, N+, and F+), oxidation (anode oxidation, titanium spraying), ion plating (TiN, alumina), and ion beam mixing (Ag, Sn, Zn, and Pt) with Ar+ were compared to polished titanium. F+-implanted specimens were significantly more effective in inhibiting the growth of both *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*. Other surface-modified specimens showed comparable responses with polished titanium for adhesion and bacterial growth [208].

In an in vitro study, three surfaces (turned titanium (TU), nanoporous TiO_2 coated (SG), and anodized Ca^{2+} modified (OC) surfaces) were evaluated for their ability to adhere bacterial colonies of *Streptococcus sanguinis* and *Actinomyces naeslundii* [220]. The presence of nanotopographic alterations of the implant surfaces did not cause significantly greater accumulations of bacteria compared to smooth surfaces. The OC surface has greater microtopographical structures than the others, although all three are classified as smooth (i.e., Sa $< 0.5\,\mu$m) [23]. In addition, at

the OC surface, the bacterial biofilm volume was, to some extent, greater than the other two surfaces. The presence of saliva, with its own glycoproteins, increased 11-times the capacity of the tested bacteria needed to adhere. The complexity of the microbiotic ecosystem likely increases the bacterial capacity of adhesion to surfaces due to the possible intensive action among different bacterial species [221].

Titanium surfaces modified with poly(methacrylic acid) brushes and silk sericin have been shown to reduce the adhesion of *S. aureus* and *S. epidermidis* and also promote osteoblast adhesion, proliferation, and alkaline phosphatase (ALP) activity [222].

A recent study on ZnO and TiO_2 nanoscale surfaces showed a reduction in the adhesion of *S. epidermidis* compared with microstructured surfaces. Moreover, increased osteoblast adhesion, ALP activity, and calcium mineral depositions were also observed [155]. Similarly, Colon et al. found that *S. epidermidis* adhesion on nanostructured ZnO and TiO_2 was less than that observed on the microphase formulations [155]. Reduction of *S. epidermidis* adhesion to 80 nm nanotube surfaces was reported in other studies [223].

In recent experiments, biofilm development on newly designed laser-modified titanium implant surfaces was evaluated and compared with that of conventional sandblasted titanium used in implant dentistry. Results showed a significantly lower total volume of the biomass on laser-modified surfaces, while no significant changes in live/dead bacteria were noticed between materials [224].

Results contradicting these findings were reported by Singh et al. [225] in studies examining protein adsorption, bacterial adhesion, and biofilm formation on nanostructured titanium thin films with a root-mean-square surface roughness (Rq) ranging from 16 to 32 nm. The increase in surface roughness presented a positive correlation with protein absorption and was inversely proportional to bacterial adhesion and biofilm formation. As a possible explanation of these findings, it was postulated that the rougher surfaces may increase the amount of adsorbed proteins, passivating the surfaces and inhibiting cell attachment. This would imply that the mechanisms underlying protein adhesion enhancement are more complex than a simple geometrical amplification of increased available surface being due to increased surface roughness.

Lin et al. [226] examined the bacterial adhesion and biofilm formation on quaternized chitosan-loaded titanium nanotubes of 200 nm length and various diameters (80, 120, 160, and 200 nm). Chitosan, and in particular quaternized chitosan, has been unequivocally proven to be inherently antibacterial, due to its chemical structure (cationic nature) [227–229].

The authors found that quaternized chitosan-loaded surfaces can significantly inhibit bacterial adhesion and biofilm formation compared with smooth surfaces. A major limitation in this study is the remarkable solubility of quaternized chitosan, in that more than half of the initially loaded quaternized chitosan dose was released within five days, while antibacterial activity should be extended over several years to be clinically effective.

A silver-modified surface and a silanized titanium nanofeatured surface were evaluated in a dog model of experimental peri-implantitis induced by ligature placement. The two surfaces showed a superior response with regard to bacterial accumulation compared with traditional titanium surfaces [67].

3. Regulatory and Safety Issues

A large variety of products obtained through nanotechnology, including more than 50 medicinal agents of pharmaceuticals, diagnostics, and biomaterials, have already been approved by regulatory authorities for clinical use. This indicates that a consolidated regulatory framework is applicable for assessing the biological and clinical profiles of these agents, as well as their safety and biocompatibility.

Current regulation of nanomedicines, including medical devices, is essentially based on criteria and study design requirements similar to those used for conventional materials. According to these regulatory bodies, there is no need for a new set of guidelines specific to nanomedicines. If research identifies toxicological risks that are unique to nanomaterials, however, additional studies and applications of ad hoc methods may become necessary. In addition, a risk-management system should be implemented, including risk minimization and postmarketing surveillance programs. These should become mandatory requirements for all kinds of medical applications using nanotechnology [230].

The regulatory status of titanium implants falls within the borderline of tools for regenerative medicine and medical devices, especially those devices used for materials in which a nanoscale surface coating is incorporated to promote bone growth and osseointegration. Currently, there are several knowledge gaps regarding the long-term effects of nanoscale titanium implants and uncertainties on how the safety characteristics of these implants should be evaluated.

3.1. Toxic Effects of Titanium-Based Nanomaterials. Several studies have indicated that titanium dioxide nanoparticles, when absorbed in the body, can interact with cell constituents in a manner different than conventional molecules [231]. These nanoparticles were proven to possess pronounced surface reactivities and unique modes of action in biological systems, due to their propensity to generate free radicals and induce oxidative stress. Consequently, a range of cellular and molecular modifications may develop, including proinflammatory gene activation, apoptotic changes, genotoxicity, abnormalities in cell adhesion and migration processes, cell proliferation, immunogenic effects, and prothrombotic effects.

Pure titanium and titanium alloys have inherent corrosion resistance, and they are considered nontoxic. However, limited information exists on the corrosion characteristics of nanoscale titanium implants. There is some evidence indicating that metallic implants may undergo surface degradation and may become the site of reactions that lead to the release of metal ions to surrounding tissues. This process may cause peri-implant inflammatory reactions and cytotoxicity [232, 233]. It has been suggested that a combined action of

oxidative stress and "foreign-body" phenomena associated with the release of titanium nanoparticles from surface coatings may induce host immune responses, autophagic changes, and lysosomal dysfunction [234, 235]. Furthermore, high concentrations of titanium dioxide nanoparticles have been shown to cause dose-dependent proinflammatory effects on human gingival fibroblasts [236]. Internalization of titanium dioxide nanoparticles associated with cellular changes has been observed in studies on human osteoblast cells [237].

Systemic effects manifested as cardiac alterations have recently been described in rats treated with nanotitanium [238]. Certainly, the clinical relevance of the toxicological data is still to be demonstrated. Most observations come from studies using cell cultures or from in vivo assays with laboratory animals exposed to extremely high doses of titanium dioxide nanoparticles by oral gavage or inhalation [231]. Furthermore, most experiments on the potential of nanotitanium to induce toxicity have addressed specific situations unrelated to dental implantology, for example, risks associated with the pulmonary absorption of titanium nanoparticles in occupational settings or the dermal application of sunscreens containing nanotitanium. This is a relevant point since the toxic reaction occurring at the material-tissue interface is a function of the specific tissue where the interface is created.

Isolated reports of allergic reactions to titanium dental implants have been published [239]. Based on current knowledge, however, there is no scientific evidence of nanodependent toxicity occurring in humans after the application of titanium implants.

3.2. Safety Testing. In a recent monograph [240] TiO2 is referred to be possibly carcinogenic to humans based on sufficient evidence in animal model and insufficient evidence in human model. However these considerations refer exclusively to titanium particles used as pigment. Dental implant is not nanoparticles inserted in human body; conversely it is a macroscopic titanium bulk with nanometric surfaces. Although it is possible that mechanical wear and/or surface corrosion produce solid particulate wear debris and soluble forms, this occurrence is reported only from the bearing site of orthopedic prosthesis [63, 241, 242]. There is no evidence of particulate release from dental implants, which by definition are immobile within the bone.

Because of the peculiar biological properties of titanium nanoparticles, it is not completely clear how experimental studies should be designed in order to assess the safety and biocompatibility of nanoscale implants. Regulatory documents on the assessment of the adverse effects of nanomaterials used in medical devices have recently been published [243, 244].

Biocompatibility testing standards exist that specifically cover dental materials, such as ISO 7405 and ISO 10993 [245]. Several types of short-term or long-term tests, including cell culture assays, in vivo studies in animals, implantation tests, and clinical trials, have been proposed to assess the safety and biocompatibility of new dental materials.

Current evidence suggests that the biological response to dental implants may vary depending on the device category, the tissue contact location, and the contact time. Implantation assays in which the test material remains in place for 1-2 years before being examined have been proposed to assess the possible long-term effects of permanent implants such as chronic inflammation, propensity of infection, blood interaction, fibrogenic cell function, and oncogenicity [245–247]. It is not clear if these evaluation methods may be suitable for assessing the biocompatibility of nanoenabled dental implants.

4. Conclusions

Nanotechnology can lead to significant changes in the physicochemical and topographical characteristics of titanium surfaces by influencing the surface free energy, wettability, and absorption of proteins. Different technologies have been developed to control the surface characteristics of titanium at nanometric levels. The validity of these methods is not easily comparable because of the great discrepancies in processes and mechanisms implicated in the surface modifications. Biological molecules, ions, and cells can interact with these nanomodified surfaces by complex mechanisms and can affect the osseointegration process in different ways. Nanoscale surface modifications can increase the affinity to the bone at the bone-implant interface by promoting bone formation and enhancing BIC. Studies on cell cultures have identified nanoscale topographical features that promote adhesion and bone induction in osteoprogenitor cells. Titanium nanomaterials possessing such characteristics could represent a valuable tool for increasing osseointegration, particularly in patients considered at risk for bone qualitative deficits. Some modified surfaces with nanoscale features are already available, namely, those modified with hydrofluoric acid or with calcium phosphate.

The application of nanotechnology to implant surfaces may significantly contribute to the prevention of peri-implant infections. Studies have demonstrated that surface characteristics can influence the adhesion and growth of the bacterial microflora, and convincing experimental evidence is currently available suggesting that nanometric changes can modulate these properties. In perspective, the introduction of nanoenabled implant surfaces can be expected to exert a positive influence in implantology, by improving osseointegration and reducing peri-implant infection rates.

Although the advantages associated with the use of nanoscale titanium are supported by a large body of experimental observations, the potential risks and risk-benefit balance of nanotitanium have yet to be evaluated, especially in long-term studies. The development of new test methods is needed to assess the modes of action, biocompatibility, and long-term effects possibly associated with prolonged tissue contact of nanoscale titanium implants. Research priorities should also include detailed studies to demonstrate the superiority and real clinical advantages of nanoscale titanium as a cost-effective tool compared with traditional materials.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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