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REVIEW

Titanium oxide antibacterial surfaces in biomedical devices

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ABSTRACT

Titanium oxide is a heterogeneous catalyst whose efficient photoinduced activity, related to some of its allotropic forms, paved the way for its widespread technological use. Here, we offer a comparative analysis of the use of titanium oxide as coating for materials in biomedical devices. First, we introduce the photoinduced catalytic mechanisms of TiO_2 and their action on biological environment and bacteria. Second, we overview the main physical and chemical technologies for structuring suitable TiO_2 coatings on biomedical devices. We then present the approaches for in vitro characterization of these surfaces. Finally, we discuss the main aspects of TiO_2 photoactivated antimicrobial activity on medical devices and limitations for these types of applications.

KEY WORDS: *Biomaterial surface modifications, Titanium oxide, Antibacterial properties, Infection-resistant materials*

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TITANIUM OXIDE AND ANTIBACTERIAL SURFACES IN BIOMEDICAL DEVICES

Device-related infections: a clinical demand driving material science research

An increasing number of clinical procedures requires the use of biomedical devices, whose widespread presence in modern therapeutic treatments is driving the demand for better performances and longer reliability. One of the major issues of both short-term devices and implantable prostheses is represented by device-related infections (DRIs) due to bacterial colonization and proliferation (1). About half of the 2 million cases of nosocomial infections that occur each year in the United States are associated with indwelling devices (2): these infections generally require a longer period of antibiotic therapy and repeated surgical procedures, resulting in potential risks for the patient and increased costs for the healthcare system.

The planktonic bacteria that colonize a device surface tend to form a biofilm and the sessile bacterial cells, enclosed in a self-produced polymeric matrix of this kind, can withstand host immune responses and generally show extraordinary antibiotic resistance (3). Eventually, bacteria rapid multiply and disperse in planktonic form, giving rise

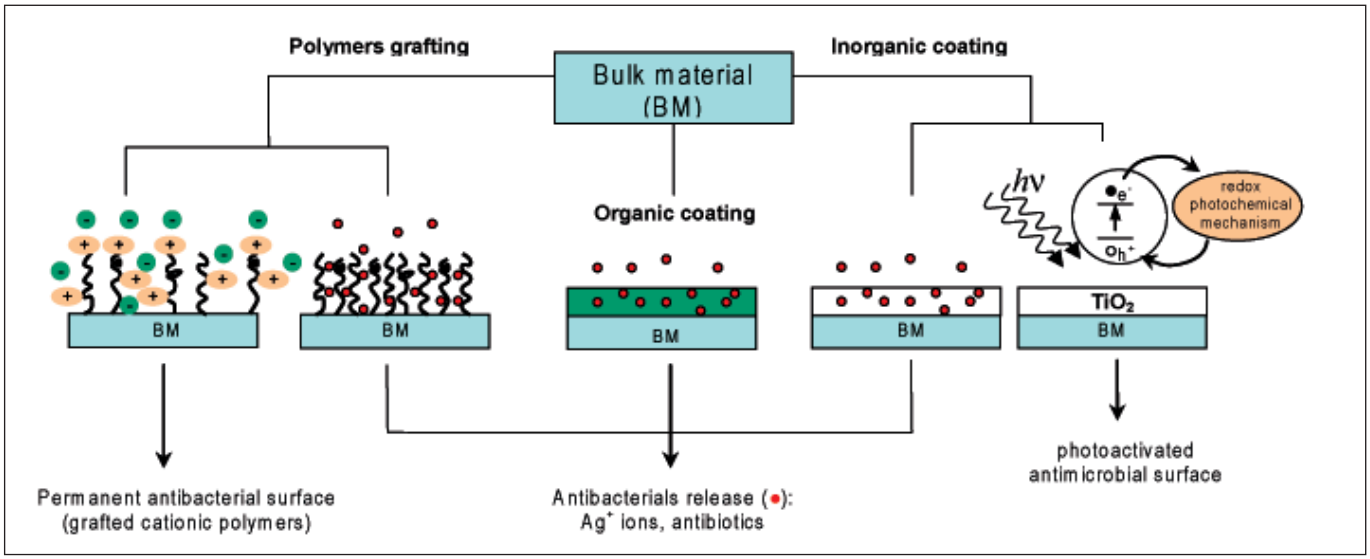

Fig. 1 - *Surface modification approaches in medical devices aimed at obtaining antibacterial properties.*

to a chronic infection that is not easy to eradicate by conventional antibiotic therapy. In some cases, there are no early signs of bacterial infections and their outbreaks are masked by the ongoing tissue inflammation due to the surgical procedure. Hence, DRI diagnosis often occurs when a full-blown infection has already damaged tissues of the host organism (1), thus making reoperation of infected implants mandatory.

Specific guideline practices have been designed with the aim of minimizing the risks of infection on biomedical devices (1-3). However, implantation procedures are increasingly performed in a population that comprises relatively older and more immunocompromised patients, with growing risks of morbidity and mortality. Although systemic therapies can be used in DRI treatment, these methods are potentially not effective due to impaired blood circulation and the need for a high concentration of antibiotics (4). A promising alternative to a conventional systemic therapy is represented by the design of device surface properties aimed at locally modulating interfacial interactions between implanted devices and host tissues. Bacterial colonization of medical devices leading to DRIs is, in fact, a surface-mediated process, one that poses challenging problems to biomaterial scientists.

The modulation of device properties via chemical composition and structure modification of the surface represents the focus of several ongoing research programs (5) aimed at improving current bulk biomaterials with a wide variety of chemical and physical patterns in order to control *in vivo* cell size, shape, spatial organization, and proliferation (6). It is important to note that for some applications the study of new surfaces is also a distinctive departure from "standard" products (7), which are mainly marketing-driven.

Several surface modification approaches aimed at introducing surface-assisted antibacterial properties have been described so far, and comprehensive and excellent reviews have been published (1, 4, 8-10): a scheme of the possible strategies for antibacterial surface coatings is shown in Figure 1.

There is a number of efforts addressed at designing surfaces that provide the release of an antibacterial agent: polymers and polymer coatings represent a potentially interesting approach when controlled drug release of organic molecules or inorganic antimicrobial compounds is envisaged (11-15). Drug release from a loaded matrix has intrinsic disadvantages involving the duration and effectiveness of the antibacterial action, since they are limited by loading and release kinetics (1). An alternative is represented by covalent immobilization of antibiotic molecules, which show the ability to prevent bacteria adhesion (16). A completely different approach is based on inorganic (or hybrid organic-inorganic) coatings, in

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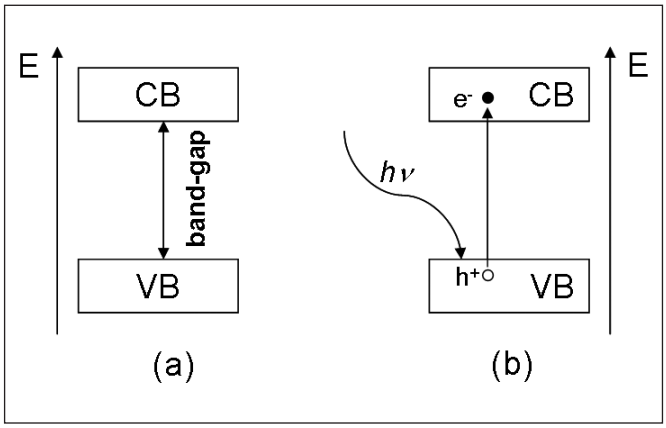

Fig. 2 - *Band energetic structure **(a)** and mechanism of photo-irradiation **(b)** of a semiconductor.*

which both an antimicrobial compound release and an intrinsic antibacterial activity are possible. In this review, we will focus our attention on the latter approach, based particularly on titanium oxide, whose antibacterial properties primarily relate to the semiconductor nature of this oxide.

Photocatalytic activity in titanium oxide surfaces

Photocatalytic activity in semiconductors

In the last three decades, photocatalytic processes promoted by semiconductors (SC) have found a widespread application in many fields, including water splitting, energy production, air and water purification, surface sterilization and organic synthesis (17-23). The reasons for this increasing interest in photocatalytic protocols are the growing demand for reducing environmental pollution and for developing more eco-friendly and cheaper processes. It is well known that a SC is characterized by a band energetic structure, with a band gap between the lower valence band (VB), entirely filled with electrons, and the unoccupied, higher energetic conduction band (CB) (Fig. 2a). When the SC is photo-irradiated by light with photon energy ($h\nu$) at least equal to the band gap, electrons present in the occupied band are photoexcited and move to the CB, leaving a positive charged hole in the VB (Fig. 2b) (24). Once formed, the electron and hole pair (e^--h^+) may undergo either fast recombination, in a time range of picoseconds, or charge trapping, if in the presence of suitable e^- or h^+ scavengers (for example molecular oxygen and water, respectively). In the latter case, a photocatalytic reaction occurs, leading to the development of a wide range of useful processes.

Titanium dioxide (titania, TiO_2) is undoubtedly the most commonly employed of the n-type semiconductors, thanks to its high photoactivity and stability, relatively low cost, and non-toxicity. Often metal-doped in order to increase the λ radiation adsorption, it is the photocatalyst of choice in organic synthesis (25-29) for the preparation of smart-materials with self-cleaning and self-sterilizing surfaces, and for environmental purification (air and water) (24, 30-32).

TiO_2 exists in three main polymorphs: anatase, rutile, and brookite. Anatase shows a band gap of 3.2 eV, corresponding to a UV wavelength adsorption of 385 nm. In contrast, rutile has a smaller band gap (3.0 eV), with excitation wavelengths that extend into the visible light range (410 nm). Nevertheless, anatase is generally considered the most photochemically active phase of titania. The reason for this higher activity should be attributed to the combined effect of the higher surface adsorptive capacity of anatase and its higher rate of hole trapping. Recently, studies have shown that mixtures of anatase-rutile or brookite-anatase were more active than anatase alone (33, 34).

Photooxidations promoted by TiO_2: the role of O_2 and H_2O

When TiO_2-surfaces are irradiated with suitable photon energies under aerobic conditions, leading to the formation of the e^--h^+ pairs (Eq. 1), molecular oxygen can act as an electron scavenger, providing the corresponding superoxide radical (Eq. 2). This reaction, which competes with the fast electron-hole recombination, represents the initiation phase of a free-radical chain promoted by water (Eq. 3), leading to the formation of hydroxyl (•OH) radicals (Eqs. 4 and 5, Scheme 1).

•OH radicals, which can also be generated via direct oxidation of water and adsorbed hydroxide ion (Eqs. 7 and 8, Scheme 2) by means of photo-generated holes (35, 36), were found to be present on irradiated TiO_2 surfaces by spin trapping experiments with EPR spectroscopy (32).

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$$TiO_2 + h\nu \longrightarrow e^-_{CB} + h^+_{VB} \quad (1)$$
$$O_2 + e^- \longrightarrow O_2^{\bullet -} \quad (2)$$
$$H_2O \longrightarrow OH^- + H^+ \quad (3)$$
$$O_2^{\bullet -} + H^+ \longrightarrow HOO\bullet \quad (4)$$
$$HOO\bullet + H^+ \longrightarrow H_2O_2 \quad (5)$$
$$H_2O_2 + e^- \longrightarrow (\bullet OH)_{ads} + OH^- \quad (6)$$

ads = adsorbed

Scheme 1 - *Mechanism of photoinduced formation of hydroxyl radical from O_2.*

$$H_2O + h^+ \longrightarrow (\bullet OH)_{ads} + H^+ \quad (7)$$
$$OH^- + h^+ \longrightarrow (\bullet OH)_{ads} \quad (8)$$

Scheme 2 - *Mechanism of photoinduced formation of hydroxyl radical from water.*

Once formed, these highly reactive radical species seem to play a key role in promoting the indirect oxidation of organic substances (Eqs. 9-11, Scheme 3).

$$(\bullet OH)_{ads} + RH \longrightarrow R\bullet + H_2O \quad (9)$$
$$R\bullet + (\bullet OH)_{ads} \longrightarrow (ROH)_{ads} \quad (10)$$
$$(ROH)_{ads} + (\bullet OH)_{ads} \longrightarrow RO\bullet + H_2O \quad (11)$$

Scheme 3 - *Indirect oxidation of organic substances promoted •OH radicals.*

Nevertheless, more recently Fox (37, 38) and Tachikawa (39, 40) have demonstrated that in many cases the same products could also be formed via direct oxidation by holes (Eq. 12), partially reconsidering the overestimated role of •OH radicals.

$$RH + h^+ \longrightarrow R\bullet + H^+ \quad (12)$$

In this context, O_2 has not only the role of electron scavenger in the initiation phase (Eq. 2), it is also involved in further reactions with organic radical species, leading to products of partial or total oxidation (Eq. 13-15, Scheme 4) (41).

$$R\bullet + O_2 \longrightarrow ROO\bullet \quad (13)$$
$$ROO\bullet + RH \longrightarrow ROOH + R\bullet \quad (14)$$
$$ROOH + e^- \longrightarrow RO\bullet + OH^- \quad (15)$$

Scheme 4 - *Formation of active oxygen-centered radicals by means of O_2.*

The role of water in photocatalysis on TiO_2 is no less important. If the promotion of photoxidative processes appears clear from its capability to generate hydroxyl radicals on TiO_2-surfaces, its inhibition action by site blocking must be stressed as well (42). Site blocking may occur through two different routes, one according to which water occupies key adsorption and/or reaction sites on the surface, and the other one in which the access of reactants on the surface is inhibited by a solvent effect (24).

Mechanisms of self-disinfection activity on TiO_2

One of the main advantages of photocatalytic sterilizing surface is the fact that they operate without electrical power or chemical reagents, since light, oxygen and water are the only required ingredients. TiO_2 surfaces are not poisonous and do not cause environmental pollution. These characteristics make self-sterilizing TiO_2 materials the products of choice for future medical applications.

The antibacterial properties of irradiated TiO_2-surfaces derive from the combination of two different characteristics of these materials, namely, their self-cleaning and self-disinfection properties. Self-cleaning can be considered an indirect action against bacteria. In fact, degradation of organic substances (Eq. 16) by total oxidation (43), according to the mechanism previously disclosed, prevents from bacteria and biofilm adhesion on material surfaces.

$$\text{organic contaminant} \xrightarrow[h\nu > \text{band-gap}]{TiO_2,\ O_2} \text{intermediate radical species} \longrightarrow CO_2 + H_2O \quad (16)$$

As far as the direct antibacterial activity by irradiated TiO_2-surfaces is concerned, the real mechanisms which regulate bacterial killing are still under debate.

Matsunaga et al (44, 45) explained the mechanism of sterilization by TiO_2 powders on *Escherichia coli* cells evaluating the concentrations of coenzyme A (CoA). It is known that CoA mediates an electron transfer between the cell and an electrode or a semiconductor. They showed that the de-

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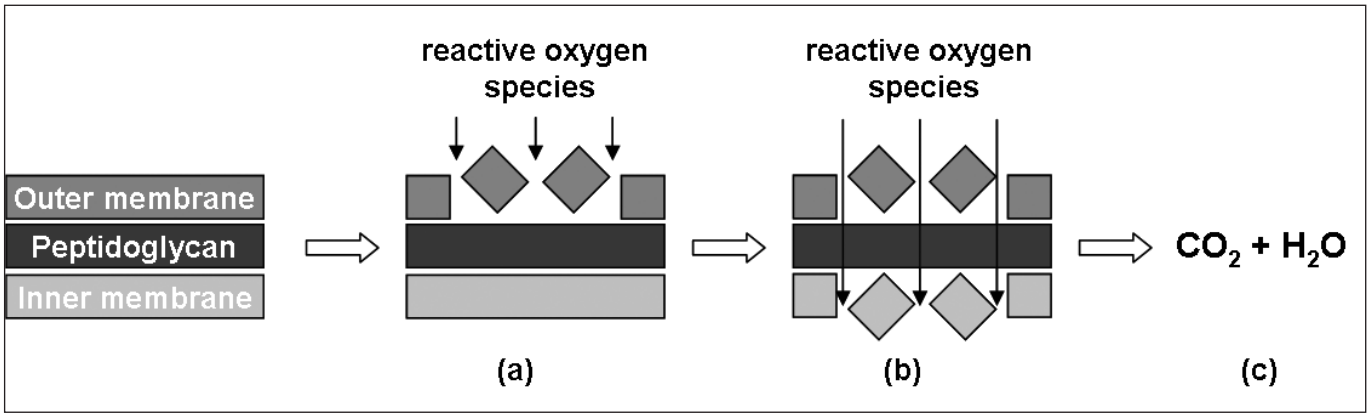

Fig. 3 - *Three-step proposed mechanism for photokilling of bacteria on irradiated TiO_2-surfaces.*

crease of CoA in the bacterial cells is related to the irradiation time and to the increase of the dimeric CoA concentration, causing inhibition of respiration. However, different types of experimental evidence have shown that the killing action of irradiated TiO_2 is primarily due to membrane and cell wall damage. In a recent review Dalrymple et al (46) have presented details of the kinetic aspects of the killing mechanism and explored the theoretical development of a model for photocatalytic disinfection based on lipid peroxidation.

In particular, Sunada et al (47) proposed a three-step mechanism for photokilling of bacteria on irradiated TiO_2-surfaces (Fig. 3): (a) attack of cell walls by reactive oxygen species; (b) disordering of the inner cytoplasmic membrane and killing of the cell; (c) decomposition of the toxic ingredients of bacteria.

In the first stage the outer membrane is partially damaged. This first attack does not particularly affect the vitality of the cell, but it changes the permeability of bacterium towards reactive species, which can enter the cell and attack the cytoplasmic membrane by lipid peroxidation (second step). It is at this stage that the structural and functional disordering of the inner membrane leads to cell death.

The third step is what makes photocatalytic sterilization the most effective technique when compared with classical antibacterial treatments. At longer reaction times, dead bacteria were found to be completely mineralized mainly to CO_2 and H_2O, once again due to the before-mentioned self-cleaning property of photo-irradiated TiO_2-surfaces (Eq. 16).

STRATEGIES TO OBTAIN TITANIUM OXIDE SURFACES ON MEDICAL DEVICES

Surface modification technologies for antibacterial properties in biomedical devices

The interest in titanium oxide as biomaterial surface is originally due to the performances of Ti and its alloys (Ti alloys, hereafter) commonly used in biomedical device manufacturing. Ti alloys are covered by a thin (nanometric) TiO_2 film, that spontaneously grows on their surfaces in air. This nanometric layer preserves the properties of the bulk material and interacts with biological fluids, resulting in a modulation of specific responses with the contact biological tissues (48). The comprehension of the role of TiO_2 film on biomedical Ti alloys paved the way to intense research activity focused on technologies for modifying and structuring the natural oxide layer in order to increase its resistance and give rise to novel properties (see, for instance, the results obtained in increasing the corrosion resistance of Ti alloys by increasing the oxide layer thickness in (49)) (48, 50-52).

Industrial applications of photoactive materials, such as antifogging and self-cleaning surfaces, became popular in the last decade: however, their medical applications are still limited and seem to be still under development (4). To take advantages of the photo-activated processes of TiO_2 over the wide range of bulk biomaterials, several studies have been conducted in order to deposit thin and adherent

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films, with specific crystallographic phases (i.e., anatase or rutile). It is generally accepted that crystalline phases (even in their nanostructured forms) possess photo-activated antimicrobial behavior. However, a study from Choi and coworkers showed that even the natural oxide layer covering commercially pure Ti possess antimicrobial behavior, with no significant differences among non-treated, anodic, and thermal-treated specimens (53). This finding could be explained by taking into account that in a native, essentially amorphous, titanium oxide film a certain degree of short-range order in the nanometer range is likely to occur (54).
It becomes evident that a tuning of the surface properties of this oxide paves the way for the exploitation of structured-TiO_2 properties, by maintaining the bulk material properties of Ti alloys and, mainly, of materials other than Ti alloys. In the following section we offer an overview of the available surface technologies allowing the deposition and/or structuring of TiO_2 films with antibacterial properties. A particular emphasis is placed on the methods that can be easily scaled-up to large production of medical implants. For the sake of simplicity, we will classify these surface modifications according to the involved technologies.

Chemical and electrochemical methods

Sol-gel

Sol-gel is a wet chemical technology based on a sequence of synthesis steps similar to an organic polymerization for the synthesis of inorganic and organic-inorganic hybrid oxides (55). It is generally realized by means of hydrolysis and condensation of metallorganic alkoxide precursors, although inorganic precursor synthesis is also commonly used (55). Using this technique, bioactive silica-based thin films for biomedical applications have been produced (56-58). By using suitable Ti precursors, thin TiO_2 coatings, based on either conventional (59) or the Stepwise Surface process (60), can be obtained on different substrates. Surface modifications aimed at obtaining antibacterial properties using sol gel TiO_2 coatings have been realized by several research groups (12, 61-71), even if in some cases it is difficult to compare the results due to scarce homogeneity in surface preparation, chemico-physical characterization, and the selected bacteria strains and methods (this point will be further elucidated in the next section).
Chun et al (64) proposed the modification of orthodontic wires made of stainless steel as bulk material and tested its photo-activated antiadherent properties (on *Streptococcus mutans*, which are the cause of dental caries) and its antibacterial properties (on *S. mutans* and *Porphyromonas gingivalis*, the former causing periodontitis) of these surfaces. They found that the bacterial mass that bound to the TiO_2-coated orthodontic wires remained unchanged after adhesion tests, whereas uncoated wires increased their mass by 4.97%. Furthermore, the TiO_2-coated orthodontic wires had a bactericidal effect on both strains; since one of the main causes of failure in orthodontic treatment is the development of dental plaque initiated by the adhesion of *S. mutans* to the tooth surface or orthodontic devices, this treatment appears of particular interest for this specific device (64).
This example allows us to point out that materials other than Ti alloys can be treated in order to add a thin titanium oxide layer onto their surface: results of this sort have been achieved by other authors on different organic and inorganic substrates (4, 70-73). Another advantage of using the sol-gel technique is the possibility of incorporating metal ions, nanometric clusters, and bactericidal molecules that in most cases have antibacterial properties *per se*, namely Ag ions/nanoparticles (61, 63, 74-76), or Cu ions (69, 73). The presence of these dopants on TiO_2 structure plays a synergistic role in photokilling bacteria, since they can act as: (i) *antimicrobial species*, when directly released in concentration enough to directly kill the bacteria species; (ii) *charge separator enhancers*, enhancing the efficiency of the redox reactions; (iii) *oxidants* (e.g., Cu^{2+} has been demonstrated to reduce to Cu^{+} and react with H_2O_2 in a Fenton-Type reaction, resulting in the production of •OH radicals). Obviously, these synergistic effects, deriving from doping TiO_2 structures are not peculiar of sol-gel technology, and have hence been explored also using other technologies, as discussed below.
Several examples other than those reported here can be found in literature (4, 61, 63, 69-77), and we suggest the reader refer to each of these works to have an overall idea about ongoing research studies. However, some general conclusions on this technique can be drawn. Sol-gel allows the treatment of different bulk materials, without affecting the morphology at the micrometric scale. Different allotropic forms can be deposited and their tuning depends on the chemical-physical steps involved in the preparation of sol and following film deposition and curing. Moreover, it is quite an economic solution, providing that surfaces are

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correctly prepared and devices have a simple 3D shape (sol-gel coatings can be deposited mainly either *via* dip or spray coating).
As a general suggestion to the reader, this technique appears particularly useful in producing advanced antibacterial coatings: due to the simple industrial scale-up and the esthetic quality of the resulting film, it represents a powerful tool in medical device design that has yet to be fully exploited.

Electrochemical technologies

Electrochemical surface modifications are based on electric polarization of electrical conductive substrates in three different modes: anodic oxidation, cathodic polarization (or Electrolytic Deposition (ELD)), and electrophoretic deposition (EPD). Cathodic polarization is generally used for the deposition of calcium phosphate coatings and for this reason will not be further discussed in this review.

Anodic oxidation

Anodic polarization allows thick and conveniently structured Ti oxide films to be achieved only over the surface of Ti alloys: electrode reactions in combination with electrical field-driven metal and oxygen ion diffusion lead to the formation of an oxide film at the anodic surface (48, 78). (The explanation for the electrochemical processes involved in anodization can be found in several works (48-50, 79-84).) The anodic oxide film growth is a two-stage process that results in either a thin or thick titanium oxide film: up to 160 V of applied voltage drop in the electrochemical cell, a linear growth in the nanometric range of the TiO_2 film is achieved (50); when anodization is carried out at higher voltages, an increased gas evolution and often sparking are obtained, resulting in titanium oxide films up to tenth μm thickness. The latter process is generally called Micro-arc oxidation (MAO) or Anodic Spark deposition (ASD) (48, 78).
Morphological and structural features of oxides can be hence tuned using anodization over a wide range, resulting in films of increased oxide thickness, porous coatings, and selected crystallographic forms. Moreover, ion insertion in TiO_2 structure is also possible, providing that a suitable electrochemical bath is selected, although TiO_2 ion doping at low voltage anodization is actually a controversial issue (80, 81, 85, 86). Diffraction studies showed the oxide films to be either totally amorphous or in some cases partially crystalline (82).
The presence of the anatase phase has been shown to play an important role in the reduction of bacterial colonization on low-voltage anodized surfaces (87). Del Curto et al proposed an anodization followed by a heat treatment to induce phase conversion of the TiO_2 layer; the resulting surfaces exhibited a remarkable reduction in the attachment of three streptococcal strains.
When anodization is carried out at higher voltages local melting and re-crystallization processes result in the incorporation of ionic species with a concentration gradient along the oxide: the electrolytic bath affects the final chemical composition of the grown oxide. An interesting treatment has been proposed by Song et al (88): they obtained Ag- or Pt-containing coatings on Ti-based implants by performing MAO in a bath composed of silver and platinum salts. Antibacterial properties were evaluated by using *S. aureus* and *E. coli*. Lower Ag-containing coatings exhibited *in vitro* antibacterial activity with no cytotoxicity, while higher Ag concentrations had a cytotoxic effect.
These two examples allow us to draw some main conclusions: the anodization process can be effectively applied for titanium modification and coating preparation for biomedical applications, taking advantage of both the morphological and chemical results of such treatments, since these films show controlled porosity, morphology, chemical composition, and allotropic structure (50, 89-92). Anodic modification technology thus appears interesting when complex devices made of Ti alloys need to be treated in order to obtain antibacterial properties.

Electrophoretic deposition

Electrophoretic deposition allows the deposition of coating from almost any material class, including metals, polymers, and ceramics (93-96). The mechanism of electrophoretic deposition involves electrophoresis, in which charged particles move toward an electrode of opposite charge, and deposition, due to the motion of charged particles and their local accumulation. Coating formation is achieved via particle coagulation (93-96).
EPD appears of particular interest in surface modifications of materials for biomedical application (48, 78) because the deposition of either inorganic or hybrid coatings with high purity is possible on complex geometries. Promising results have also been obtained in TiO_2 antibacterial

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coatings: Santillán et al (97) studied the synthesis of TiO_2–Ag composite nanoparticles and the fabrication of TiO_2 and TiO_2–Ag coatings on titanium substrates by EPD. Ag nanoparticles ($_{NP}$Ag) were directly formed and grown on the surface of TiO_2 nanoparticles ($_{NP}TiO_2$) from nucleophilic reaction catalyzed by alkalis. They were able to demonstrate the feasibility of EPD of TiO_2–Ag coatings on titanium substrate and that all the developed coatings showed *in vitro* bioactivity, with a degree of bioactivity qualitatively decreasing as Ag content in TiO_2 coatings increased. However, the authors did not evaluate the bacterial response of these coatings.

Chemical Vapour Depositions (CVD)

The growth of TiO_2 films can also be pursued by using an RF plasma enhanced chemical vapor deposition method (PECVD), which is a variant of the chemical vapor deposition (CVD). This technique allows the deposition of thin films of different materials (for example silicon dioxide or silicon nitride) starting from their precursors and operating at lower temperatures due to the plasma action that facilitates the occurrence of the chemical reactions. For example, with regard to the titanium dioxide, Szymanowski et al (98) reported the deposition of TiO_2 onto glass slides and Rashig rings in order to create TiO_2 film having photo-induced antibacterial properties. They used titanium tetrachloride diluted in argon as source of Ti and gaseous O_2 as source of oxygen. The gaseous mixture was injected in a vacuum chamber (79.8 Pa) and irradiated with a RF power of 20 W to 300 W for 30 minutes. The TiO_2 film thickness grew up to more than 500 nm depending on the deposition power. The antibacterial capability was tested with *E. coli* strains (DH5α) showing good bacterial inhibition while increasing the TiO_2 film thickness. Other techniques frequently used are based on magnetron sputtering (99-101), ion beam sputtering (102) and spray pyrolisis (103).

Physical methods

Plasma Immersion Ion Implantation

One of the most widely used physical techniques for the generation of TiO_2 thin film on the surface of a large class of biomedical items is based on the immersion of the material to be treated in a *plasma* under a high voltage bias (104-106). The term *plasma* is used to define a state of matter ("the fourth state of matter") characterized by the presence of a significant number of electrically charged or ionized atoms and/or molecules having the fundamental characteristic of exhibiting a collective behavior due to long-range Coulomb interactions (107). The surface of the materials treated with the plasma can be modified by implanting new ionic species into the original materials. In this way a thin layer of the material surface is doped, allowing modification of mechanical, chemical and/or electrical properties. This process is generally called Plasma Immersion Ion Implantation (PIII or PI^3). When a deposition of matter is performed by forming a thin layer of material, the process is called Plasma Immersion Ion Implantation and Deposition (PIII&D or PI^3&D). The plasma techniques have a wide field of application, such as wear and corrosion protection, microelectronics, and medical-device manufacturing (108).

A number of authors have achievd the coating of different materials like metals, glasses, silicon wafers, and ceramics with TiO_2 with PIII&D techniques. Baba and Hatada (109) reported the deposition of a TiO_2 thin film onto fused quartz glass and silicon wafer through PIII&D using titanium tetraisopropoxide as precursor. The plasma was generated by a 50 W antenna irradiating RF power at 13.56 MHz at 1.3 Pa pressure. Negative pulses of 20 kV having a duration of 50 μs and a repetition rate of 100 Hz gave TiO_2 films with a thickness of 318 nm after annealing at temperatures between 673 K and 1023 K for 1 h. Similar results were obtained by Jing et al (110).

Shiraishi et al (111) described the preparation of antibacterial metal implant (titanium and SUS316 stainless steel) with a TiO_2 using a plasma source ion implantation followed by annealing. The TiO_2 layer conferred photocatalytic bactericidal effect against *S. aureus* under UVA irradiation.

The bactericidal effects of TiO_2 under UV-light irradiation is particularly useful for sterilizing the contaminated surface of dental implants as reported by Suketa et al (112). The authors used a plasma source ion implantation to deposit a layer of TiO_2 onto metallic titanium and tested the photobactericidal effectiveness of the resulting surfaces on *Actinobacillus actinomycetemcomitans* and *Fusobacterium nucleatum*, two microorganisms responsible for infections of the oral cavity.

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Plasma spray

Plasma spraying is a different and also widely used technique to form ceramic and oxide coatings on a wide class of inorganic substrates. The process is based on the action of an electric arc that melts and sprays materials onto a solid surface. Generally the material to be deposited is injected in powder form using an inert gas (argon) as carrier. Both nanometric and micrometric powders can be used in order to obtain a different surface roughness (113). This technique presents several advantages, such as high deposition rates (80 g/min), thick deposits (hundreds of micrometers to several millimeters), and low costs. Moreover, the resulting coatings have a surface morphology characterized by a good degree of roughness that is particularly suitable in the orthopedic applications of titanium because it favors osteoblast adhesion (114).

Mixtures of TiO_2 and other oxides, like as Al_2O_3, can also be deposited on stainless steel surfaces by atmospheric plasma spraying technique as described in (115). Often the TiO_2 powders are mixed with silver powders in order to have antibacterial activity even without light irradiation (116). The biocompatibility and the antibacterial activity of the TiO_2 coating onto titanium slides can also be improved by grafting collagen and eventually loading it with *gentamicin*, an aminoglycoside antibiotic (117).

IN VITRO EVALUATION OF TIO_2 ANTIBACTERIAL ACTIVITY

Since the demonstration of its antimicrobial activity, UV-activated TiO_2 has been used in suspension, in liquids, or immobilized on surfaces for the destruction of Gram-negative and Gram-positive bacteria, including endospores, fungi, algae, protozoa, viruses as well as for the inactivation of microbial toxins and prions (45, 72, 118-127). Ions such as Cu^{2+} and Ag^{+} in combination with TiO_2 were reported to enhance its antimicrobial activity (72). Furthermore, the photocatalytic activity of TiO_2 was reported as performed against pure bacterial cultures, mixed cultures (128) and natural communities (129-131).

As a general concept, photocatalytic surfaces or suspensions are referred to as being self-disinfecting rather than self-sterilizing. Photocatalysis has been shown to be capable of killing more Gram-negative than Gram-positive bacteria as reported in an excellent mini-review by Howard et al (72). The difference is usually ascribed to the difference in cell wall structure and peptidoglycan thickness between Gram-positive and Gram-negative bacteria. The cell wall in Gram-positive bacteria is essentially composed of a very thick peptidoglycan layer whereas in Gram-negative bacteria it has a thin inner peptidoglycan layer externally surrounded by an outer membrane mainly enriched of lipopolysaccharide (LPS) and lipoprotein (Fig. 4).

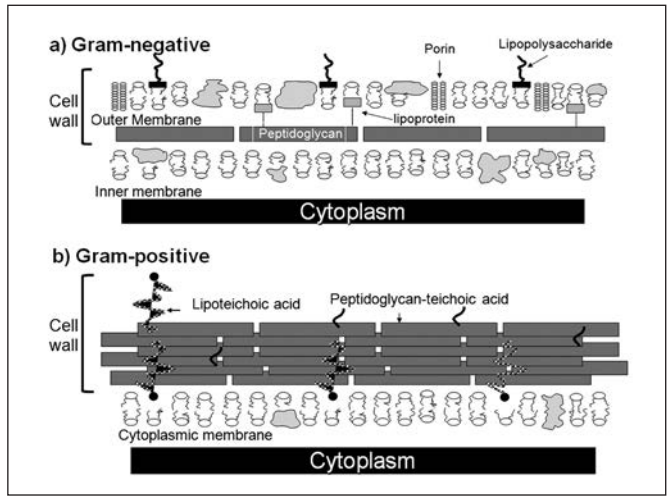

Fig. 4 - *Outer layers of bacteria:* ***(a)*** *Gram-negative* ***(b)*** *Gram-positive.*

Peptidoglycan is a peptide-crosslinked polysaccharide which surrounds bacterial cells and confers rigidity to maintain shape and internal pressure: in Gram-positive bacteria it accounts for as much as 90% of the cell wall whereas in Gram-negative bacteria it accounts for only 10%. But in Gram-negative bacteria, the outer membrane is the first external line of defense whereas in Gram-positive bacteria it is the thick peptidoglycan layer. However, it is not clear if the peptidoglycan layer is the critical target of attack by radicals or simply retards the diffusion of oxidants to the underlying vital sites (phosholipid bilayers) of bacterial cells.

Up to now, most studies have been performed against *E. coli* strains, but other Gram-negative bacteria have also been reported to be susceptible to TiO_2 photocatalytic activity. Regarding Gram-positive bacteria, species of 17 different genera, including aerobic and anaerobic endospore formers, were shown to be killed by TiO_2 photocatalytic activity (details in ref. (72)). Furthermore, the planctonic cells were less resistant to photocatalytic disinfection than endospores. The killing mechanism of photoactivated TiO_2

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has been studied in Gram-positive and Gram-negative bacteria.

In vitro evaluation techniques to asses antibacterial properties

Different type of methods can be used to assess killing of microorganisms by photocatalysis such as *i)* evaluation of bacterial survivability, *ii)* determination of intercellular components leakage (cations, RNA and protein), *iii)* microscopy observations, up to *iv)* determination of lipid peroxidation products by spectroscopic studies and *v)* evaluation of killing of bacterial cells throughout biofilm.

Evaluation of bacterial survivability

Adhesions and proliferation assays are the first methods to perform for evaluating the killing efficacy of titanium oxide surfaces or suspensions. Cell survival can be expressed as the ratio of the Colony Forming Units (CFU) from bacteria treated with irradiated TiO_2 surfaces/suspensions and CFU from untreated bacteria (87, 92). Furthermore, a test with 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) can be performed at different times of incubation to evaluate the mitochondrial activity of the bacterial cells. The former method is the most reliable it is time consuming whereas the latter is more rapid.

Evaluation of changes in membrane permeability

The leakage of intercellular components can be an indirect method for the assessment of bacterial membrane damage. Various studies performed on *Streptococcus sobrinus*, *E. coli* and *S. aureus* with TiO_2 showed leakage of potassium ions from the bacteria and the parallel decrease in cell viability (70, 72, 132-135).

Microscopy observations

Trasmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM) images of TiO_2-treated bacterial cells such as *S. mutans* (132), *Pseudomonas aeruginosa* (136), *E. coli* (47, 137-142), *S. aureus* (143), *MRSA* and *methicillin-sensitive S. aureus* (137), and *Streptococcus pneumonia* (143) were used to show membrane damage and progressive destructions of cells if compared to untreated cells (72). In general, microscopic studies showed morphological changes suggestive of cell wall disruption of UVA irradiated TiO_2 surfaces/suspensions. On *E. coli* cells, various degrees of cell disruption including plasmolysis, intracellular vacuoles ghost and cell debris was observed; in particular, the outer membrane is the first to be attacked, followed by the complete degradation of the cytoplasmic membrane. On *S. aureus* treated cells, a separation of cytoplasmic membrane from the peptidoglycan layer or cell distortion was observed. Photocatalytic killing for Gram-negative and Gram-positive bacteria occurred without substantial visible degradation of peptidoglycan.

Confocal Laser Scanning Microscopy (CLSM) can be effectively performed to show cell membrane damage using LIVE-DEAD® BacLight™ Bacterial Viability Kit. The kit includes two fluorescent nucleic acid stains: SYTO9 that penetrates both viable and nonviable bacteria, and propidium iodide that penetrates bacteria with damaged membranes and quenches SYTO9 fluorescence. Dead cells, which take up propidium iodide, fluoresce red, and cells fluorescing green are deemed viable.

Spectroscopic studies

X-ray diffraction (144), laser kinetic spectroscopy and attenuated total reflection Fourier Transform Infrared Spectroscopy (FTIR) (134, 145-147) were used to show cell disruption due to lipid peroxidation or direct oxidation. Lipid peroxidation occurs when polyunsaturated fatty acids (linoleic acid) are attacked by ROS such as hydroxyl radicals and H_2O_2 produced by irradiated TiO_2 surfaces. Malondialdehyde (MDA) is released as a product of membrane degradation. It has been suggested that the free radicals •OH and H_2O_2 were responsible for killing close to the TiO_2, with H_2O_2 acting at a distance (118, 148-152). Direct oxidation of bacterial components is feasible when cells are in direct contact with the surface of TiO_2. The oxidation occurs directly on the membrane components without production of reactive oxygen species as intermediates.

Evaluation of killing of bacterial cells throughout biofilm

Bacterial biofilms are associated with various pathological conditions in humans such as cystic fibrosis (*P. aeruginosa*), colonization of indwelling devices (*S. aureus* and *Staphylococcus epidermidis*) and dental plaque (oral streptococ-

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cal strains) formation involved in caries and periodontitis. The possibility of evaluating the role of TiO_2 in the reduction of biofilm formation is becoming extremely important. A biofilm is defined as a microbial-derived sessile community and is characterized by cells that are embedded within an amorphous slimy material comprising mainly the polysaccharides and/or proteins secreted by the bacteria (153). For biofilm assays, bacterial cell cultures are statically incubated with the TiO_2 surfaces/suspensions overnight at 37°C. At the end of incubation, the cells are fixed and stained with crystal violet and then air dried. For a quantitative estimation of biofilms, the absorbance of the solubilized crystal violet is determined at 590 nm in a microplate reader (154). Recently, a TiO_2 catalyst was used for killing *S. epidermidis* cells throughout its biofilm (155). On the other hand, the photocatalytic activity was reduced by *P. aeruginosa* biofilm (156) and by a natural biofilm (157). The type of catalysts used and the different chemical composition of the bacterial biofilms can explain these differences. Furthermore, nanoporous TiO_2 was shown to not cause greater biofilm formation by oral commensal bacteria compared to other tested surfaces (90).

As mentioned above, different standard methods for testing antimicrobial efficiency of photocatalytic processes are available. Unfortunately, it is very difficult to compare results from different research groups, mainly because different bacterial strains have been used with different growth media and experimental conditions (128). A rigorous and scientific approach would be very helpful in order to produce comparable results and evaluate the real efficacy of photocatalysts in killing bacteria cells.

CONCLUSIONS

Antibacterial surface functionalization represents the most effective way to reduce DRIs. Despite huge efforts on the part of health care systems, prophylactic measures have succeeded mainly in reducing the frequency of early-stage infections, with minor results in late-stage processes of bacteria surface colonization (1). Several surface-mediated antibacterial approaches have been proposed, however, none of them have received widespread acceptance in clinics.

In this short review, we described one of these, namely, photo-induced bactericidal activity in TiO_2, and related technologies aimed at obtaining TiO_2 coatings on biomedical devices. The reasons for a widespread interest in photocatalytic antibacterial activity are evident and supported by several research and literature articles (72). In particular, TiO_2 represents an ideal candidate as a photocatalyst (43): it is easy to produce and use, it is efficient in catalyzing reactions and has a wide spectrum of antimicrobial activity, since it is capable of killing a wide range of organisms including bacteria and endospores (72). Moreover, its biocompatibility is clinically proven, since a huge number of medical devices are made of Ti alloys (48, 158-162).

Both chemical and physical methods can be exploited in order to design and realize efficient photocatalytic surfaces, since materials science and technology now offer several ways to obtain TiO_2 surface coatings with suitable structures. Chemical approachs are, in our opinion, the most powerful, due to the possibility of coupling TiO_2 surfaces with organic molecules. However, the surface technology should be selected according to the device function and bulk material, providing that the process is compatible with the preservation of the overall device functionality.

There are some major limitations in TiO_2 coating applications used in biomedical devices as antibacterial coating. A main point that has been stressed in the previous section is that the coating should be designed for specific applications, taking into account the bacteria involved, their attachment and growth mechanisms, and the biological environment envisaged for the application. Unfortunately, testing of antibacterial coating efficacy is conducted in simplified environments *in vitro*; moreover, there are no common procedures, and clinical studies are costly and complex.

A second limitation in using TiO_2 coating applications as an antibacterial surface regards the activation mechanism: photons with enough energy have to reach the semiconductor surface in order to activate it and promote the catalytic processes. The problem of activating energy can be solved by doping TiO_2: the development of photocatalysts exhibiting high reactivity under visible light ($\lambda > 400$ nm) is actively promoted and structures can be prepared by metal-ion addition/implantation, reducing of TiO_2, or nonmetal doping (163). In the case of metal ion doping, a synergistic antibacterial effect can be achieved via the local release of antibacterial ions.

Despite some limitations, therefore, TiO_2 surface coatings appear to be a promising, powerful approach in the fight against transmission of infectious diseases.

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