CHAPTER FOUR

Toward novel antibacterial surfaces used for medical implants

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Abstract

In the present roadmap, recent advances in antibacterial properties of nanostructured surfaces on metal substrates used in medical applications are presented. The importance of antibacterial surfaces for their application in medicine is described and the most commonly applied synthesis methods for fabrication of nanostructured antibacterial surfaces are disclosed. Further on, the antibacterial mechanisms of nanostructured/biomimetic surfaces are discussed. The examples of antibacterial efficiency of nanostructured TiO_2 oxide layer on Ti substrate are presented and discussed with the emphasis on future advances in this field.

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1. The importance of antibacterial surfaces in biomedical applications

Metals such as stainless steel, titanium, cobalt-based materials and their alloys, etc. have been widely used in medical applications as implant materials, e.g., in dental, cardiovascular and orthopedic applications, and with recent advances in 3D printing technologies, the use of metal materials even increased, as they can be designed according to the patient-specific anatomy (custom-made implants). In Fig. 1 the example of titanium intervertebral implant designed based on a patient CT scan, printed by 3D printing technologies, is presented. Although metal implants offer good mechanical properties (high strength, high fracture toughness, hardness), their drawback is that they can evoke acute or chronic inflammatory responses, that can ultimately lead to implant failure and removal. Designing the implant with the optimal combination of mechanical properties, biocompatibility, corrosion resistance and also antibacterial activity, that would allow a long lifespan of implant in the human body, is therefore highly challenging and usually could not be fully achieved. In recent years many research efforts have been directed toward the development of nanostructured surfaces, or biomimetic surfaces, which were shown to have a significant influence on bacterial adhesion and biofilm formation.

In this roadmap, the state of the art of ***antibacterial activity*** of nanostructured metal surfaces for application in medicine is presented. Bacterial infections present a serious concern as they can lead to post-surgical complications or even to implant failure, which is connected with the high

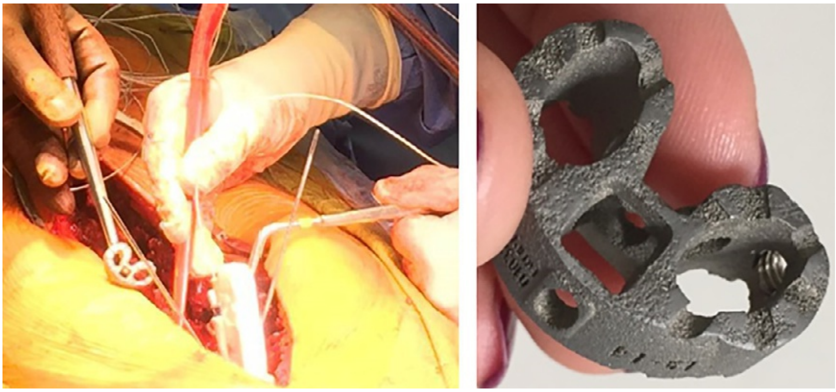

Fig. 1 3D printed titanium intervertebral implant (kindly donated by Ekliptik Ltd.).

number of revision surgeries and high hospital costs [1,2]. Implant-related infection can result from the external environment (operating environment, surgical equipment, human skin, etc.) as well as from bacteria in the human body [3]. The infections are commonly treated by antibiotics, but serious problems occur due to biofilm formation, which highly reduces the effectiveness of antibiotic therapy. Biofilm is a self-produced polysaccharide matrix adhering to the surface of the implant and acts as bacterial insulation from external stress [4], which ultimately reduces the efficiency of antibiotic treatment and increases bacterial shelf life. In addition, the extensive use of antibiotics led to an increased number of antibiotic-resistant bacteria strains, which nowadays presents a serious health concern. The post-surgical complications cause immense health care costs due to prolonged antimicrobial therapy, multiple surgical interventions, chronic pain, immobility and even implant removal. Thus, there is an increasing demand to develop novel medical implants that would prevent bacterial adhesion and biofilm formation, preferably without the use of antibiotics.

It is important to consider that bacterial adhesion and biofilm formation of metal surfaces highly depends on surface characteristics of materials as well as the involved bacteria strain. Various factors like electrostatic interaction [5,6], steric hindrance, hydrophobicity [7] and van der Waals forces govern bacterial attachment to the surface [3]. With the development of nanotechnology, nanostructured/biomimetic biomaterials have been fabricated to prevent bacterial infections. By the use of various surface modification techniques surface topography and roughness can be altered, which influences on bacteria attachment [8] as well as biofilm formation.

Various natural nanostructures indicate that surface nanotopography may play an important role in the design of new generation of medical devices with superior properties. For instance Lotus leaves consist of micro and nanoscale hierarchical structures that provide self-cleaning and superhydrophobic properties [9]. The nanoscale pillar structure on the surface of dragonfly and cicada wings provides these surfaces with bactericidal properties [10], while the conical nanopillars on the surface of cicada wings can also lyse the bacterial cell along with its self-cleaning property via a mechanical action [11]. It was postulated that the main driving force of the unique bactericidal activity of nanostructures is due to the contact killing mechanism, where nano-features cause ruptures on the cell wall leading to bacteria's membrane lysis and death [10,12]. However recent studies by Jenkins *et al.* [13] imply that the killing can be also correlated with the initiation of oxidative stress caused by the nanostructured surface. Thus the approach to fabricate biomimetic surfaces with antibacterial properties presents an intriguing way to solve the current issues of implant-related infections, biofilm formation and misuse and/or overuse of antibiotics.

2. Engineering approaches to create antibacterial nanostructured surfaces

In recent years, researchers have widely investigated nanostructured materials for various applications because of their exceptional plasmonic, physicochemical and biological properties [5,14].

As mentioned in the previous section, for medical applications specific properties such as surface roughness, electrostatic interaction [5,6], steric hindrance, hydrophobicity [7] or van der Waals forces are desired. As the implant surface, with its specific surface features, is the primary source that interacts with biological material and initiates biological response and dictates bacterial attachment and biofilm formation, the appropriately nanostructured surface could present an elegant solution to combat bacterial infections on biomaterials. For this purpose, various "bottom-up approaches" are proposed where surface modification is achieved via physical (thermal spray, physical vapor deposition, etc.), chemical (sol-gel, etching, etc.), thermal (sintering, thermal oxidation, hydrothermal etc.), electrochemical (micro-arc oxidation, anodization, etc.) or mechanical (grinding, machining, polishing, etc.) methods [15–17]. Different physical and chemical methods are used for fabrication of nanostructures and these fabrication methods can be broadly categorized into "Top-down" and "Bottom up" approaches. In "Top-down" approach, through physical and chemical methods bulk materials disintegrate into smaller particles to form thin films and nanoparticles. In "Bottom-up" approach ions, atoms and molecules are self-assembled to fabricate short rage ordered nanostructures. This approach consists of mechanical, physical, and chemical methods that in short provide regulated and hence this approach is used for applications where specific properties such as changes in surface topography [2,18] homogenized structures [19].

Herein, commonly applied methods for preparation of nanostructured surfaces with improved antibacterial activity are presented.

Hydrothermal method has emanated as a profitable and simple process to achieve well-defined crystallography and morphology. In the case of titanium oxide formation by hydrothermal treatment, thin and firmly attached anatase layers which enhance bioactivity are formed [20]. Through hydrothermal method, Sun *et al.* [21] immobilized TiO_2 nanotubes (TiO_2 NTs) on carbon fibers (CFs) by alkali treatment of TiO_2 NWs. Initially authors grew TiO_2 nanowalls (NWs) on CFs using solvothermal method and later CFs/TiO_2-NWs was hydrothermally treated with NaOH in order to obtain TiO_2-NTs on CFs. The synthesized NTs had inner and outer diameter of 4.6 and 8.7 nm respectively, and overall coverage of carbon fibers was achieved. From X-ray photoelectron spectroscopy (XPS) and Energy-dispersive X-ray spectroscopy (EDS) analysis, it has been confirmed that C, O and Ti was present on the surface of carbon fibers/ TiO_2 NTs. This method provides an interesting approach to prepare TiO_2-NTs on flexible non-metallic substrates especially graphene which can induce oxidative stress due to its conductive character which can affect antibacterial cell viability. Depending upon the temperature, titania formed through hydrothermal method can be fabricated in different structures; from nanoparticles to nano-flower like structures [22]. Also, Lorenzetti *et al.* [23] fabricated anatase crystals on titanium using hydrothermal method and observed that nanostructured coating leads to changes in surface topography and crystal morphology which results in increased hydrophilicity. Upon irradiation of HT synthesized titanium samples with UV light, superhydrophilicity (water contact angle less than 4°) was observed. This photosensitive coating can prevent bacterial infections before and during implantation due to photocatalytic properties of material and release of reactive oxygen species (ROS). Authors also studied electrochemical properties of polycrystalline anatase TiO_2 coating prepared by hydrothermal (HT) method in Hank's solution for bone implants and observed that due to coating thickness, crystal morphology and porosity of HT anatase coating the corrosion resistance was improved [24], which may increase the lifetime of medical device [24]. For biomimetic nanostructures hydrothermal method is primarily used due to its reliability, environmentally friendly nature, efficiency and ability to control pressure and temperature during the process. Using hydrothermal method different type of naturally occurring protruding nanoscale features can be mimicked on the surface, which show adverse effect to bacteria. Upon the contact of bacterial cell membrane with the nanostructured surface the bacteria membrane is ruptured which also leads to oxidative stress which further promotes cell death. Combination of various parameters such as temperature, alkaline etchant and primary etching duration affect the morphology of the resultant nanostructure. Bright *et al.* [25] studied the effect of synthesis duration and type of alkaline etchant for fabrication of nanotopographic features obtained on titanium alloy discs. Authors used potassium hydroxide (KOH) and sodium hydroxide (NaOH) as etchants and duration was varied for 1, 3, 4, and 5 h. From these different parameters, two favorable conditions were achieved with KOH etching for 5h and NaOH etching for 4 h. Treatment with NaOH for 4 h led to the formation of densely packed nanospikes (75 spikes per μm^2) compared to scarcely distributed nanospikes obtained after treatment with KOH for 5 h. Bactericidal effect against gram negative bacteria is achieved using higher density of spikes whereas for gram negative bacteria opposite, i.e., sparser nanostructure is preferred.

In sol-gel method, a sol or colloidal suspension is produced through either polymerization or hydrolysis of inorganic metal salts or metal-organic compounds. TiO_2 nanorods and nanotubes have been synthesized via controlled sol-gel hydrolysis of titania-based precursor in addition to templating agents followed by polymerization of TiO_2 by depositing on template surface or self-assembled templates [26]. This process does not provide single-crystalline Ti—O based nanocrystals as they are composed of nanoparticles, thus modification of this process is required, especially for biological applications. Guo *et al.* [27] synthesized silver (Ag) containing hybrid coating on titanium using sol–gel process. The growth of *S. aureus* on the fabricated hybrid coating was significantly reduced (percentage reduction was 99.3% after 24 h incubation) due to the favorable release of Ag ions which inhibit the bacterial adhesion on the surface. Ahmad *et al.* [28] produced $ZnO/SrZnO_2$ nanocomposite using sol-gel method that acts as an efficient visible light-triggered photocatalyst. Authors also showed that the fabricated nanocomposite poses an antibacterial action against *E. coli*. Reactive oxygen species (such as HO•, O_2• and H_2O_2) are produced in large number due to confined electron/hole recombination and optimal band gap of $ZnO/SrZnO_2$ nanocomposite. The hydroxyl holes and radicals destroy carbohydrated, lipids and proteins present in cell wall of *E. coli*. Moreover, they restrict reproduction and respiration of *E. coli* by degrading nucleic acids and inactivating respiratory enzymes. Hence the impaired cell wall does not maintain the increased K+ ion gradient in protoplasm which further affects processes such as cell cell divisions and respiration and ultimately leads to cell death. This method is commonly used in association with other processes in order to form a larger nanofabrication process. For example, Asgarian *et al.* [29] combined sol-gel process with electrophoretic deposition method for producing a bioceramic coating for implants. They applied hardystonite coating ($Ca_2ZnSi_2O_7$) which was produced by sol–gel method onto a titanium substrate (Ti_6Al_4V) using electrophoretic deposition method. *In vitro* analysis by MTT assay of the produced bioceramic coating increased the reproduction and viability of bone marrow stem cells making it suitable candidate for clinical applications.

In the last few years, various antimicrobial surfaces have been reported based on the contact-killing mechanism, reactive agent release, and anti-biofouling surface [30,31]. Especially, gaseous plasma-based surface modification for antimicrobial surfaces have gained significant attention [32,33] as they present rapid, simple, eco-friendly and substrate independent technique, unlike conventional approaches. In addition, interaction between microorganisms and species from gaseous plasma revealed that non-equilibrium plasma (low or cold temperature) can disrupt multi-layered biofilms and act as phsico-chemical tool for biomedical decontamination [34,35]. The major role for the inactivation of microorganisms was attributed to reactive oxygen and nitrogen specifies which are generated by plasma [36,37]. Through plasma deposition method, an antibacterial coating can be obtained from gaseous form of a selected precursor wherein molecules are electrically excited to a plasma state. This method is fast, free of any solvents and provides different functional group coatings depending upon their penultimate usage. Nanoscale coatings prepared by plasma deposition attach considerably to different type of substrate material without the need for substrate pre-modification. Using plasma deposition metals, ceramics, polymers and composites can be coated by using the same process and eliminating process optimization needed to suit a particular substrate material. On the other hand, wet techniques such as Layer-by-Layer (LbL) or Self Assembled Monolayers (SAMs), are limited to the specific type of substrate material that is required. Whereas, plasma polymers can be deposited on substrates of complex shapes, such as porous materials or those having complex nanotopography, as well as micro and nanoparticles. Wang *et al.* [38] fabricated a nanocapsule containing antibacterial coating, in which controlled release of Ag+ ions was obtained by aerosol assisted atmospheric pressure plasma deposition. The fabricated coating exhibits antibacterial efficiency against *E.scherichia coli* and *Staphylococcus aureus* and tolerable cytotoxicity for murine fibroblasts. Through argon plasma etching Hirano *coli* and *Staphylococcus aureus* and tolerable cytotoxicity for murine fibroblasts. Through argon plasma etching Hirano *et al.* [39] obtained nanopillar structure on surface of stainless steel. Nanopillars due to their biocidal property, i.e., mechanically rupturing the bacterial cell wall exhibit antibacterial effect against both Gram positive and Gram negative bacteria.

The most conventionally used method to achieve an oxide layer on the metal substrate is electrochemical method [40,41]. This method uses electrochemical cell where metal acts as an anode and after the process of anodization the crystal structure and microscopic texture of the metal surface is altered. Different morphologies have been obtained using electrochemical methods such as nanoparticles, nanorods, nanopores, nanowires and nanotubes [5,6]. Among all these morphologies, TiO_2 NTs have been extensively synthesized and studied in much detail. For example, Kulkarni *et al.* [42] fabricated TiO_2 NTs by anodization of Ti foil in ethylene glycol based electrolyte containing deionized water and NH_4F. Their work also reported the influence of water content and anodization voltage on the transition of nanopores to nanotubes [18,42]. Herath *et al.* produced nanostructures by anodization of 316 L grade stainless steel via electrochemical etching method [43]. This method was optimized using two different electrolyte solutions: HNO_3:H_2SO_4 (1,1) and HNO_3, by changing electrolyte concentration, applied potential and anodization time. Nanosacke surface roughness was produced by both processes with varying corrosion susceptibility. By using 50% HNO_3 hierarchical roughness was obtained with dense spikes (10–20 nm in diameter) that covered the candy like protrusions (10–15 μm diameter). On the other hand, a terrace like single scale morphology with nanoscale ridges of 34.8 ± 1.2 nm in width were achieved from other electrolytes. Knowledge related to fabrication of nanostructured surfaces will be essential for the advancement of antiviral and antibacterial testing in implant and hospital applications. In other study by Jakubowicz *et al.* [44] biofunctionalization of titanium surface was carried out using electrochemical anodization. It was performed using electrolyte consisting of H_3PO_4 and HF which leads to the formation of anatase-TiO_2 on Ti surface, moreover traces of $Ti(HPO_4)_2$ can support osseointegration. The surface consists of porous oxide morphology, wettability and good corrosion resistance depicting improved bioadhesion and osteoblast cell proliferation and attachment. As discussed previously, considerable attention has recently been focused on fabrication of biomimetic surfaces with antibacterial properties. For this purpose various micro and nano-fabrication methods are used to reproduce the antibacterial behavior of certain naturally occurring antibacterial surfaces. Nano-structured pattern of cicada wings was replicated on poly methylmethacrylate (PMMA) using nano-imprint lithography (NIL) method by Dickson *et al.* [45]. In the study, cicada wings were used as stamps for transferring the pattern onto PMMA substrate. The fabricated nanopillared surface had relatively less growth of *E. coli* compared to flat surface.

3. Mechanisms of antibacterial properties of nanostructured surfaces

For years, researchers have struggled to improve the efficacy of implants surface to prevent bacterial adhesion and biofilm formation. Since the antiadhesive and bactericidal effects of metal surfaces are highly correlated with the physico-chemical characteristics of the surface, various surface modification techniques have been used to alter biomaterial surface properties, such as wettability, surface chemistry, morphology, surface charge, etc. However, when studying the antibacterial activity on surfaces, it is important to consider that different types of bacteria react differently to the specific surface. Among others, the surface roughness/texture affects the adhesion of different types of bacteria [8,46]. It has been shown that the adherence of oral streptococci on TiO_2 nanotubular surface can be reduced by decreasing nanotube diameters [8]. The lowest adhesion of *Streptococcus sanguinis* and *Streptococcus mutans* on TiO_2 nanostructured surfaces was observed for small diameter nanoporous TiO_2 surface [8], which coincides with the highest osteoblast adhesion on small diameter nanotubular/nanoporous TiO_2 surface [47,48]. It was also shown that Gram-positive *S. aureus* is able to adhere to metal surfaces with the roughness below 0.5 nm, however Gram-negative *P. aueruginosa* cannot colonize smooth surfaces (roughness below 1.0 nm) [11,49]. It has also been shown that *E. coli* rather colonize the surfaces with 120-nm in depth and 1.3 μm width than surfaces composed of grooves with 50 nm in depth and 1.6 μm spacing [11,50,51]. It is therefore highly recommended to perform antibacterial test with different bacteria strains on the same surface with different nanotopography.

The antibacterial activity of metal surfaces has been described by three possible mechanisms: (i) ***repelling bacterial adhesion** (**antifouling**)*, (ii) ***mechano-bactericidal** (**contact killing**)* and (iii) ***releasing of bactericidal substances** (antibiotics, reactive oxygen species (ROS), metals such as Ag, Zn, Cu,* etc.). Antibacterial surfaces can be obtained by either alteration of surface chemistry (e.g., coatings, incorporation of antibacterial nanoparticles) or surface morphology/roughness.

3.1 Antifouling effect

Antifouling coatings on the surface of metal oxides have been applied to inhibit bacterial infections of implants by mitigating colonization. These mechanisms of antibacterial activity are known from nature, for instance, lotus and taro leaves exhibit highly hydrophobic surface which can repel bacteria and contamination [52,53]. Similarly, the shark skin has self-cleaning, anti-biofouling, superoleophobic properties due to micro-structured riblets, while nanostructured hair-like structures present on Gecko feet are super-hydrophobic (contact angle of 150°) and act antibacterial against various gram-negative and gram-positive bacteria [3,54,55].

According to Freschauf *et al.* [56], superhydrophobic surfaces exhibit antibacterial properties due to their "minimal solid-liquid contact at the surface, weak surface interactions with bacteria, and low sliding angle." Such antibiofouling surfaces repel bacteria due to reduced strength of adhesion.

These examples found in nature have inspired researchers to develop surfaces with advanced characteristics that can be applied in biomedical applications. For instance, Mukherjee *et al.* [57] extracted waxes extracted from lotus and taro leaves and prepared a superhydrophobic water-repellent nano-structured layer on silicon substrates. According to Freschauf *et al.* [56], superhydrophobic surfaces exhibit antibacterial properties due to their minimal solid-liquid contact at the surface, weak surface interactions with bacteria, and low sliding angle. Such anti-biofouling surfaces repel bacteria due to reduced strength of adhesion, attributed by the air traps between the surface structure and bacterial cell membrane.

3.2 Mechano-bactericidal effect

Research in the field of nanomaterials has revealed that the material's topography is a crucial factor for appropriate biological response. Hayles *et al.* [58] showed that sharp, spikelike nanostructures synthesized on commercially pure titanium surfaces using hydrothermal etching effectively eliminate dental pathogens (*S. mutans*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis*) in anaerobic conditions. Iglič and collaborators [7] prepared TiO_2 nanotubes on the surface of Ti substrate and studied antibacterial properties of TiO_2 nanotubular layer; authors performed tests with oral bacterial species, *S. sanguinis and S. mutans*, and showed that the adherence of oral streptococci can be modified by TiO_2 nanotube diameter [8] as already mentioned above.

The bactericidal activity of nanostructured surfaces originates from its physical [8] instead of its chemical properties; the cell membranes are stretched by the nanostructured surface, which causes cell damage/break. For example, it was suggested among others by Linklater *et al.* [11] that there may be two types of mechano-bactericidal mechanisms of nanostructured surfaces; the first one is associated with a surface composed of nanopillars that induce the stretching beyond the elastic limit of the membrane and its' rupture, while the other type is associated with surfaces composed of sharp nano-edges (for example graphene nanosheets) that induces the reorientation of the lipid tails of the phospholipid bilayer and extraction of the lipids, resulting in pore formation and bacterial cell death.

3.3 Formation of reactive oxygen species (ROS)

The antibacterial influence of ROS formation in some crystalline inorganic oxides, such as TiO_2, ZnO, Mn_2O_3, that are often formed on the surface of metal implants to improve their biocompatibility, should not be neglected. Antibacterial activity of certain semiconductors arise from photo-induced ROS under light irradiation, with the energy equal to or greater than the band gap of the material. The antibacterial properties of TiO_2 are strongly dependent on material's crystalline structure since it is known that crystalline phases affect the formation of ROS (see [59], and references therein). Also, enhanced photogeneration of ROS can arise from material's defects, as shown for ZnO nanoplates by Joe *et al.* [60]. Besides, ROS generation is influenced by the modification of band structure through the introduction of various dopant materials into them [61]. ROS induce a use oxidative cellular damage to the bacterial cells, however the exact antibacterial mechanisms as well as the bacterial response to them remain not fully understood [62].

4. Example: Antibacterial activity of hydrothermally synthesized TiO_2 surfaces

Antibacterial effect of Ti substrate (control) and Ti substrate covered by TiO_2 oxide layer (hydrothermally treated Ti) were studied in order to examine the effect of nanostructured surface of bacterial cells attachment. The testing method used in this study was based on ISO 22196 for evaluation of the antibacterial effect of Ti substrate and hydrothermally treated Ti [58].

In Fig. 2, the morphology of as-purchased Ti substrate and hydrothermally treated Ti surfaces is presented. Ti substrate (foil of a 0.10 mm thickness) is micro-structured (Fig. 2A), however hydrothermally treated sample is nano-structured (Fig. 2B). As shown in Ref. [63], the hydrothermal synthesis resulted in the formation of TiO_2 oxide layer on the surface of

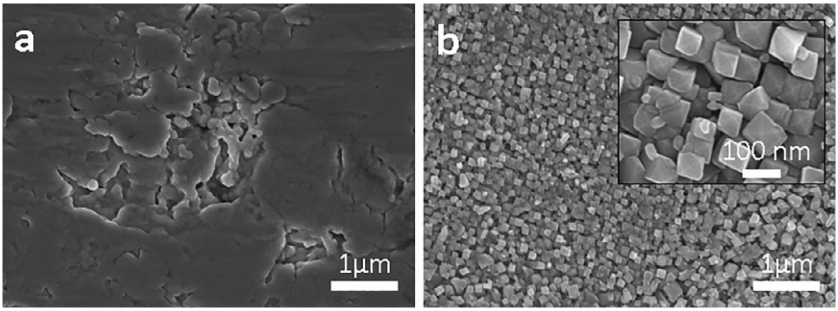

Fig. 2 Scanning electron morphology (SEM) micrographs of the (A) untreated Ti foil and (B) Ti foil subjected to hydrothermal treatment.

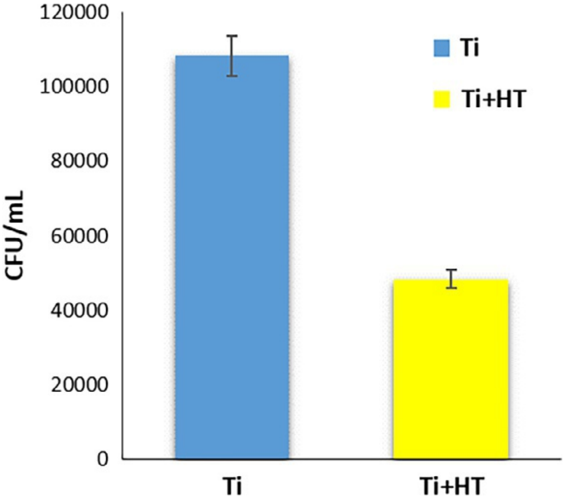

Fig. 3 CFU/mL of *E. coli* against untreated Ti (Ti) and hydrothermal treated Ti (Ti + HT).

Ti substrate. The thickness of the oxide layer after hydrothermal treatment was >55 nm [63]. Water contact angle (WCA) measurements revealed that Ti substrate is hydrophobic (WCA = 97.6°), while hydrothermally treated Ti substrate is superhydrophilic (<5°). Additional Energy-dispersive X-ray spectroscopy (EDX) analysis performed revealed that the surface of hydrothermally treated Ti consists of O—56.5 at.%; Ti—43.4 at.%; K—0.1 at.%, while the 100 at.% of Ti was detected on the untreated Ti foil.

In Fig. 3, differences among the two surfaces (untreated Ti substrate and hydrothermally treated Ti) against Gram-negative bacteria (*E. coli*) are summarized. A significant decrease in the bacterial count was observed for hydrothermally treated Ti in comparison with untreated Ti substrate (Fig. 3).

Results of antibacterial activity tests showed that hydrothermally treated Ti substrate possess higher antibacterial activity as compared to untreated Ti. This could be due to nano-sized features (sharp edges) that can mechanically rupture the bacterial cell membrane, or increased surface electric charge density which may influence the adhesion of bacteria (see also Refs. [5,6,8,48]). However the possible mechanism can also include the formation of ROS [59], since the hydrothermally treated Ti substrate has TiO_2 nano-structured surface. UV treatment of the samples prior to antibacterial tests can improve the antibacterial response of nanostructured samples. Since TiO_2 is semiconducting material with bandgap of 3.2 eV (the bandgap depends on the crystal structure of the material; anatase, rutile), which is equivalent to an excitation wavelength of 388 nm, it is possible that UV irradiation of the samples could trigger the formation of ROS. As pure Ti substrate express higher hydrophobic characteristics than hydrothermally treated Ti surface, the antifouling effect is less possible as an antibacterial mechanism.

5. Future of antibacterial surfaces in biomedicine

The present roadmap clearly shows that bacterial infections pose a serious concern to all implantable materials. To this day implant-related infections present a high risk for implant failure and revision surgery, which may be life-threatening for the patient and is connected to high medical costs. An additional issue is also the high rise of antibiotic-resistant bacteria strains, which are mainly linked to the excessive use of antibiotics, also for the treatment of implant-related bacterial infections. Thus there is an immense demand to develop new strategies to overcome implant-related bacteria infections and find new approaches for the development of antibacterial surfaces, which will prevent the use of antibiotics and lower the risk of implant-related infections. Designing specific nanotopographies and studying the interaction mechanisms with different types of bacteria and biofilms could provide new insights into the complex mechanisms taking place between the biomaterial and the biological environment. This will bring new knowledge highly relevant for the development of surfaces with specific surface features that would elicit desired biological response, not only prevent bacterial infection but at the same time also provide for good proliferation of desired cell type (multifunctional surface). By mimicking the perfect examples found in nature and with recent advances in nanotechnology novel, nanostructured surfaces with superior properties could be developed.

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Appendix

A.1 Synthesis of TiO_2 oxide layer on the surface of Ti substrate

Titanium substrate (Ti foil = 0.50 mm, Advent, 99.6 + %) has been subjected to hydrothermal treatment as described in Ref. [63]. Briefly, Titanium (IV) isopropoxide ($\geq$97.0%, Sigma-Aldrich) has been used as a precursor of Ti ions and potassium hydroxide (90%, flakes, Sigma-Aldrich) was used to adjust the pH of the aqueous suspension to 10. Ti substrate (10 × 10 mm) was heated in a prepared Titanium (IV) isopropoxide suspension in a stainless steel autoclave (Parr Instrument Company, Illinois, USA) at 200 °C for 24 h. After the synthesis, Ti substrate was washed with deionized H_2O, dried under a stream of N_2 and additionally dried in an oven in an air atmosphere at 70 °C for 2 h. Then, Ti foil was ultrasonicated for 5 min, and the washing/drying process with deionized H_2O, N_2 and air was repeated.

A.2 Scanning electron microscope (SEM) analysis

The morphological and compositional analysis of the materials was conducted by Scanning electron microscope (SEM—JEOL JSM-7600F) and Energy dispersive X-ray spectroscopy (EDX—Oxford Instruments).

A.3 Evaluation of antibacterial activity

The pathogenic strain of *Escherichia coli* (*E. coli*) was first prepared in Luria-Bertani broth for 24 h at 37 °C. A suspension of *E. coli* (10^5 colony forming unit (CFU)/mL) was prepared, from which 0.1 mL was pipetted onto the surface of hydrothermally treated Ti and untreated Ti. The samples were then incubated in Incubator (I-105 CK UV, Kambič) for 24 h at 37 °C in a humidity box in order to maintain relative humidity at 90%. After incubation, *E. coli* on the surface was removed using 2.5 mL of sterilized phosphate buffered saline (PBS) and 0.2 mL of this solution was taken for inoculation of *E. coli* in the Nutrient agar plate at 37 °C for 24 h. Then the number of CFUs can be determined. For convenient counting of CFUs, before inoculating *E. coli* in the Nutrient agar plate, the initial solution was diluted further with PBS by factor of 10^0–10^5. The CFU/mL were calculated using automated colony counter (Acolyte 3, Synbiosis).

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