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Evidence of antibacterial activity on titanium surfaces through nanotextures

O. Seddiki [a], C. Harnagea [a], L. Levesque [b], D. Mantovani [b], F. Rosei [a,c,*]

[a] INRS – Centre Énergie, Matériaux et Télécommunications, Boulevard Lionel-Boulet, Varennes, Québec J3X 1S2, Canada
[b] Laboratory for Biomaterials and Bioengineering (CRC-I), Dept Min-Met-Materials Engineering & Research Center CHU-Quebec, Laval University, Quebec City, Canada
[c] Center for Self-Assembled Chemical Structures, McGill University, H3A 2K6 Montreal, Quebec, Canada

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ABSTRACT

Nosocomial infections (Nis) are a major concern for public health. As more and more of the pathogens responsible for these infections are antibiotic resistant, finding new ways to overcome them is a major challenge for biomedical research. We present a method to reduce Nis spreading by hindering bacterial adhesion in its very early stage. This is achieved by reducing the contact interface area between the bacterium and the surface by nanoengineering the surface topography. In particular, we studied the *Escheria Coli* adhesion on titanium surfaces exhibiting different morphologies, that were obtained by a combination of mechanical polishing and chemical etching. Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM) characterization revealed that the titanium surface is modified at both micro- and nano-scale. X-ray Photoelectron Spectroscopy (XPS) revealed that the surfaces have the same composition before and after piranha treatment, consisting mainly of TiO_2. Adhesion tests showed a significant reduction in bacterial accumulation on nanostructured surfaces that had the lowest roughness over large areas. SEM images acquired after bacterial culture on different titanium substrates confirmed that the polished titanium surface treated one hour in a piranha solution at a temperature of 25 °C has the lowest bacterial accumulation among all the surfaces tested. This suggests that the difference observed in bacterial adhesion between the different surfaces is due primarily to surface topography.
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1. Introduction

Nosocomial infections (Nis), also referred to as healthcare associated infections, are defined by the U.S. Centers for Disease Control and Prevention (CDC) as infections contracted by a patient after 48 h or more subsequent to his admission in a hospital or health care facility [1]. According to this definition, it is thus mandatory that the patient should show no symptom of infections at the time of admission [1].

According to the World Health Organization (WHO) [2], the proportion of hospitalized patients between 1995 and 2010 who suffered from Nis in developed countries varied by country from 3.6% to 12%, whereas in less-developed countries, this ratio ranges from 5.7% to 19.1%. In the USA, 3–5% of health care facility users contract Nis, and since the 1980s, an increasing proportion of infections are due to pathogens resistant to therapies [3]. Nis are a major concern for public health. In 2002 alone, about 1.7 million of these infections were reported to cause 99,000 deaths in the United States alone [4]. This placed Nis as the main source of deaths ahead of other infectious diseases like the Acquired Immunodeficiency Syndrome (AIDS) [5]. Furthermore, the annual direct medical costs induced by Nis, in U.S. Hospitals, have been estimated between 35.7 and 45 billion dollars [6].

A majority of Nis are caused by bacteria [7]. The infectious agents can be transmitted by patient care personnel, mostly caused by lack of hand hygiene [8]. Another major channel of transmission is the healthcare equipment, which is estimated to contribute to up to one-third of all Nis [9]. In addition, medical devices and the health care environment may act as exogenous sources of these infections [1].

To control the spreading of Nis, standard cleaning and sanitizing protocols based on chemical treatments are routinely applied [10,11]. Alcohol 70% is very efficient in reducing the levels of bacterial contamination by up to 82% on health care equipment [9]. This is

* Corresponding author at: INRS – Centre Énergie, Matériaux et Télécommunications, Boulevard Lionel-Boulet, Varennes, Québec J3X 1S2, Canada.
Tel.: +1 514 228 6905; fax: +1 450 929 8198.
E-mail address: rosei@emt.inrs.ca (F. Rosei).

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a significant result for thwarting Nis, since health care equipment is a major transmission channel for these diseases. Also, surface sanitation by disinfectant gases such as formaldehyde, ethylene oxide and hydrogen peroxide is another method used to reduce the spreading of these infections [12,13].

Bacterial adhesion to a surface results in the formation of a biofilm which confers resistance to antimicrobial agents [14,15]. Therefore, effective methods to prevent the development of biofilms should act at an early stage [16]. This "prevention rather than cure" approach is founded on the use of materials with antibacterial properties. Accordingly, surfaces subject to bacterial contamination (surgical tools, medical devices, doorknobs, etc.) are coated with materials that prevent such bacterial colonization.

The antibacterial activity of a surface is induced by inserting bactericidal elements in a durable coating. For example, elements such as Cu, Ag and V embedded in a diamond like carbon film (DLC) [17] render the resulting surface antimicrobial. Similar results are achieved when different materials used for coating are impregnated with elements such as antibiotics, quaternary ammonium compounds, iodine, fluorine, phenols, or heavy metals [11,16,18–29]. The lethal effect of these bacteria contact killing surfaces requires continuous release of bactericidal elements in the environment. This can lead to the increase of bacterial resistance to such elements [19], and makes it challenging to maintain the efficiency of these surfaces over a long period of time [18,19].

A different approach to overcome these issues is to design surfaces that do not necessarily kill bacteria, but rather simply hinder bacterial adhesion in its very early stages. This approach is based on the well-known fact that the adhesion of bacteria to a surface occurs in two steps: (i) a physically reversible adhesion phase followed by (ii) an irreversible molecular and cellular attachment phase [30]. Hence, acting at the first stage of bacterial colonization (physical adhesion) minimizes the chances of biofilm formation [31].

The adhesion of bacteria on a surface depends on many factors [30,32]: conditions prevailing in the medium close to the surface like the temperature, duration of exposure, or concentration of the bacteria; the properties of bacteria like hydrophobicity or surface charge [30] and the characteristics of the surface such as its chemical composition, roughness, surface topography, or wettability.

In this work, we focus on the effect of surface topography on bacterial adhesion. It is already well-known that surface morphology affects the development of microorganisms [33], as widely exploited in tissue engineering applications [34]. It is now well established that features with sizes below few micrometers present on the surface (like grooves, ridges, and wells) influence the adhesion, orientation, motility and morphology of eukaryotic cells [35–37]. However, several differences occur between bacteria (prokaryotic cells) and eukaryotic cells, the most relevant for this work being the higher rigidity of bacteria, making them less prone to change their morphology to conform to surface features, as eukaryotic cells are known to do [38,39]. Nevertheless, the topographic structures on the surface also influence bacterial adhesion. For example, it was reported that bacteria tend to align preferably along existing scratches, when the width of the grooves (~700 nm) is comparable to the transversal diameter of the bacterium (~500 nm), but shorter than its length (~2 μm) [40]. Therefore, if the size and shape of such scratches match that of the bacterium, its adhesion is enhanced markedly, since the cell – surface contact is maximized [41]. In contrast, when the structures on the surface are smaller, the bacterium may not be able to conform to these asperities. The contact interface between the bacterium and the substrate being reduced considerably, they interact only at the top of the surface features [42]. Micro-structured surfaces with a design achieved by biomimicry have been proven to reduce significantly the colonization of bacteria only due to their microscale topography [43,44].

There are several ways to induce topographic features on a surface [38]. The technique of choice depends on the nature of the material, the complexity of the targeted structures, and the desired accuracy. Here we report a surface modification that hinders bacterial adhesion by using an effective, simple and low cost method. The latter consists of etching micro and nanoscale topographic features at the surface of a material by using a simple chemical treatment. This technique has the additional benefit of being easily scalable in industrial environments for efficiently treating objects independent of their size or shape [45].

Here we selected titanium, because it is widely used in biomedical implants and bone-contact applications [46,47]. Previous work focusing on orthopedic implant materials showed that the piranha solution can act as etchant to induce micro and nano features on titanium and titanium alloy substrates, thereby improving biological response [48–54]. The aim of this work was to regulate cell-substrate interactions, for better host-tissue integration of medical implants, by accelerating osteoblast growth while inhibiting the proliferation of fibroblasts. Our current efforts intend to demonstrate that piranha etching can also be used to induce antibacterial properties. Despite its importance in the confinement of nosocomial infections, our finding is also important in the field of biomedical implants because bacterial infections may occur in 22–66% of cases with a morbidity rate of up to 2% [55].

2. Materials and methods

2.1. Substrate preparation

The study of the dependence of bacterial adhesion on nanotextured titanium surfaces was performed on four types of surfaces: (i) as received surface, untreated, (ii) polished only, and polished and etched at two different temperatures, (iii) 25 °C and (iv) 80 °C.

The substrates were titanium sheets (99.7% purity, 0.89 mm thick from Alfa Aesar) cut into 10 mm × 10 mm specimens. The type (i) substrates were used without further processing, except the standard cleaning procedure, consisting of washing in an ultrasonic bath for 1 h with distilled water, then for 15 min with acetone and finally drying under nitrogen flow.

2.1.1. Polishing

The untreated substrates were mechanically polished to a mirror finish using an automatic grinding machine (Abramin™, Struers) by following several steps [51]. First, the samples were polished using SiC grinding paper (grain size 36 μm), step repeated until the sample showed a homogeneous surface appearance to the naked eye. The samples were then further polished for 5 min using a diamond suspension (grain size 9 μm). Finally, the mirror finish was obtained by a treatment with colloidal silica suspension (SiO_2 with a grain size of 0.05 μm) for 10 min. After polishing, the samples were cleaned using the same procedure as for type (i) substrates specified above.

2.1.2. Chemical etching

The piranha solution was prepared by mixing an equal volume of 30% aqueous hydrogen peroxide H_2O_2 with sulfuric acid H_2SO_4 (95–97%) in a beaker under continuous stirring. The container was kept immersed in a water bath held on a stirring hot plate. The temperature of the solution was controlled by placing the temperature probe of the hot plate controller (EchoTherm™ HS40, Torrey Pines Scientific) in the container.

When the piranha solution reached the desired temperature, the titanium samples, previously placed in an appropriate sample holder, were chemically etched by immersing them in the solution for 1 h. After etching, the treated samples were rinsed with distilled water, then cleaned for 20 min in an ultrasonic bath with ethanol

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