

Feasibility of a Chronic Foreign Body Infection Model Studying the Influence of TiO₂ Nanotube Layers on Bacterial Contamination

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Abstract

Bacterial infections on the surface of medical devices are a significant problem in therapeutic approach, especially when implants are used in the living. In cardiology, pacemaker generator pocket surfaces, made in titanium alloy can be colonized by pathogen microorganism. This contamination represents a major risk of sepsis, endocarditis and localized infections for patients. A way to limit this bacterial contamination is to modify the surface topography using nano-structuration process of the titanium alloy surface of the implanted devices. The aim of this study is to evaluate the influence of TiO₂ nanotube layers on bacterial infection in the living, considering the feasibility of an animal model of chronic foreign body infection. TiO₂ nanotube layers prepared by electrochemical anodization of Ti foil in 0.4 wt% hydrofluoric acid solution were implanted subcutaneously in Wistar rats. Three weeks after implantation, TiO₂ implants were contaminated by a *Staphylococcus epidermidis* strain using two different concentrations at 10⁶ and 10⁸ colony forming unit (CFU) in order to induce a sufficient infection level and to avoid unwanted over infection consequences on rats health during the experiments. After 28 days in the living, 75% of nanotube layers initially submitted to the 10⁸ CFU inoculum were contaminated while only 25% nanotube layers initially submitted to the 10⁶ CFU inoculum remained infected. This significant result underlines the influence of TiO₂ nanotube layers in decreasing the infection level. Our *in vitro* experiments showed that the synthesized TiO₂ nanotubes indeed decreased the *Staphylococcus epidermidis* adhesion compared to unanodized Ti foil.

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Keywords

Titanium Implants, Titanium Dioxide Nanotubes, Bacterial Adhesion, Cardiac Device Implantable

1. Introduction

Due to the evolution of medical indications, we are witnessing a cardiac device implantable (CDI) increase estimated in France at 2 percent per year [1] [2]. Infections due to contamination of CDI are relatively common and dangerous. The incidence varies between 0.13 and 19.9 percent depending on the series [3]. We distinguish localized infections at the pulse generator pocket which are the most frequent (89 percent), sepsis or endocarditis infectious, less frequent but more severe with a mortality rate around 25 percent. The majority of infections are caused by staphylococci (71 to 93.5 percent) with 33 to 75 percent of coagulase-negative Staphylococci strain and 14 to 44 percent of *Staphylococci aureus*. For this last specific strain, 15 to 30 percent are methicillin-resistant. Retrospective studies on the management of these infections have shown the superiority in terms of morbidity-mortality rate of an association of antibiotics and extraction of the entire CDI (pulse generator and probes) versus antibiotics alone. Indeed, in contact with the cardiac device, these bacteria create in the early hours a biofilm, increasing antibiotic resistance and mechanisms of host and can also cause chronic infection [4]. The synergistic multiple antibiotics are binding, toxic and ineffective. The extraction of CDI is complex and risky [5].

Pulse generator is composed of a titanium alloy: Ti-6Al-4V. It has a high corrosion resistance, high biological inertia and good tissue integration (good biocompatibility). These characteristics are superior to those of pure titanium [6]. Pure titanium or titanium alloy can be nanostructured using electrochemical techniques such as anodization. It is then possible to obtain titanium dioxide nanotube arrays of controlled and homogeneous size distribution [7]-[10]. Previous studies have demonstrated an antibacterial effect, usually by reducing bacterial adhesion, but also bactericidal effect of titanium dioxide nanotube arrays [11]. In order to decrease CDI infections by using a method of nanostructuring of the device, it is necessary to confirm that the *in vitro* data can be extended to an *in vivo* animal model.

The main objective of our study was to investigate the feasibility of a chronic foreign body infection animal model and to qualify the influence of titanium dioxide nanotube arrays in decreasing the adhesion of *S. epidermidis in vitro*.

2. Materials and Methods

2.1. TiO₂ Nanotubes Layer Synthesis

To fabricate anodic TiO₂ nanotube layers, we used Ti foil (Goodfellow, 99.6% purity) with a thickness of 0.025 mm. The Ti foils were cut 8 mm × 8 mm squares by shear and degreased by successive sonication in trichloroethylene, acetone, and methanol, followed by rinsing with deionized water and blown dry with nitrogen. Afterward, the cleaned Ti samples were etched with a HF:HNO₃:HCl:H₂O (1:10:20:69) solution for 5 min to remove the naturally occurring oxide layer and subsequently rinsed with deionized water and blown dry with nitrogen gas. Finally, the Ti foils were dried in an oven at 100°C and cooled in a desiccator. Anodization was carried out at room temperature (20°C) in 0.4 wt% HF aqueous solution with the anodizing voltage maintained at 20 V during 20 min. After the anodization process, the samples were cleaned with deionized water and blown dry with nitrogen. They were finally dried in an oven at 100°C and cooled in a desiccator.

2.2. Scanning Electron Microscopy

Samples were characterized by a Field Emission Scanning Electron Microscope (FESEM) ZEISS Supra 55 VP (ZEISS-Germany) used with a low voltage (3 kV) to limit charge effects. The working distance was 5 mm. Pictures were obtained with the secondary electron detector in-lens.

2.3. Bacterial Culture

An American Type Culture Collection (ATCC) 35,984 *Staphylococcus epidermidis* strain is used. This strain

was previously modified with the introduction of a plasmid carrying the gene for tetracycline resistance and that encoding a fluorescent protein (GFP) (pCN40- Δ erm-Tet-GFP). The strain is stored at -80°C .

2.4. Assay for *in Vivo* Study

The study was approved by the Ethics Committee for Animal Experimentation Auvergne (CEMEAA, authorization number EC 19-13). An animal model previously described [12] and already used on rats [13] was chosen with some modifications. Male Wistar rats (Janvier, Mayenne, France) from 220 to 240 g weight were used. Rats were anesthetized by intraperitoneal injections of ketamine (50 mg/kg, Panpharma, France) with Xylazine (15 mg/kg, Rumpun[®], Bayer, France). During the whole experiment they receive analgesia if needed by Buprenorphine (0.05 mg/kg, Vetergesic[®] multidosage, Alstoe, UK). For simple anesthesia for subcutaneous punctures, they receive isoflurane (2% - 3% Forane[®], Baxter, USA). The rats were sacrificed by intra cardiac injection of thiopental (50 mg/kg, Rotexmedica/Panpharma, France). We implanted subcutaneously for each rats 2 multi perforated tubes of polytetrafluoroethylene (Teflon) closed at the ends and having the dimensions: 10 mm in inner diameter, 12 mm in outer diameter and 32 mm long (Figure 1). These tubes contain two Ti samples of the same nature and the same origin (either native or TiO₂ nanotube arrays). A sample is for making a quantitative analysis and a second for a qualitative analysis by fluorescence. Each rat has a tube containing TiO₂ nanotube arrays and a tube containing native Ti foils, thereby forming its own control. Three weeks after implantation of tubes in sterile condition of 4 rats, a puncture of the tubes is carried out to verify sterility. Animals with contaminated tubes are excluded. Four tubes (2 rats) are infected with 10⁶ CFU (inoculum “low”) and the other 4 tubes with CFU 10⁸ (inoculum “high”), by subcutaneous injection.

The kinetic profile of the planktonic infection (non-adherent bacteria) of the tubes is determined by performing sampling every 4 days until day 20 (Figure 2). Two hundred microliters of the fluid from the tubes are removed at each sampling. Decimal dilutions of the samples are made in saline, and then 25 μL are plated on LB agar containing tetracycline at 12 $\mu\text{g}/\text{mL}$. After 48 h incubation at 37°C , a quantification of bacterial colonies is done.

After 28 days, the two samples are removed from each tube (Figure 3). The number of adherent bacteria (bacteria of the corresponding biofilm) of each sample is determined as described below (*in vitro* study). The second sample is analyzed qualitatively, using a confocal microscope (Leica TCS-LSI Solism, Germany).

2.5. Assay for *in Vitro* Study and Bacterial Adhesion

Twelve nanostructured samples and 12 pure Ti plates are disposed in wells of a 24 well plate (Falcon[®], BD Biosciences, California, USA) containing 1 mL of TSB (Formula per liter: Pancreatic digest of casein, 17.0 g, Papaic digest of soybean, 3.0 g, Dextrose, 2.5 g, Sodium chloride, 5.0 g, pH 7.3) and 0.5 mL of the inoculum suspension (*S. epidermidis*), containing about 5.10⁶ CFU. Suspensions are incubated at (37°C , 5% CO₂, humidified environment), under stirring at 25 rpm. Different incubation times are predefined: 1, 2, 6, 24 hours. At the end of incubation time, 3 plates of each assay are rinsed two times in physiological saline to remove non-adherent bacteria. Each plate is sonicated for 15 min in 1.5 mL of physiological saline to detach and individualize adherent bacteria. Fifty microliters of the suspension thus created are then seeded (EasySpiral[®] Dilute, Interscience, Yvelines, France) onto agar plates of lysogeny Broth (LB; DifcoTM LB Agar Miller, Becton Dickinson



Figure 1. Photograph of a multi perforated Teflon tube containing nanostructured Ti foil.

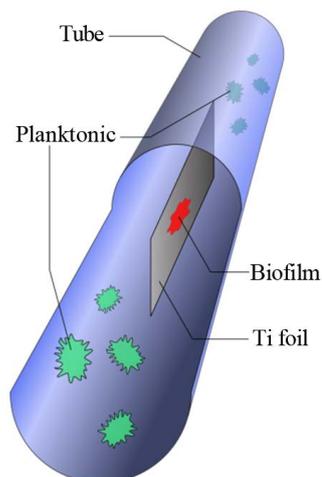


Figure 2. Schematic representation of the tubes distribution containing Ti foils (native or nanostructured) with *S. epidermidis* (planktonic and biofilm).

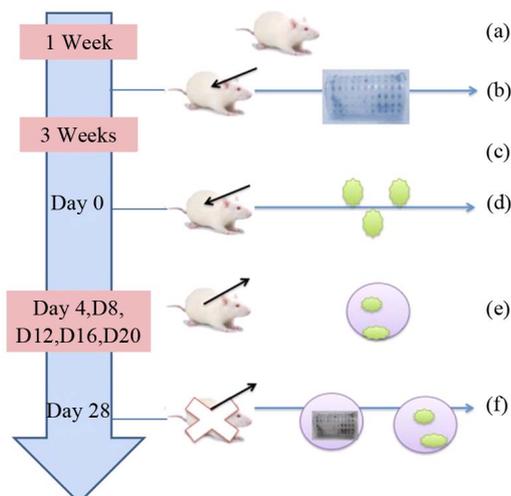


Figure 3. General scheme of the experiment steps (a) stalling. (b) Implantation of the two tubes containing respectively native or nanostructured Ti foils (c) wound healing, formation of serous fluid in the tubes. (d) *Staphylococcus epidermidis* inoculation to sterile tubes in rats (e) sampling in the tubes every 4 days for bacteriological analysis of the planktonic phase (f) euthanasia of rats. Extraction of the plates contained in the tubes for bacteriological analysis of biofilm.

and Comany Sparks, MD 21152, USA) containing Tétracycline at 12 $\mu\text{g/mL}$. These agar plates were incubated for 24 h at 37°C. The quantification is done automatically by counting colonies (Scan[®] 300, Interscience, Yvelines, France). This experiment was performed in triplicate.

2.6. Statistic Study

Numerical data were analyzed using analysis of variance (ANOVA) standard as the Student test for paired samples. Indeed, we studied bacterial quantification (quantitative variable) depending on the nature of titanium surface (qualitative variable). In addition, for each incubation of a TiO_2 nanotube arrays is included in the same conditions, a control sample of pure Ti. The number n is 9, so we have a degree of freedom of 8. The significant difference is seen as $p < 0.05$.

3. Result and Discussion

3.1. Fabrication of Ordered Nanotube Arrays

Figure 4 shows FESEM top down images of the ordered array of TiO₂ nanotubes obtained by anodizing a Ti foil at 15 V (**Figure 4(a)**) and at 20 V (**Figure 4(b)**). We observe ordered nanotube arrays grown on top of the Ti foil.

From the FESEM images, measurements of the diameter and length of the TiO₂ nanotubes were done. The results are shown in **Table 1**. The increase in the anodization voltage induces a rise of the length and diameter of the TiO₂ nanotubes.

Figure 5 shows the EDX spectra of the samples anodized at 15V (**Figure 5(a)**) and 20 V (**Figure 5(b)**) and their elemental composition. The both samples had similar elemental composition. The fluorine detected on the surface of the TiO₂ nanotube layers are originated from residual electrolyte in the TiO₂ nanotubes.

3.2. Quantitative Results of Bacterial Adhesion

Rats are normally fed, they all continued to gain weight throughout the experiment (average weight 405 g on day 28, with minimum weight and maximum weight respectively 392 g and 419 g. We did not notice any post-operative healing problem. The two inocula used were sufficient to ensure a localized infection without altering the rats health during the experiment. After 28 days post inoculation surveillance, no rat died prematurely or had clinical signs of sepsis. Planktonic bacterial profile during the experiment is summarized in **Figure 6**. We have plotted Log₁₀ (CFU/mL) versus days after the sample contamination.

With both inocula (“low” and “high”), we see a decrease in the number of bacteria over time. At the end of the experiment on day 28, a tendency to the disappearance of planktonic bacteria was observed with the inoculum

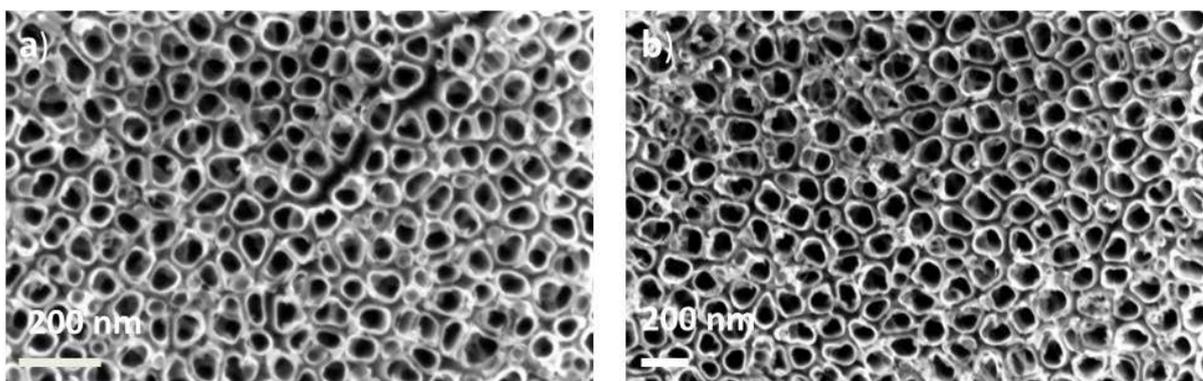


Figure 4. Top down FESEM images of TiO₂ nanotube arrays nanotubes obtained by anodization at 15 V (a) and at 20 V (b), magnification 100 K.

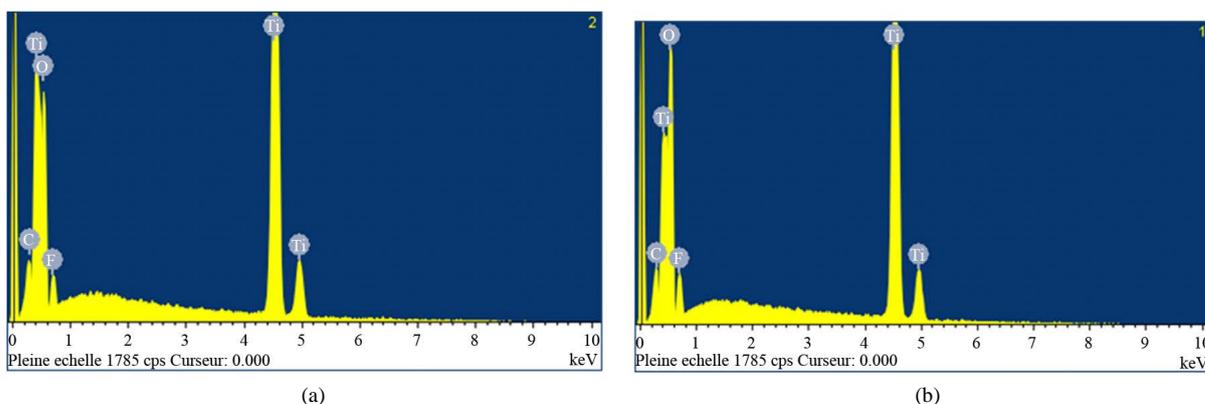
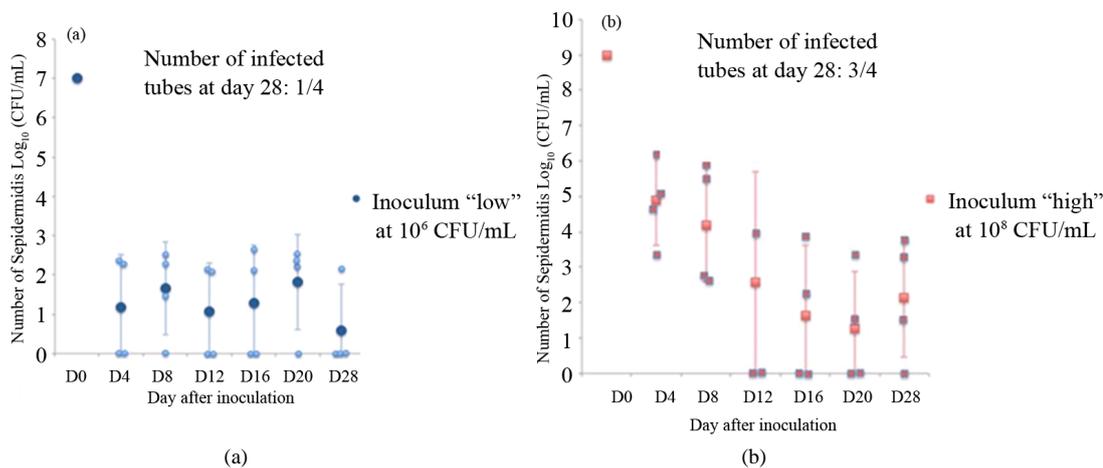


Figure 5. EDX spectra and TiO₂ nanotube arrays nanotubes obtained at 15 V (a) and at 20 V (b). Abbreviation: EDX, energie dispersive X-ray.

Table 1. Influence of the anodization voltage on the length and diameter of the TiO₂ nanotubes.

Anodization voltage	TiO ₂ NT diameter	TiO ₂ NT height
15 V	64.5 (±9.8)	279.3 (±28)
20 V	84.1 (±15.4)	371 (±31.8)

**Figure 6.** Planktonic bacterial profile during the experiment: (a) Inoculum “low” at 10⁶ CFU/mL (N = 4 light blue and mean dark blue); (b) Inoculum “high” at 10⁸ CFU/mL (N = 4 dark red and mean light red).

“low”: in three out of four tubes, we did not detect bacteria. On the contrary, with the inoculum “high” we find bacteria in three out of four tubes (75%).

Analysis of adherent bacteria to the samples (the biofilm forming) confirms the results of planktonic bacteria. It is summarized in **Table 2**.

A decrease in the amount of adherent bacteria in relation to the amount of bacteria in planktonic phase is observed when the tubes contained TiO₂ nanotube arrays (**Table 2**) while the tubes with native Ti foils have the same amounts of adherent bacteria and planktonic bacteria.

3.3. *In Vitro* Bacterial Adhesion

Bacterial growth on TiO₂ nanotube compared with native Ti, depending on the incubation time is shown in **Figure 7**. Globally, the amount of adherent bacteria increases with time in every condition. At each time of culture, numbers of alive bacteria able to form colonies are lower in TiO₂ nanotubes condition, with significant difference after 1 and 6 hours. The tendency seems to decrease later.

3.4. Discussion

This study is, for our knowledge, the first to investigate the feasibility of *in vivo* evaluation of the antibacterial properties of TiO₂ nanotube arrays. The results of *in vitro* manipulations have revealed a decrease in the adhesion of *S. epidermidis* in the presence of TiO₂ nanotube arrays.

In a previous study [14], a decrease in bacterial adherence was observed when Titanium substrate was coated by evaporation technique using an electron beam. In contrast, when the anodization process was used, there was, in spite of a higher adhesion of fibronectin, an increase in bacterial adhesion to the TiO₂ nanotube arrays. We can suggest two explanations: first, the role of fluorine coming from the electrolyte. Even if the fluorine allows a reduction in percentage living bacteria with a clean anti-bacterial effect, it helps to increase the adhesion of dead bacteria, promoting themselves the adhesion of living bacteria [15]. Secondly, anodization provides an amorphous TiO₂ layer which, in comparison with a crystalline layer increases bacterial adhesion. We chose the anodizing process because this technique allows the formation of TiO₂ nanotube arrays. In further works, the nanocavities may be used as nanocarrier for drug delivery.

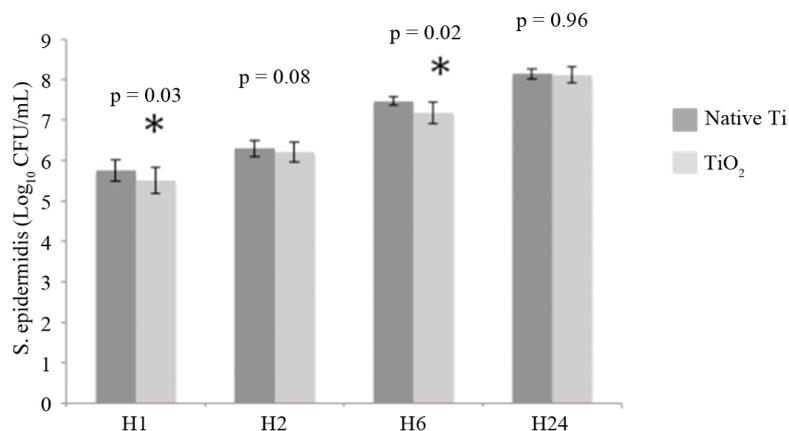


Figure 7. Bacterial on growth on native Ti vs. TiO₂ nanotube arrays.

Table 2. Planktonic and adherent bacteria quantification on day 28.

Tubes	Adherent bacteria (Log ₁₀ CFU/mL)	Planktonic bacteria (Log ₁₀ CFU/mL)
TiO ₂ NT-1	N.D.	N.D.
TiO ₂ NT-2	2.8	5.3
TiO ₂ NT-3	N.D.	N.D.
TiO ₂ NT-4	2.8	3.7
Pure Ti-1	N.D.	N.D.
Pure Ti-2	N.D.	N.D.
Pure Ti-3	3.1	3.3
Pure Ti-4	1.6	1.6

Thus, our experimental results are opposite to others studies which revealed an increase in bacteria (even living) vs native Ti. Anodization process parameters are responsible for these changes in bacterial adhesion. In other study, higher concentration of HF (1.5% vs 0.4%), and a shorter duration of anodization (10 min vs 20 min) are used, it increases the content of fluorine ion and reduce the size of the tube diameter (70 vs 84 nm), which are two factors that promote bacterial attachment.

Concerning the nanotube diameter influence on cell adhesion, a previous study [16] showed that depending on the diameter of the TiO₂ nanotube arrays, an adherence decrease for *S. epidermidis* and *S. aureus* were found. As in our work, it has been found a reduction of adhesion from H1, especially when the nanotube diameter was between 60 and 80 nm. The nanotubes would lead to a stress response of bacterial, with membrane rupture, thus reducing the number of living bacteria. For diameters of 80 nm, a 20 V voltage anodization was needed for 20 minutes with 0.4% HF, whereas for the diameters of 60 nm, a voltage of 15 V was enough. The voltage variation also causes a variation of the Ti/O ratio as regards the composition of the TiO₂ nanotube arrays. This Ti/O ratio can influence the cell adhesion.

In the same study [16], the authors found a decrease in the adhesion of dead bacteria when TiO₂ nanotube arrays were annealed at high temperature (500°C). Indeed, this technique can remove fluoride ions coming from the anodization and make the TiO₂ layer crystallized. Also, it was shown that a crystalline layer decreased bacterial growth [17]. We chose initially not to anneal our nanostructured samples.

TiO₂ nanotube arrays coupled or not to an electrical stimulation of 15 to 30 V also permit a decrease in the formation of the biofilm of *S. aureus* after 2 days of culture compared with the native Titanium [18]. Like in our study, bacterial adhesion without stimulation H24 was almost identical between the TiO₂ nanotube arrays and the native Titanium. On the other side, authors noted an increase of the bacterial biomass to H1, H4 and H8 in the presence of TiO₂ nanotube arrays, measured by the crystal violet staining. However, the authors could not

clarify whether this was an increase in adhesion (due to overproduction of the fibronectin promoted by the TiO₂ nanotube arrays), or an increase in the amount of biofilm, or both. The technique used did not distinguish between live and dead bacteria. They specified that a cell count (live bacteria) was necessary to make sense of things considering the increase in density of staining. Our study (using the determination of the number of viable bacteria, CFU) told us that bacterial adhesion to H1 and H6 was lower on TiO₂ nanotube arrays.

In order to reduce bacterial adhesion, other studies have been conducted. Impregnating TiO₂ nanotube arrays with antibiotic (Gentamycin), could also decrease the adherence of *S. epidermidis* [19]. The authors also found an increase in the adhesion of *S. epidermidis* to H1 on TiO₂ nanotube arrays without antibiotics vs. conventional Ti, whereas for H4 there was a decrease.

More recently, the idea of incorporating silver nanoparticles into the TiO₂ nanotube arrays was developed [11]. After 4 days of culture, all planktonic bacteria had been destroyed, and that this association nanoparticles and nanotubes prevent bacterial adhesion. In our study, we did not choose to use these alternative techniques. These experimental data will also have to be checked *in vivo*.

We conducted our manipulations on sheets of Ti Goodfellow[®] brand, which is—as we have seen—similar to native Ti. X-ray study of a PM has shown that it was not native Ti. The material used is an alloy of Ti:Ti-6Al-4V. Other studies focusing directly on the evaluation of *in vitro* antibacterial properties of the alloy TiO₂ nanotube arrays had reached the same conclusions as for the TiO₂ nanotube arrays obtained from pure Titanium. So we could extrapolate our results on Ti Goodfellow[®] sheets to those we could get on the surface of a real Pacemaker.

Considering our *in vitro* results, the feasibility of an animal model of chronic foreign body infection had all his senses to find a possible reduction in the adhesion of *S. epidermidis* on TiO₂ nanotube arrays versus native Ti *in vivo*.

We chose an experimental model, first described in guinea pig, which was initially developed to evaluate the effectiveness of antibiotics in localized chronic foreign body infection. Contrary to the rat, this animal is similar to humans in how to resist to infection, but it is also very fragile, more expensive and difficult to handle.

For these reasons we decided to adapt this model in rats as previously described with the idea that the infected polytetrafluoroethylene tube could accept a Titanium foil in an infected environment. This is a quiet convenient method because a biofilm can develop easily on the material surface, and the surrounding planktonic phase is always accessible transcutaneously to monitor the evolution of infection. At the same time, with this model it is difficult to insure the development of a chronic infection as the inoculum quantity necessary to do that is not known and is probably strain related. We found that the introduction of Ti foil (whether native or nanostructured) into tubes were well tolerated by rats. The wound indeed, healed normally and there were no clinical signs of poor tolerance of the foreign body. In addition, it was necessary to determine the “ideal” inoculum. It must be sufficient to induce a chronic infection in the tube, but it must also not be too large to avoid the risk of inducing sepsis, which may skew the results. Previous trials using this model had consistently confirmed this preliminary study. We chose two inocula: a “low” CFU 10⁶ and a “high” CFU 10⁸. This choice was guided by the fact that the rat’s natural defenses against staphylococci are more effective than those of the guinea pig and also because we used a strain of *S. epidermidis* not a *S. aureus* strain, known to be more resistant to the natural defenses of the rat. The injection of a volume of 100 μL was intended not to create pressure inside the tube that can induce capillary damage and also not to disseminate the inoculum outside the tubes. The results of this *in vivo* study have shown that chronic infection localized foreign body was feasible in 75% of cases, with a “high” inoculum CFU 10⁸. The results at Day 28 were also encouraging. Indeed, when we compared the amount of planktonic and adherent bacteria, we found that they were identical in tubes containing native Ti foils, and different for those with TiO₂ nanotube arrays. The amounts of adherent bacteria were then lower than those determined in planktonic phase. These results should be confirmed by a study with a sufficient number of rats to determine whether or not there is a significant difference between the two types of support.

4. Conclusion

The feasibility of a chronic foreign body infection model has been investigated in this work. The animal experiments results on wistar rats indicate that infection is still present 28 days after the samples contamination in a quarter of the implants infected at 10⁶ CFU while 75% of the samples infected remain infected with an inoculum at 10⁸ CFU. The adaptation of this previously described animal model of chronic foreign body infection to a new

one, where the tube is considered as a cell dedicated to test infection of different materials seems to make sense. The final infection at day 28 seems to be dose-dependent. With both inocula, the number of bacteria decreases versus time, showing the tendency of the TiO₂ nanotubes layer in reducing the infection more efficiently than pure titanium surface. By comparison of confocal cliché we emit the hypothesis that bacterial adhesion is lower on the nanostructured samples versus the unanodized foil. This difference concerning biofilm adhesion is confirmed by our *in vitro* experiments. A significant difference in bacterial adhesion is seen between native titanium foil and TiO₂ nanotube arrays using the analysis of variance: bacterial adhesion is lower on nanostructured samples compared to unanodized titanium surfaces.

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