Materials Science and Engineering C 45 (2014) 176–183

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Role of protein environment and bioactive polymer grafting in the *S. epidermidis* response to titanium alloy for biomedical applications

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ARTICLE INFO

Article history:
Received 2 January 2014
Received in revised form 15 July 2014
Accepted 29 August 2014
Available online 6 September 2014

Keywords:
Bacterial adhesion
Bioactive polymer
Poly(sodium styrene sulfonate) (polyNaSS)
Protein adsorption
Surface modification
Titanium alloy

ABSTRACT

Joint implant-related infections, namely by *Staphylococci*, are a worldwide problem, whose consequences are dramatic. Various methods are studied to fight against these infections. Here, the proposed solution consists in grafting a bioactive polymer on joint implant surfaces in order to allow the control of the interactions with the living system. In this study, sodium styrene sulfonate, bearing sulfonate groups, was grafted on the surface of titanium alloys. Scanning Electron Microscopy, colorimetric method, Fourier-transformed infrared spectroscopy and contact angle measurements were applied to characterize the surfaces. Bacterial adhesion studies were studied on poly(sodium styrene sulfonate) grafted Ti_6Al_4V and Ti_6Al_4V surfaces previously adsorbed by proteins involved in the bacteria adhesion process. Fibrinogen and fibronectin were demonstrated to increase staphylococcal adhesion on Ti_6Al_4V surfaces. Ti_6Al_4V grafted sodium styrene sulfonate surfaces inhibited the adhesion of *Staphylococcus epidermidis* in 37% and 13% on pre-adsorbed surfaces with fibrinogen and fibronectin, respectively. The mechanism of the observed inhibiting bacteria adhesion properties is related to the differences of proteic conformations induced by poly(sodium styrene sulfonate) grafting.

1. Introduction

The growing human life span and the continuous demand for a better quality of life are the main motivations behind all the developments in biomaterials industry and research. Despite all the advances in the quality of healthcare, the probability of infection during a surgical procedure is still high. In the orthopedics field, where biomaterials find a high variety and number of applications, the risk of infection is of about 2–5% [1]. Currently implanted medical devices are still unable to actively resist bacterial adhesion, colonization, and biofilm formation. Ideally, it would be possible to eradicate implant-related infections with effective antibiotics, but this strategy faces two major problems: the bacterial biofilm resistance to the antibiotic penetration and the development of microbial resistance [2,3].

When a biomaterial is introduced in the body its surface is instantaneously covered by plasma proteins, thus becoming the interface that will contact with the surrounding biological environment [4]. Bacteria, namely *Staphylococcus aureus* and *Staphylococcus epidermidis*, the most important microorganisms causing implant-associated infections, present, on their surface, receptors to domains present in the adhesive proteins, such as fibrinogen (Fg) and fibronectin (Fn), called adhesins, which can quickly promote their adhesion [5,6]. After adhering, bacteria may colonize and change their phenotype in order to start the biofilm production. Inside the polymeric matrix of the biofilm, a bacterial community is developed and its elimination either by the host immune system or by the usage of antibiotics becomes extremely difficult [7].

With the aim of avoiding bacterial adhesion and the consequent biofilm formation, biomaterial surface properties can be improved [8,9]. In addition, the protein adsorption process as well as the composition of the adsorbed protein layer and the conformation of adsorbed proteins are also dependent on the surface properties, namely chemistry, hydrophilicity and roughness by allowing the exposure or not of protein domains involved in bacterial adhesion [9,10]. Covalent grafting is one type of surface modification that offers a strongest link between biomaterial implant and its coating, producing a more durable interface [11]. Grafting of poly(sodium styrene sulfonate) (poly(NaSS)) on titanium surfaces has been investigated and shown to enhance bone cells' adhesion and also has antibacterial properties to some extent [9,12–15].

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http://dx.doi.org/10.1016/j.msec.2014.08.054
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This work aims to study the potential application of a poly(NaSS) grafting process of medical titanium alloys to prevent bacterial colonization.

2. Materials and methods

2.1. Materials

13 mm diameter Ti_6Al_4V discs (CERAVER, Roissy France) were used as substrates. The grafting process was conducted on Ti_6Al_4V surfaces by CERAVER company at Plailly, France. The details of the process have been described [16,17]. Prior to antibacterial adhesion test, all discs were extensively washed: 1.5 M, 0.15 M NaCl water solutions (sodium chloride, Fischer), distilled water, phosphate buffer saline solution (PBS, Gibco). This procedure was repeated 3 times. The samples were then sterilized by using ultraviolet light (UV, 30 W) for 15 min, each side. For bacterial adhesion assays, bovine serum albumin (BSA; Sigma, ref. A4503) and bovine plasma fibrinogen — fraction I, type I-S (Fg; Sigma, ref. F8630) solutions were prepared in Dulbecco's phosphate-buffered saline (DPBS) at concentrations of 400 and 4000 μg/mL for BSA, and at 300 μg/mL for Fg. Human plasma fibronectin (Fn; Chemicon International, ref. FC010) was dissolved in a 1% (w/v) BSA solution at a concentration of 20 μg/mL, in order to stabilize the structure of this high molecular weight protein. The concentrations of the prepared protein solutions were 10% of their serum concentration.

2.2. Surface characterization

2.2.1. Surface topography by Scanning Electron Microscopy (SEM)

Titanium alloy surfaces were analyzed with support of an environmental Scanning Electron Microscope (SEM, Hitachi TM-3000) in secondary electron mode. The microscope was operated at 15 kV under 10^{-5} Torr vacuum. The working distance between the microscope sensor and the samples ranged between 8.7 and 11.2 mm. The observation was performed at ×100 and ×1000 magnifications. The ungrafted and grafted samples that were not inoculated with bacteria were dried and were not subjected to any pre-treatment before SEM observation. After 1 h of incubation with the bacteria, the cells were fixed on ungrafted and grafted samples overnight with 4% formaldehyde solution at 4 °C. Before SEM observation, samples were rinsed twice with DPBS.

2.2.2. Contact angle measurements

The contact angle formed between a 2 μL drop of distilled water and the samples was measured by KRUSS DSA10 measuring system (Germany). For each sample 6 contact angle measurements were performed to determine the mean value.

2.2.3. Toluidine blue colorimetric assay

Grafted Ti_6Al_4V samples were individually immersed in a toluidine blue (TB) aqueous solution (5×10^{-4} M) for 6 h at 30 °C to induce complexation of TB with sulfonate groups. After that period, the samples were rinsed with 1×10^{-3} M sodium hydroxide aqueous solution until the excess dye was completely removed. The stained surfaces were then immersed in a 50% acetic acid solution for 24 h, to induce decomplexation. The decomplexed TB solutions were measured by visible spectroscopy using a Perkin-Elmer spectrometer lambda 25 at 633 nm [12].

2.2.4. Fourier-transformed infrared spectroscopy/Attenuated Total Reflectance (FTIR/ATR)

FTIR spectra, recorded in an attenuated total reflection (ATR) mode, were obtained using a Thermo Nicolet Avatar 370 Spectrometer. The presented data comes from 128 spectra, which were obtained with a 4 cm^{-1} resolution, using a 45° Ge crystal, and a wavenumber from 600 cm^{-1} to 4000 cm^{-1}.

2.3. Bacterial adhesion studies

2.3.1. Bacterial adhesion

S. epidermidis RP62A (ATTC 35984) was cultured in trypticase soy broth (TSB) (Bio-Rad, ref. 64144), at 100 rpm and 37 °C, for 12–16 h (overnight culture) and suspended in DPBS to 3×10^5, 3×10^6 or 3×10^7 bacteria/mL after quantification by Multisizer III-Coulter counter (Beckman).

Prior to bacterial inoculation, the sterilized Ti_6Al_4V discs were immersed in a 400 μg/mL BSA solution for 30 min, in a 24-well tissue plate. Then, the discs were immersed in a protein–PBS solution (Fg or Fn) at 10% its plasmatic concentration, for 1 h at 37 °C and under agitation (100 rpm) to promote their adsorption to the surface. Before the bacteria inoculation, the protein solution was removed and the discs were washed three times with 400 μg/mL BSA solution. Afterwards, 1 mL of the bacterial suspension at the desirable concentration was added to each well, and incubated at 37 °C for 1 h, under agitation.

2.3.2. Quantification by the detachment method

After 1 h of incubation with bacteria suspensions, the excess of bacteria was removed by washing the discs three times with DPBS. Afterwards, a trypsin–EDTA solution at 0.05% (w/v) (Gibco, ref. 25300) was added to detach the bacteria adhered to the surface. When trypsin solution was added, 40 aspirations were made with the micropipette, before it was removed, and a short wash with DPBS was performed. The adhered bacteria (now in the trypsin–DPBS solution) were then quantified using the Coulter counter. Triplicates were used to evaluate each condition.

2.3.3. Quantification by the imaging-based method

Acridin orange (AO), as used by Levon et al. [18], was used to stain adhered *S. epidermidis* on Ti_6Al_4V disc surfaces. Briefly, after the incubation with bacteria and they being rinsed twice with DPBS, the Ti_6Al_4V discs were immersed for 20 min in 1:10,000 (w/v) AO stain (Sigma A-6014) in 0.2 M acetate buffer (pH 3.8), in the dark at room temperature. Afterwards, the samples were rinsed twice with distilled water. The observation of the stained bacteria was carried out using a fluorescence microscope (Carl Zeiss Axiolab E re).

Using the staining method previously described, nine photos of each disc ($n = 2$) were taken using a digital camera (1×HRD 100-NIK, Diagnostic Instruments Inc.) mounted on a fluorescence microscope (Carl Zeiss Axiolab E re) at ×100 magnification. From the digital images obtained, and using Matlab, it was possible to create a color filter to quantify the number of adhered bacteria and determine the average percentage of surface colonized by the bacteria. The threshold values that characterize the orange filter were empirically determined, and defined by the 3 conditions in RGB color space (Fig. 1). Briefly, in the RGB color space each pixel has intensity in red, green and blue components. Combining the intensity and the ratio of the intensity values in each component, it was possible to define the orange color. All the pixels that fulfill the stated conditions were counted and used to calculate the percentage of colonized surface.

2.4. Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad, San Diego, CA, USA). *T* test was used to assess the difference between contact angles. One-way analysis of variance (ANOVA), followed by Tukey's post hoc test was performed on bacterial adhesion studies. Differences were considered statistically significant at $p \leq 0.05$. Results were expressed as mean ± standard deviation (error bars).

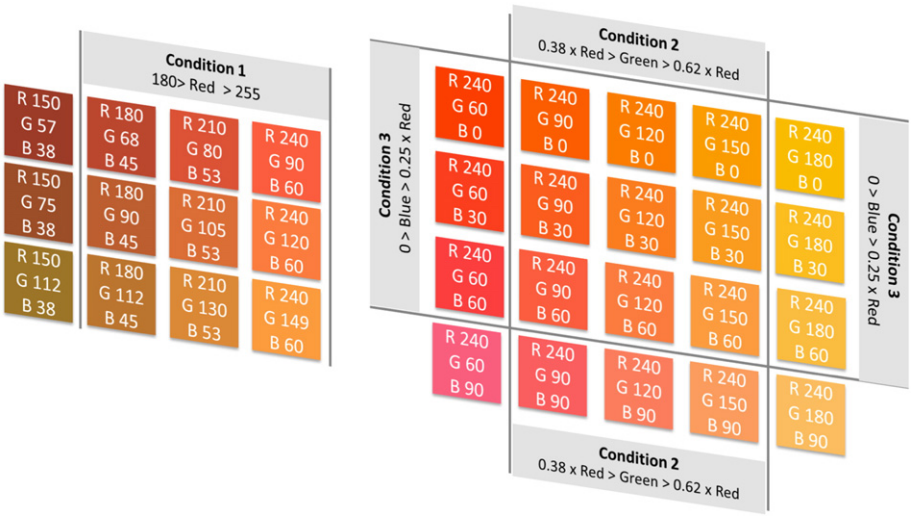

Fig. 1. Color filter — conditions underlying the definition of the orange color.

3. Results and discussion

3.1. Characterization of the Ti_6Al_4V samples

3.1.1. Morphological characterization

The surface topography of the two different surfaces was evaluated by SEM (Fig. 2). Both Ti_6Al_4V surfaces presented ring shaped valleys; the most easily observed have 20 μm of width but valleys down to 1 μm of width were also measured. Comparing the surface of ungrafted Ti_6Al_4V with grafted Ti_6Al_4V discs a similar macroscopic roughness was observed. However, grafting of poly(NaSS) induces smoothening of the surface.

The surface roughness is an important factor in cell adhesion, proliferation and differentiation and also in bacterial colonization [19,20]. The fact that both ungrafted Ti_6Al_4V and grafted Ti_6Al_4V surfaces present valleys with dimensions closer to bacteria size (about 1 μm) might facilitate the bacterial adhesion, providing excellent points for bacterial anchorage [20].

3.1.2. Contact angle analysis

The surface wettability was determined by measuring the contact angles (θ) of a polar liquid (dH_2O) deposited on the surfaces. The obtained θ values (average ± standard deviation), for each assessed disc type (n = 6), are presented in Fig. 3.

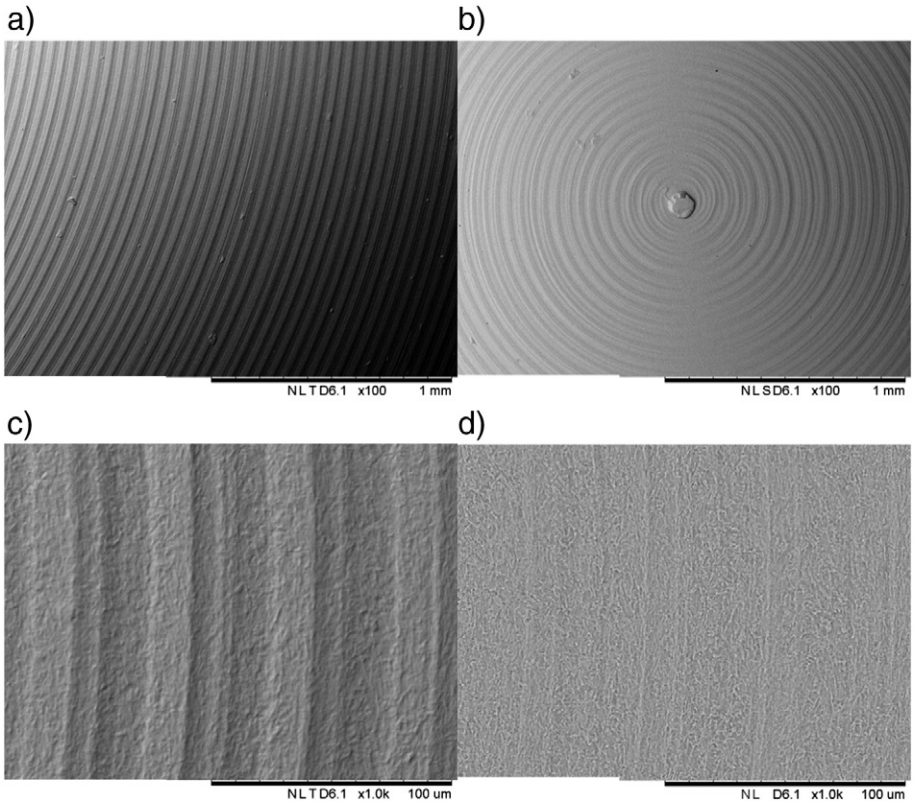

Fig. 2. SEM images of the Ti_6Al_4V discs supplied by CERAVER. The two types of discs were analyzed: (a, c) ungrafted Ti_6Al_4V, and (b, d) grafted Ti_6Al_4V surfaces, at low magnification (×100), and at high magnification (×1000).

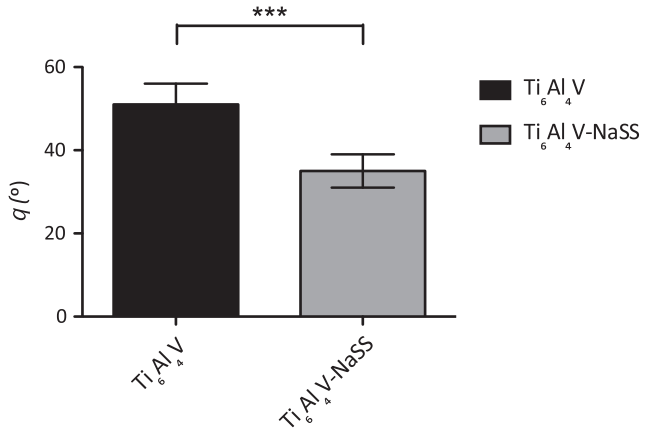

Fig. 3. Contact angle of dH_2O on different Ti_6Al_4V surfaces at t = 0 s (n = 6). Grafting polyNaSS on the surface of Ti_6Al_4V statistically significantly reduced the contact angle of the dH_2O drop (***, $p < 0.0001$).

In previous studies the measured contact angles of water drops on Ti_6Al_4V surfaces were found in the range between 58° and 79° [21–23]. The obtained contact angle for Ti_6Al_4V (51°) seems to be close to these values. The initial surface preparation (e.g. washing with Kroll's solution) has been previously pointed out as responsible for increasing the wettability of the metallic surface [12,24]. Moreover, in high energy surfaces such as the tested ones, the roughness was demonstrated to increase the wettability of the surface [25].

It was previously reported by the present team that electrochemical oxidation induces an increase of the hydrophilic character [13]. The same influence of grafting in the hydrophilic character of the surface was confirmed for the Ti_6Al_4V-NaSS. The average contact angle obtained for Ti_6Al_4V-NaSS is quite similar to the value previously obtained by the group (31 ± 3°) [12]. The hydrophilic character of the surface is well known to play a key role in protein adsorption [10,26] and cell adhesion. In the case of bioactive polymers and surfaces grafting leads to an increase of the wettability of the surface and specific interactions between surface and proteins, which allow a modulation of cell and bacteria responses [27–29].

3.1.3. Determination of the grafting density (toluidine blue)

The toluidine blue (TB) colorimetric method allowed the quantification of the poly(NaSS) present on the grafted surfaces, after a 15 h process. The average result was 1.09 μg cm^{-2} (±0.2 μg cm^{-2}), attesting to the reproducibility of the grafting process. Ungrafted surfaces did not react with TB and were used as control.

3.1.4. Structural characterization by Fourier-transformed infrared (FTIR) spectroscopy

Modifications of the chemical composition of the Ti_6Al_4V surfaces were detected after the poly(NaSS) grafting. FTIR/ATR spectra show intense peaks at 1009 and 1038 cm^{-1}, which reveal the presence of the sulfonate group (SO_3^-), compared to the absence of these peaks on Ti_6Al_4V surface (Fig. 4). The presence of the sulfonate group on grafted samples confirms the success of the grafting process. No peak was detected on control samples Ti_6Al_4V and Ti_6Al_4V oxidized.

3.2. Bacterial adhesion studies

S. epidermidis RP62A (ATTC 35984) was the selected bacterial strain to assess the bacterial response to the tested Ti_6Al_4V discs due to its clinical relevance [2,30].

3.2.1. Influence of the protein environment

Fibrinogen (Fg) and fibronectin (Fn) have been described as proteins that interact with Gram positive bacteria and are involved in the adhesion and colonization processes [5,8,31]. Thus, two protein solutions were used to pre-adsorb adhesive proteins on Ti_6Al_4V discs in order to evaluate the influence exerted by each protein on *S. epidermidis* adhesion. Using trypsin to detach the adhered bacteria, the differences related with the protein environment and the amount of inoculated bacteria were assessed. Additionally, the quantification of the bacterial adhesion to the tested biomaterials was also performed using an imaging-based method (Fig. 5). This technique does not change the original state of the biological system in opposition to the detachment using trypsin followed by cell counting (detachment method). Adhered bacteria are directly stained and observed on the surface of the discs. Shortly, after the adhered bacteria were stained, nine photos of the discs' surface were captured and the number of bacteria was quantified using a color-based filter.

Comparing the adhesion observed on the surfaces coated with Fg (Fig. 6a) with the ones coated with Fn (Fig. 6b), it is clear that Fg actively promotes bacterial adhesion. Imaging-based method and detachment method were applied for similar experimental conditions in order to compare both methods (Fig. 6).

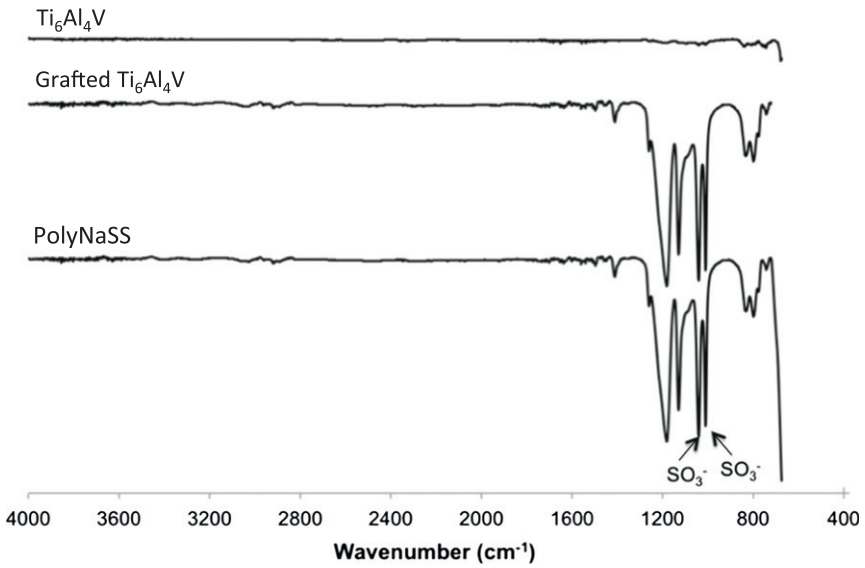

Fig. 4. FTIR/ATR spectra of ungrafted Ti_6Al_4V discs, poly(NaSS) powder and grafted Ti_6Al_4V discs.

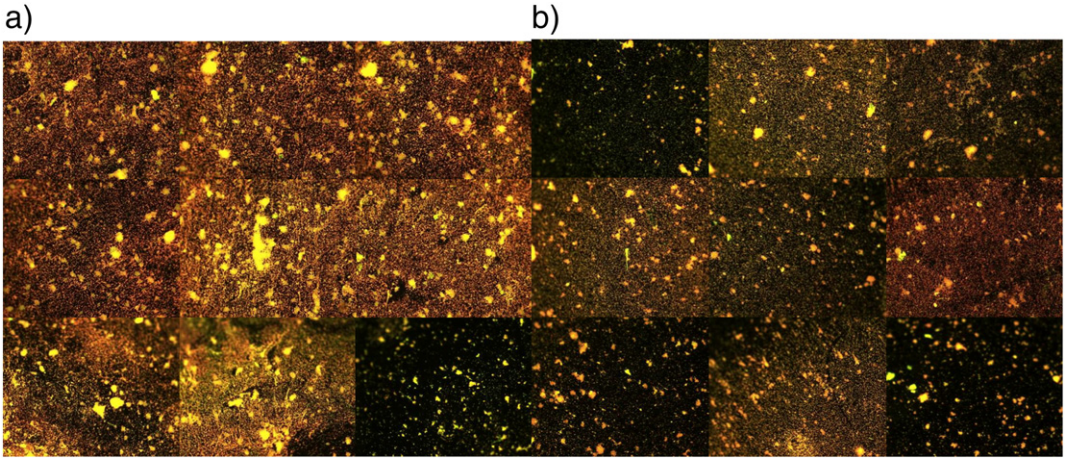

Fig. 5. Images after AO staining of the adhered *S. epidermidis* on Ti_6Al_4V discs where Fg (a) and Fn (b) were pre-adsorbed. An initial bacterial concentration of 3×10^7 bacteria/mL was used. Images were captured at ×100 magnification.

Both used methods pointed that Fn and Fg are clearly associated with an increase in Staphylococcal adhesion. Both proteins are present in blood plasma and their positive influence on bacterial adhesion was previously reported [5,22,32,33]. The higher bacterial adhesion was observed on the surfaces with Fg for the two applied methods. However, the results obtained by the imaging-based method do not permit a clear identification of a statistically significant adhesive effect to one of them. A major problem of this method is related with the high variation of the data. As can be observed in Fig. 5, the *S. epidermidis* colonization was not uniform, with regions with much more bacteria than others.

From the imaging-method results, BSA seems to act as an anti-bacterial adhesion coating. Henry-Stanley et al. [34] have reported that pre-coating or the pre-incubation with Alb was demonstrated to decrease the Staphylococcal capacity to adhere to a surface. On the other hand, *S. epidermidis* present adhesins with high affinity to other proteins such as Fn [32]. The Fn binding adhesion Embp, presented on the *S. epidermidis* surface, binds not only Fn type III domains but also Fg and other adhesive proteins [32].

The influence of the initial amount of inoculum to be used to test bacterial adhesion was assessed. More extensive differences related with bacterial adhesion were only verified for the bacterial inoculum of 3×10^7 bacteria/mL. This concentration seems to be critical to promote a more extensive bacterial response to the biomaterial. There is no standard bacteria concentration to be used in testing bacterial adhesion, with values ranging from 1×10^7 to 5×10^9 bacteria/mL [5,18,33,35,36]. Henry-Stanley et al. [34] used a bacterial inoculum of 1×10^8 bacteria/mL and they referred that the number of bacteria which initiate a catheter infection is likely far lower than that. In the present work, three inoculum concentrations were chosen to evaluate the bacterial adhesion to Ti_6Al_4V and Ti_6Al_4V-NaSS surfaces: 3×10^5, 3×10^6, and 3×10^7 bacteria/mL. The concentration of 3×10^7 bacteria/mL was obtained after 2 h of incubation of a *S. epidermidis* culture with an initial OD_{600} of 0.1 abs. After quantification, bacteria were suspended in DPBS, in order to minimize their growth. At this time point, the early exponential phase of *S. epidermidis* growth was also achieved and it is during this period that adhesin expression occurs at maximum level. In vitro, adhesins are expressed during the exponential growth phase and then down-regulated, until they are no longer expressed in the bacterial cell surface in the stationary phase of growth [37]. However, the situation in vivo is much more complex and not so predictable. Differences in the virulence activity were reported, which reveals the limitations of the in vitro experiments [32].

3.2.2. Influence of poly(NaSS) grafting on S. epidermidis adhesion

As previously demonstrated, grafting poly(NaSS) on the Ti_6Al_4V surface leads to physicochemical modifications that influence its bioactivity [14,15]. In this work, the effect of grafted poly(NaSS) on the bacterial response to the surface was evaluated (Fig. 7).

The hydrophilicity of the surface was found to be inversely related with the registered level of bacterial adhesion. For both tested proteins, the highest adhesion was observed for the Ti_6Al_4V, which is the less

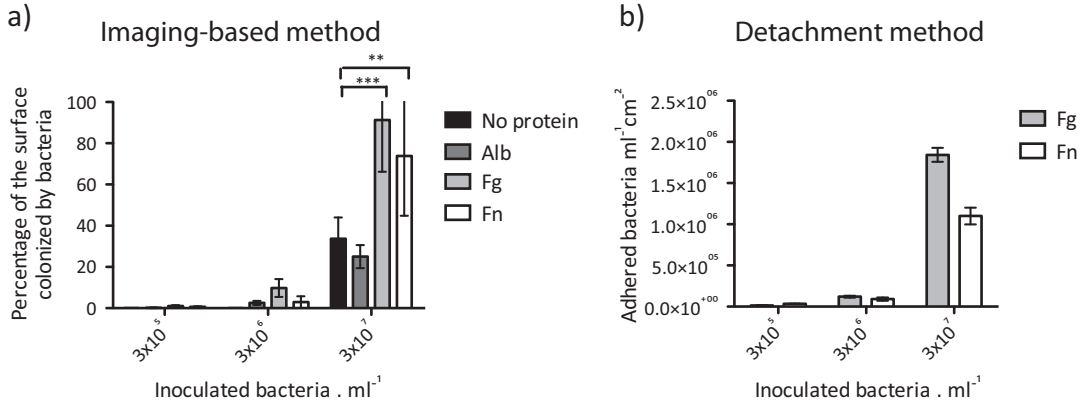

Fig. 6. Influence of the protein environment on the quantification of the *S. epidermidis* adhesion to Ti_6Al_4V discs' surface using the imaging-based method (left; n = 2) and using the detachment method (right; n = 3). The differences in the amount of adhered bacteria registered between the surfaces without adsorbed proteins and the surfaces pre-adsorbed with Fg were statistically significant (***, $p < 0.001$), for the inoculum of 3×10^7 bacteria/mL. Furthermore, for the same initial inoculum, Fn induces statistically significant (**, $p < 0.01$) increase of *S. epidermidis* adhesion.

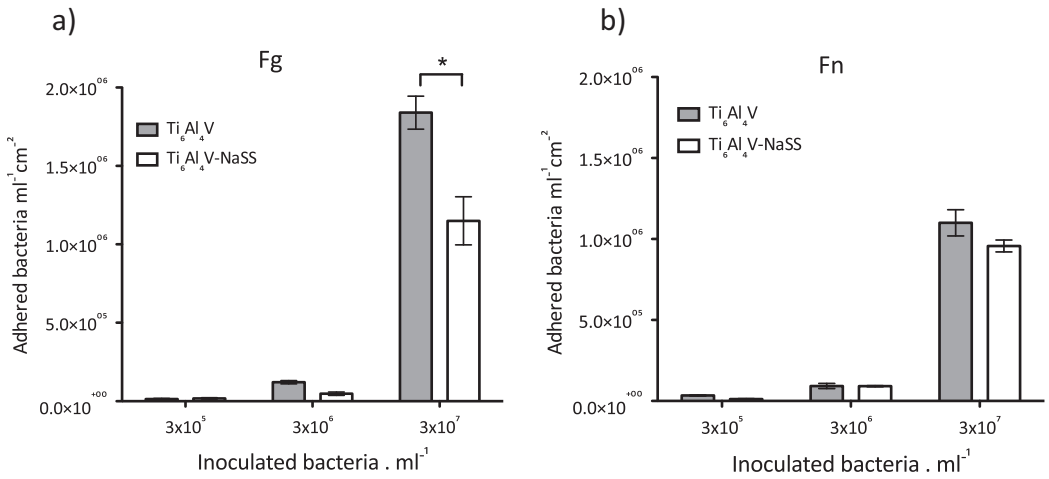

Fig. 7. *S. epidermidis* adhesion on Ti_6Al_4V surfaces pre-coated with Fg (a; n = 3) or with Fg (b; n = 3). The differences in the amount of adhered bacteria between the Ti_6Al_4V-NaSS and the Ti_6Al_4V were only statistically significant (*; $p < 0.05$) for the initial inoculums of 3×10^7 bacteria/mL, after pre-adsorption of Fg on the tested surfaces. A lower *S. epidermidis* adhesion was observed for grafted Ti_6Al_4V discs in the presence of Fg and less in the presence of Fn. *S. epidermidis* adhesion has been described to be mediated by Fg.

hydrophilic tested surface. This demonstrates the preponderance of the bioactive grafted polymer activity on the hydrophilicity property.

Comparing the obtained results between the Ti_6Al_4V and the grafted Ti_6Al_4V discs, an inhibitory effect on the bacterial adhesion can be assigned to the poly(NaSS) grafting, namely in the presence of Fg. The amount of adhered *S. epidermidis* seems to be mediated by the adsorbed proteins. Barrias et al. [10] have shown that surface hydrophilicity influences the adsorbed amount and the functional presentation of cell-binding domains of Fn and the subsequent cellular response to the surface.

Inhibition percentages of bacterial adhesion were calculated using the difference between the slopes of the calculated linear regressions for bacterial adhesion on Ti_6Al_4V-NaSS and the Ti_6Al_4V, respectively. Compared with Ti_6Al_4V, inhibitions on bacterial adhesion of 37% and 13% were verified for the grafted Ti_6Al_4V surfaces pre-adsorbed with Fg and Fn, respectively (Supplementary Fig. 1).

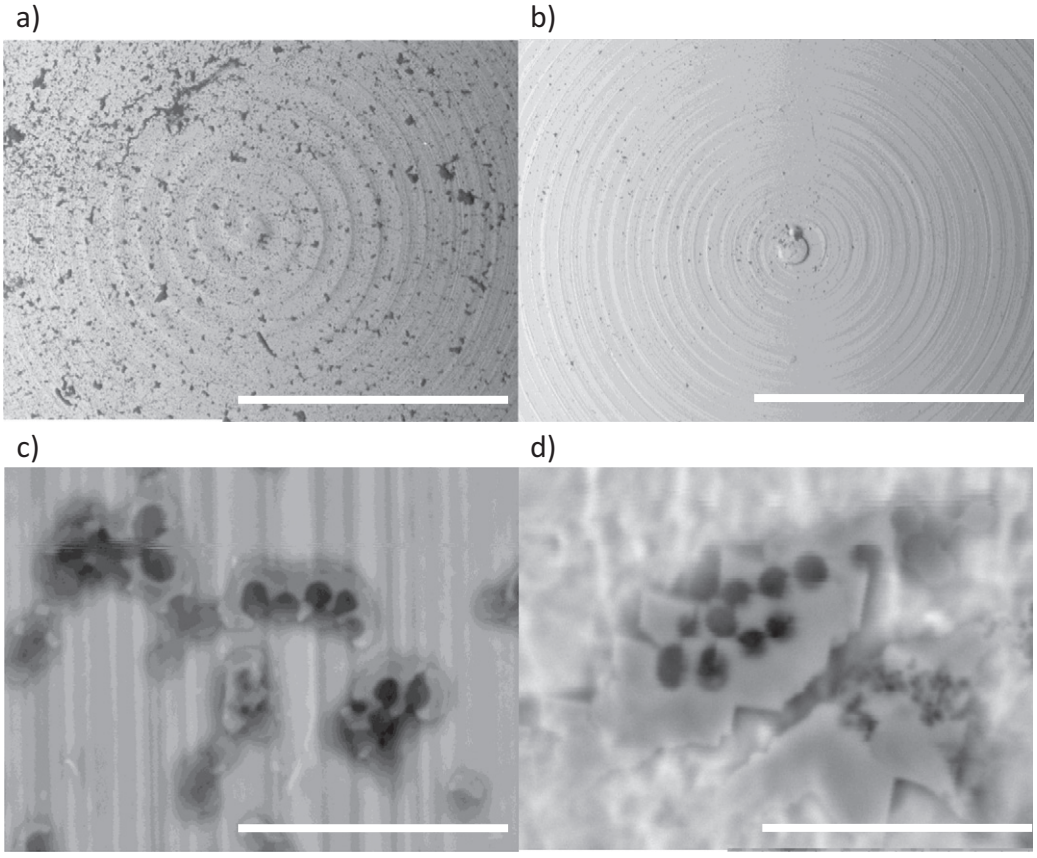

Fig. 8. SEM images of ungrafted Ti_6Al_4V (a, c) and grafted Ti_6Al_4V discs (b, d) after 1 h of incubation with *S. epidermidis* at a concentration of 3×10^7 bacteria/mL. Pre-adsorption with Fn was performed. The differences in terms of bacterial adhesion (black dots on the surface) are very clear (a, b). Scale bar: 1 mm. Higher magnifications (c, d) are presented to illustrate the bacterial anchorage on the discs' surfaces. Scale bar: 10 μm.

Inoculated ungrafted Ti_6Al_4V and grafted Ti_6Al_4V surfaces pre-coated with Fn were observed by SEM (Fig. 8). The presence of adhered bacteria is indicated by the black dots on the analyzed surfaces. Poly(NaSS) grafting was confirmed to inhibit bacterial adhesion since a much higher number of adhered *S. epidermidis* were verified for the ungrafted when compared to the grafted discs. On the other hand the surface roughness, namely the valleys with 1 μm dimension, constituted preferential spots for bacteria to attach and start colonizing the surface. Despite the fact that small clusters of *S. epidermidis* aggregated by extrapolymeric substance were observed on both types of samples, the number and the size of the observed bacterial cluster were higher on the ungrafted discs. Moreover, an increased amount of extrapolymeric substance seems to be produced by the bacteria adhered to grafted Ti_6Al_4V surfaces perhaps attempting to minimize the antibacterial effect of the grafting.

Although the grafting of the surface minimizes the bacterial adhesion, it improves the attachment of osteoblast-like cells (MG63 cells) as well as their differentiation as reported by Migonney et al. [12,14]. Hence, a surface leading to a selective protein adsorption and bacteria adhesion is the purpose of poly(NaSS) grafting, which presents both hydrophilic character and bioactivity. A kinetic study was carried out to show that polyNaSS was not released. The grafted surfaces of titanium alloy were immersed for several months in PBS at 37 °C. It was shown that the grafting density did not decrease after several months which confirmed the stability of the polyNaSS grafting [38,39]. The biocompatibility of polyNaSS grafting for bone repair was demonstrated in vitro and in vivo [12,14,40]. Animal experiments were conducted for time points until 4 weeks and no infection was detected on implants [14]. Thus, Ti_6Al_4V-NaSS surfaces are expected to present bioselectivity, preventing infection, in clinical applications such as in orthopedics or dental medicine.

4. Conclusions

During this work, the influence of grafting with poly(NaSS) on the bacterial response to Ti_6Al_4V was evaluated. The role of the protein environment in mediating the interaction between the surface and the bacteria was also investigated. Poly(NaSS) grafting increased the hydrophilic character and the bioactivity of the surface, reducing the bacterial adhesion for the surfaces pre-coated with fibrinogen and fibronectin. This inhibitory effect is assumed to be related with changes in the amount and in the conformation of the adsorbed proteins on the bioactive surface [38]. A great advantage of the poly(NaSS) coating relies in its bioactivity, which could be in part due to its moderate hydrophilic character. Although highly hydrophilic surfaces inhibit bacterial adhesion, the capacity of the biomaterial to allow host cell adhesion and further tissue regeneration may be seriously affected. An imaging-based method to evaluate the bacterial adhesion to the surfaces was developed and compared with the detachment method. This new method was demonstrated to be easier, quicker and producing similar results. The preservation of bacteria organization on the surface is a major asset of the imaging-based method.

The surface roughness seemed to influence the amount of the adhered bacteria and biofilm formed on the surface, although the mechanisms underlying this effect were not clearly elucidated. In all the performed experiments, fibrinogen and fibronectin presented a tendency to promote the adhesion of *S. epidermidis*, in contrast with the inhibitory effect of albumin. Overall, a reduction of bacterial adhesion can be attributed to poly(NaSS) grafting.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.msec.2014.08.054.

Acknowledgments

The authors thank very sincerely: the ANR for the funding of the ACTISURF project ANR-09-TECS-001 "Biologie et Santé", the University Paris 13 for the grant allocated in the Master Student Programme and Dr I. Ben Aissa, Dr A. Saleh-Mghir, Dr G. Helary and B De Lambert for their help.

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