

Influence of the Nano-micro Structure of the Surface on Bacterial Adhesion

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Biomaterials failures are frequently associated to the formation of bacterial biofilms on the surface. The aim of this work is to study the adhesion of non motile bacteria streptococci consortium and motile *Pseudomonas fluorescens*. Substrates with micro and nanopatterned topography were used. The influence of surface characteristics on bacterial adhesion was investigated using optical and epifluorescence microscopy, scanning electron microscopy (SEM) and atomic force microscopy (AFM). Results showed an important influence of the substratum nature. On microrough surfaces, initial bacterial adhesion was less significant than on smooth surfaces. In contrast, nanopatterned samples showed more bacterial attachment than the smooth control. It was also noted a remarkable difference in morphology, orientation and distribution of bacteria between the smooth and the nanostructured substrate. The results show the important effect of substratum nature and topography on bacterial adhesion which depended on the relation between roughness characteristics dimensions and bacterial size.

Keywords: biofouling, bacterial adhesion, extracellular polymeric material, micro/nanotopography

1. Introduction

Reactions at interfaces are very important in biology¹. Interfaces of medical and industrial interest include bacteria/metal surface. Biofilm-associated cells can be differentiated from their counterparts in solution by the production of an extracellular polymeric material (EPM). Basically, the most remarkable and dangerous attributes of biofilms are their ubiquity and their notorious resistance to being killed by antimicrobial agents². Attachment is a complex process regulated by diverse characteristics of the growth medium, substratum and cell surface. The initial bacterial stages of biofilm formation seem to be influenced by the motility of bacteria. The microcolonies develop into a mature biofilm with an architecture that is typically characterized by macrocolonies separated by fluid-filled channels³. It is believed that these channels transport nutrients and oxygen to the bacteria and aid in waste removal^{4,5}. Surface properties significantly govern the first steps of bacterial adhesion processes. Roughness and surface composition can be modified through appropriate micro/nanofabrication techniques to study the influence of these properties on bacterial adhesion.

Nano/microfabrication techniques enable the researcher to design with nano/micrometer-level control, the biochemical composition and topography of the substrate^{1,6}. The aim of this paper was to study the influence of the surface characteristics on the bacterial attachment during the early stages of bacterial biofilms development. Substrates with different roughness, and nanostructured metals were assayed.

2. Materials and Methods

2.1. Bacteria strains and culture conditions

To determine bacterial response to the substrates of interest, a consortium of streptococci collected from the oral cavity of several

patients with a normal periodontal status were used in the experiments. A pure culture of *Pseudomonas fluorescens* (*P. fluorescens*) isolated from an industrial environment was also used to investigate the effect of motility on bacterial attachment. *P. fluorescens* was maintained in Cetrimide agar at 28 °C. *P. fluorescens* inoculum was prepared by suspending a Cetrimide agar slant (24 hours old) in 2 mL of sterile nutrient medium. Afterwards, the inoculum was poured into an Erlenmeyer flask containing 300 mL of the nutrient broth medium and kept on a rotary shaker for 3 hours at 28 °C.

Oral microorganisms collected from the oral cavity were obtained by scraping the gingival area of buccal and lingual tooth surfaces and along the entire fissure of margin of restorations on occlusal surfaces of the patients. Each sample was dispersed by sonication for 10 s in sterile culture medium. Every 2 months they were completely replaced by new samples obtained from the same patients. The consortium was cultured in Mitis-Salivarius agar medium to isolate the streptococci consortium. Isolated microorganisms were maintained in modified Mitis-Salivarius liquid medium as described elsewhere⁷.

After 24 hours growth, the different substrates were placed into the culture so that a bacterial biofouling could be formed on them. The samples were removed after periods varying from 30 minutes to 2 hours.

2.2. Substrata

The substrates used in the experiment were: sheets of Ti polished with emery papers of different grades (320 to 800), Si (plane 100 and rough), and Cu and Au (smooth and nanostructured).

Rough Si showed features of 8 µm wide and 2 µm deep, measured with AFM (Figure 1). The smooth Si surface was used as control. Ti sheets were polished to different grades with emery paper and then

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etched with 60 g/L HCl + 30 g/L NaF + 20 g/L NaCl solution in order to generate surfaces with different roughness. Nanostructured materials: Cu and Au substrates present a nanostructure consisting in channels of 90 nm height and 900 width (Figure 2). These substrates were prepared according to Schilardi et al.⁸. Cu and Au evaporated films on glass, that present a microlevel smooth structure, were used as controls.

2.3. Microscopic observations

Biofilms were observed through optical epifluorescence microscopy. Bacteria were stained with fluorescein diacetate and ethidium bromide. SEM observations of the biofilm were also made. To preserve biological structures, biofilmed metal specimens were fixed in 2% glutaraldehyde in sterile saliva or in a phosphate buffered medium, dehydrated through an acetone series to 100% and critical point dried. The effect of substratum topography was also analysed by using a Nanoscope IIIa AFM (Digital Instruments).

3. Results and Discussion

The effect of surface composition was analyzed by comparing initial stages of biofilm formation of smooth Cu and Au. Results show (Figure 3a and 3b) that after the same exposure period copper samples show lower bacterial attachment than gold samples. As expected⁹, there was an induction or major conditioning period in copper substrates and bacterial adhesion was lower than on the rest of the materials assayed for the same immersion periods.

Copper and their alloys present a lower formation of biological deposits (biofouling) due to, probably, toxic characteristics of the Cu ions (II) coming from the metal dissolution^{10,11}. Thus, when the substrate is toxic for the microorganisms, a greater production of EPM than on no toxic samples, such as gold, was observed (Figure 4).

There were several differences in the initial stages of microbial attachment on the smooth and nanostructured surfaces. It was found a slight orientation of *P. fluorescens* into the trench of the nanopatterned surface of copper substratum whereas there was an important alignment of bacteria on gold nano-patterned surface. Motile strains, place themselves easily at trenches and crevices as shown in Figure 5. The formation of biofilms by *Pseudomonas* has been proposed to occur as a series of regulated steps¹². First, flagellar mediated motil-

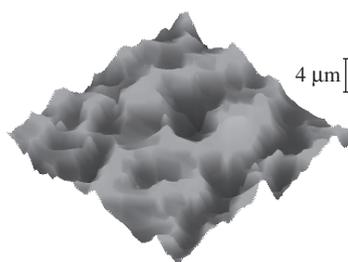


Figure 1. AFM image (contact mode, 50 x 50 μm²) of rough surface of Si showing the box-like holes.

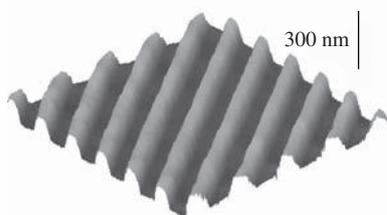
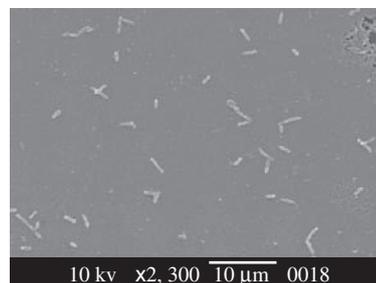
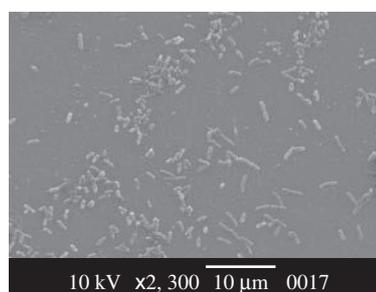


Figure 2. AFM image (contact mode, 10 x 10 μm²) of Cu nanostructured surface. The Au samples present the same superficial features.

ity may be required for a bacterium to swim toward a surface and to initiate reversible attachment¹³. A subpopulation of transiently attached bacteria become irreversibly attached to the surface to first form a monolayer, which is followed by migration and the formation of small microcolonies¹⁴⁻¹⁸. The distribution of bacteria on smooth surfaces was uniform.



(a)



(b)

Figure 3. a) SEM microphotograph of crystalline Cu surface exposed during 30 minutes to a *P. fluorescens* culture; and b) SEM microphotograph of crystalline Au surface exposed during 30 minutes to a *P. fluorescens* culture.

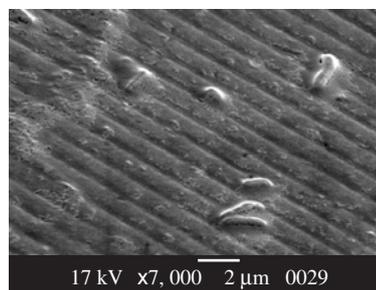


Figure 4. SEM microphotograph of Cu nanostructured surface exposed during 30 minutes to *P. fluorescens* culture.

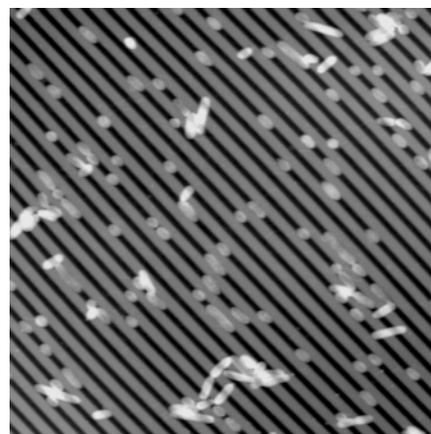


Figure 5. 40 μm x 40 μm contact mode AFM top view image of Au nanostructured surface exposed during 30 minutes to a culture of *P. fluorescens*.

Most of bacteria placed on the trench were alone and there was no microscopic evidence of production of EPM on the gold nanostructured substrate.

Change in *P. fluorescens* morphology was also noted between the nanostructured surface and the smooth control, as shown in Tables 1 and 2. Importantly, the length of the attached bacteria was shorter than those adhered to the smooth surface. We have measured the bacterial length using AFM images. On gold nanostructured surfaces the average length was $1.446 \mu\text{m} \pm 0.120 \mu\text{m}$, while on gold smooth substrate it was $1.996 \mu\text{m} \pm 0.123 \mu\text{m}$. These results were significantly different, showing cell reduction of the size on nanopatterned sample. All data were analysed by standard *t*-tests with statistical differences between means determined at $p < 0.05$. In addition, gold nanostructured surface presented more significant bacterial attachment than the control sample.

Assays on micro-rough Ti surfaces showed that *P. fluorescens* attachment was not uniform and follow preferential directions. Figure 6 shows that the Streptococci preferentially attach on the valleys.

The shape of the colonies is markedly affected by the roughness of the surface in the case of streptococci. Observations through epifluorescence microscopy showed that bacteria attached on the valleys of the rough surface and then grew following a row. After longer exposures they presented long and narrow colonies (Figure 7) different from the round shape colonies formed on smooth titanium.

In contrast with results described above related to bacterial attachment on smooth and nanostructured gold, the surface density of bacteria on smooth Si was higher than those of microrough samples (Figure 8a and 8b). Consequently, microroughness and nanostructures seem to play different roles.

Microrough Si presents a surface topography characterized by small boxes of $2 \mu\text{m}$ height and $8 \mu\text{m}$ long as shown in Figure 1. During the exposure time the boxes are progressively covered by

Table 1. Average size of *P. fluorescens* attached to Au substrates.

| Au | Smooth (μm) | Nanostructured (μm) |
|----------|--------------------------|----------------------------------|
| Length | 1.996 | 1.446 |
| Variance | 0.123 | 0.120 |
| Diameter | 1.091 | 0.909 |
| Variance | 0.032 | 0.013 |

Table 2. Average size of *P. fluorescens* attached to Cu substrates.

| CU | Smooth (μm) | Nanostructured (μm) |
|----------|--------------------------|----------------------------------|
| Length | 1.997 | 1.657 |
| Variance | 0.285 | 0.153 |
| Diameter | 0.832 | 0.898 |
| Variance | 0.012 | 0.018 |

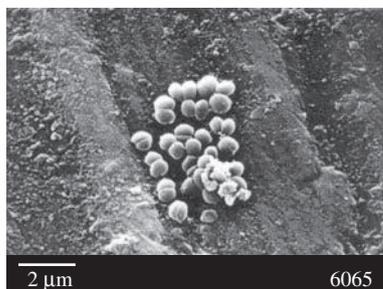


Figure 6. Adhesion of streptococci consortia to rough Ti.

a great amount of EPM in the case of *Pseudomonas* cultures. This increment in the production of polymeric material can be observed comparing Figure 9a, 9b and 9c. The extracellular polymeric material tends to smooth the microroughness of the Si substrate, as has been reported previously^{7,19}.

A comparative analysis of the results shows that dissimilar biological response was found on the different substrata used.

In the case of microrough surface of Ti, *Streptococci* preferentially attach on the valleys.

There was a significant effect of the presence of nanotopography. An important alignment of *P. fluorescens* was observed on nanopatterned Au substratum, where a great amount of isolated bacteria adhered into the trench of the pattern. It was observed a minor amount of microcolonies, and all of them were arranged in direction not parallel to the trench.

It was also observed that on nanostructured Au there was less production of EPM but more bacterial adhesion than on the control sample. In contrast, microrough Si substrate shows much more production of EPM and less *Pseudomonas* attachment than on smooth surface. When the parameters that characterize topography and roughness are in the order of bacteria dimension, it was found that bacteria easily adhered and less amount of EPM was produced.

In the case of Cu samples, the polymeric material was found surrounding and underneath the attached bacteria, maybe, in order

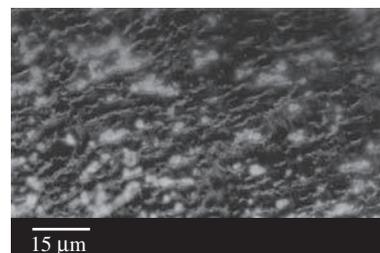
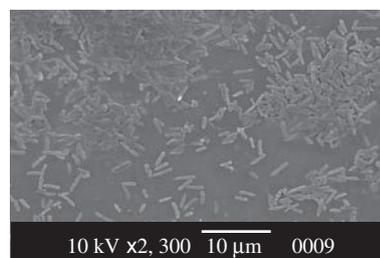
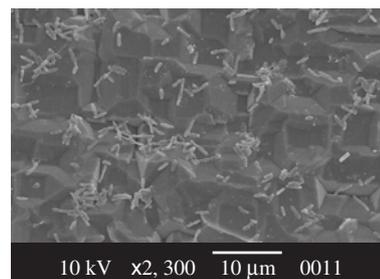


Figure 7. Epifluorescence microscopy corresponding to a rough titanium surface where rows of streptococci with preferential direction can be seen within the valleys.



(a)



(b)

Figure 8. a) SEM microphotograph of Si smooth surface exposed during 2 hours to *P. fluorescens* culture; and b) SEM microphotograph of Si rough surface exposed during 2 hours to *P. fluorescens* culture.

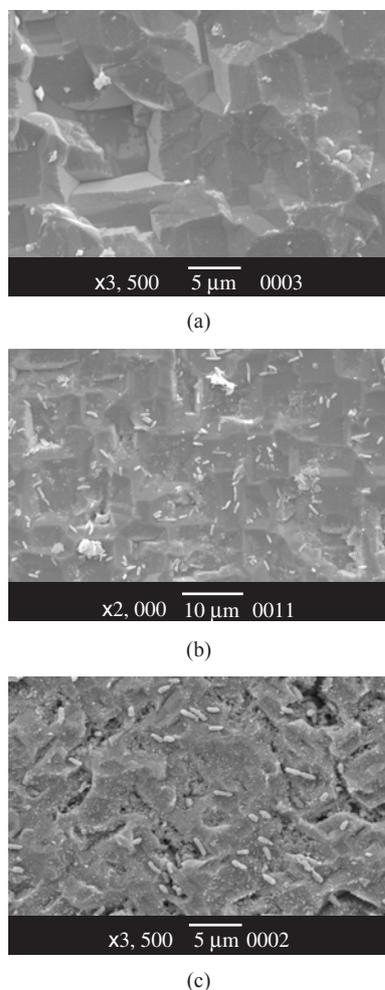


Figure 9. SEM microphotographs of Si after different exposure periods: a) $t = 0$; b) $t = 30$ minutes; and c) $t = 1$ hour.

to avoid the direct contact with toxic material as shown in Figure 4. The effect of the nature of the surface was strong during the former minutes but was less important at longer periods after the production of abundant EPM. Surfaces properties were severely modified when they were covered by the EPM.

4. Conclusions

Surface composition of the substrata, roughness and topography play important roles in the initial stages of biofilm formation.

The initial distribution of bacteria is uneven on smooth surfaces but follows some preferential directions on rough surfaces.

There is a relationship between the roughness characteristic dimension and the bacteria size which affects not only the bacterial attachment but also the production of EPM. When the parameters that characterize topography and roughness are in the order of bacteria dimension, it was found that bacteria easily adhered and less amount of EPM was produced.

It is clear that bacteria act in response to the nanotopography since they chose a preferential direction, changed their morphology and modified the production of EPM under these conditions.

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