Oral Bacterial Adhesion on Amorphous Carbon and Titanium Films: Effect of Surface Roughness and Culture Media

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Abstract: Implant infections can cause severe problems from malfunctioning to dangerous sepsis affecting the health of the patient. For many years, titanium has been the most common material used on dental implants due to their mechanical and biocompatibility properties. Recent studies suggest that amorphous carbon (a-C) films can be possible candidates for coating dental implants, improving some important features like biocompatibility and bone formation. In the oral cavity, the risk of an implant infection is high due to multiple species are capable to colonize this site and these biofilm infections can limit the use of these medical devices. The purpose of this study was to evaluate the influence of the surface chemistry, roughness, and culture media in the bacterial colonization process. To achieve this, a-C and Ti films were deposited on rough and smooth surfaces and cultured with different microorganisms belonging to the oral microbiota with mycoplasma medium (MM) or human saliva (HS). Samples were incubated for 24 h, after this, samples were sonicated and the number of attached bacteria was determined by counting the colony-forming units (CFU's) from each sample. The proportion of the species in the biofilms was determined using checkerboard DNA–DNA hybridization. Data were analyzed by Student's *t* test using Bonferroni's modification of Student's *t* test and differences on the proportion of the bacterial species attached to each surface were determined using the Mann-Whitney test. Results show an increased number of CFU's on rough surfaces, especially on the a-C surfaces. The incubation media were an important factor on the adhesion of certain taxa, whereas other species were more sensitive to surface chemistry and others to surface roughness. © 2009 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 92B: 196–204, 2010

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INTRODUCTION

The infection caused by biomaterial implants is one of their major drawbacks because it can cause severe problems from malfunctioning of the implanted device to dangerous sepsis affecting the health of the patient. Once the infection has initiated, treatment is complicated because the microorganisms adhered to the biomaterial formed a biofilm. Biofilms are structured communities of bacteria enclosed in a self-produced matrix and adherent to an inert or living surface,[1] this well-organized structure makes them more resistant to antibiotics than their planktonic counterparts.[2] In most of the cases, the solution to this problem is the removal of the infected implant, but obviously a more convenient solution should be the prevention of the biofilm formation, which could be achieved by a surface modification treatment. This issue has led to the study of biomaterials-centered infections that is of great interest for the development of new and improved biomedical devices.[3] Bacterial adhesion (first step in biofilm formation) is affected by the physicochemical properties of the microbial as well as the biomaterials' surface such as chemical composition, surface energy, hydrophilicity, and topography.[4,5] In the oral cavity, the biofilm formation process is extremely complicated because multiple species are present in the dental plaque.[6,7] In fact, there are only few studies regarding the process of bacterial adhesion and biofilm

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formation in direct contact with a dental implant surface using oral bacteria.[8–10]

Titanium and its alloys are considered important materials in the biomedical field due to their mechanical and biocompatibility properties as well as corrosion resistance and so forth that make them ideal implant materials.[11] *In vitro* studies of the biofilm formation on titanium surfaces have focused on the effects of surface roughness and surface chemistry[8,12,13] using only one or two bacterial strains.

Conversely, we have been investigating different aspects of the interaction between human cells (osteoblasts) and graphite-like carbon (GLC) films as possible candidates for coating dental implants[14–17] obtaining encouraging results. Amorphous carbon films are nanostructured materials deposited as thin films, which consist of sp^2 hybridized carbon atoms, clustered within a typical size of a few nanometers, and connected among them by sp^3 hybridized carbon atoms. Depending on the fraction of sp^2 to sp^3 hybridized C atoms, the films have been named as diamond-like carbon (DLC), GLC, or when highly hydrogenated as polymer-like carbon (PLC). The biological improvement of medical devices coated with amorphous carbon (a-C) and DLC thin films have increased their use in the biomedical field such as cardiovascular,[18] ophthalmic,[19] and orthopedic devices.[20] Biological features of these thin films include low coefficient of friction, high resistance to corrosion,[21] and antibacterial properties which might prevent implant infections.[22–24]

The purpose of this study was to evaluate how the bacterial adhesion of different oral microorganisms is affected by surface chemistry, microtopography, and culture media. The surface chemistry was modified by deposition of amorphous carbon and titanium films on both rough stainless steel and silicon substrates. The advantages of using thin films instead of roughing bulk materials are that the surface properties are not affected by the roughing method, as shown in a previous work, where we demonstrated that the surface composition of the rough stainless steel was modified by the grit blasting leading to a loss of the surface oxide.[25] The roughness of the stainless steel was significantly larger than the silicon and therefore two different roughnesses were compared. The study was performed comparing two different media: mycoplasma media (MM), which is a standard bacterial culture media, and sterilized human saliva (HS). We tested saliva because it is the major bulk fluid in the oral cavity and any surface placed in the mouth will be in contact with it and will be covered by the various saliva components (proteins, enzymes etc.). For testing materials for applications, it might be the ideal media. However, HS is a very complex and nonhomogeneous media in comparison to the MM and actually changes in the composition can be found from donor to donor. To study the surface-bacteria interactions, a more homogeneous media could be convenient. Nevertheless, it is important to determine at what extension the media affect the bacterial adhesion, subject that has not been fully studied.

MATERIALS AND METHODS

Films

The stainless steel (AISI 316L) substrates (SS) were previously sandblasted using SiO_2 particles to obtain a uniform surface roughness. Rough (r) samples of amorphous carbon (a-C r) and titanium films (Ti r) were deposited on 15 mm diameter SS. In addition, similar films were deposited on smooth (s) silicon substrates (100) of 10 × 10 mm (a-C s and Ti s). Before deposition, both substrates were ultrasonically cleaned using acetone, isopropanol, and distilled water for 30 min, respectively and then air dried.

The amorphous carbon films were produced by a hollow cathode DC magnetron sputtering system attached to a high vacuum chamber (base pressure 1.3×10^{-4} Pa), using a 4-inch diameter high-purity graphite cathode. The a-C deposits were done using 20 sccm of argon (purity 99.999%), 4 Pa of deposition pressure and 0.4 A of DC current for 30 min, leading to a film thickness around 60 nm. Ti films were deposited in a pulse DC magnetron sputtering system, using a high-purity Ti target and argon plasma at 0.2 A and 0.4 Pa for 5 min, the film thickness obtained was approximately 60 nm. X-ray diffraction and X-ray photoelectron studies shown that the film were mainly metallic titanium (data not shown).

The surfaces were characterized by scanning electron microscopy (SEM, Cambridge–Leica, Stereoscan 440 at 20 kV) and atomic force microscopy (JSPM-4210), water contact angle was measured using double-distilled water with the sessile drop technique (KSV, model Cam 101). Film thickness and surface roughness (5-mm scan length) were measured using a profilometer model Dektak II A using the contact mode.

Bacterial Strains

Nine reference strains (Table I) were tested on each surface. Lyophilized bacterial stocks (American Type Culture Collection, Rockville, MD) were rehydrated in mycoplasma broth base (BBL, Becton-Dickinson, Sparks, MD). All strains were grown on mycoplasma agar base (BBL, Becton-Dickinson) supplemented with 5% defibrinated sheep blood, 5 μg/ml hemin (Sigma-Aldrich, St. Louis, MO) and

TABLE I. Reference Strains Used for the Adhesion Assays

Species	Strain[a]
Aggregatibacter actinomycetemcomitans serotype b	43718
Actinomyces israelii	12102
Campylobacter rectus	33238
Eikenella corrodens	23834
Fusobacterium nucleatum subsp *nucleatum*	25586
Parvimonas micra	33270
Porphyromonas gingivalis	33277
Prevotella intermedia	25611
Streptococcus sanguinis	10556

[a] American Type Culture Collection, Rockville, MD.

0.3 μg/ml menadione (Sigma-Aldrich) under anaerobic conditions (80% N_2, 10% CO_2, and 10% H_2).

Bacterial Adhesion Test

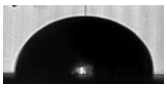
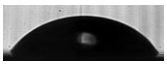
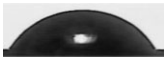
All the experiments were performed using three samples of each one: rough samples of amorphous carbon (a-C r) and titanium films (Ti r) and smooth samples of a-C s and Ti s films. Bacterial growth from 5- to 7-day cultures of each strain was harvested and the optical density (OD) in each tube was adjusted to 1 at 600 nm in a spectrophotometer. Sterile surfaces were placed individually in 12 well plates and a total of 10^6 cells/ml suspension of each reference strain was added, to obtain a mixed culture, in a total volume of 1 ml. Plates were incubated for 24 h at 35°C under anaerobic conditions using enriched mycoplasma broth media, (5 μg/ml hemin and 0.3 μg/ml menadione) MM, or HS. HS was processed as previously reported,[26] except that after centrifugation, supernatants were sterilized by filtration. After anaerobic incubation, each sample was washed twice with mycoplasma broth. After washing, 1 ml of enriched mycoplasma broth was added and samples were sonicated for 5 periods of 10 s each to deattach the adherent bacteria to each surface. Five-fold dilutions of the obtained suspension were plated on enriched agar plates. After 7 days of anaerobic incubation, a number of colony-forming units (CFU's) on the plates were visually counted to calculate the initial number of bacteria attached in each sample.

Summarizing, the method consists basically in letting the bacteria in contact to the biomaterial surface for a given incubation period of time. Then, detach them from the surface by an ultrasonic treatment and determine the number of detached bacteria. This number is obtained from the number of CFU's that grow in agar plates previously plated with dilutions of the bacteria obtained from the sonicated samples.

Biofilm Composition

To determine the proportion of each bacterial strain in the surfaces, 100 μl of the bacterial suspension obtained after sonication of each sample were placed in individual Eppendorf tubes with 100 μl of 0.5 *M* NaOH. Bacterial species were identified and quantified using the checkerboard DNA–DNA hybridization technique previously described.[27] In brief, DNA probes were prepared using the growth from 3- to 7-day cultures of the nine reference strains used for the bacterial adhesion assays (Table I). Bacterial growth was harvested and placed in tubes containing 1 ml of TE buffer. Cells were washed twice and lysed at 37°C for 1 h with either 10% sodium dodecyl sulfate (Sigma-Aldrich) (SDS) plus proteinase K (Sigma-Aldrich) (20 mg/ml) for Gram-negative strains or lysozyme (Sigma-Aldrich) (15 mg/ml) plus achromopeptidase (Sigma-Aldrich) (5 mg/ml) for Gram-positive strains. DNA was isolated and purified. Whole-genomic DNA probes were prepared for each species by labeling 1 mg DNA with digoxigenin (Roche Diagnostics, Mannheim, Germany) using a random primer technique. The specificity and sensitivity of the nine DNA probes were assessed by hybridizing each DNA probe against individual pure cultures of all of the species adjusted to 10^4, 10^5, 10^6, and 10^7 cells. The sensitivity of the assay was set to allow the detection of approximately 10^4 cells of a given species by adjusting the concentration of each individual DNA probe. For the DNA–DNA hybridization, each sample was thawed at room temperature, boiled for 10 min, and neutralized with 800 μl 5 *M* ammonium acetate (Sigma-Aldrich). The released DNA from each sample was then placed into individual lanes (Minislot-30, Immunetics, Cambridge, MA), concentrated onto a 15 × 15-cm positively charged nylon membrane (Roche Diagnosis), and fixed to the membrane by cross-linking under ultraviolet light. Two lanes on each membrane contained standards consisting of a mixture at 10^5 and 10^6 cells of each bacterial species tested. The membranes were prehybridized at 42°C for 2 h in 50% formamide (Sigma-Aldrich), 5X standard saline citrate (SSC) (1X SSC = 150 m*M* NaCl (Sigma-Aldrich) and 15 m*M* Na citrate (Sigma-Aldrich), 1% casein (Sigma-Aldrich), 5X Denhardt's solution, 25 m*M* sodium phosphate (Sigma-Aldrich) (pH 6.5), and 0.5 mg/ml yeast RNA (Roche Diagnostic). Each membrane was placed in a second device (Miniblotter-45, Immunetics) with the sample lanes rotated 90° to the channels of the apparatus. The probes were diluted to ~20 ng/ml in hybridization solution (45% formamide, 5X SSC, 1X Denhardt's solution, 20 m*M* Na phosphate (pH6.5), 0.2 mg/ml yeast RNA, 10% dextran sulfate (Sigma-Aldrich), and 1% casein), placed in individual channels of the device, and hybridized overnight at 42°C. Probes were hybridized in two sets of nine consecutive channels, leaving empty channels (hybridization solution only) to allow noise and background correction of signals. The membranes were washed twice at high stringency for 20 min each time at 68°C in phosphate buffer (0.1X SSC and 0.1% SDS). Membranes were blocked by 1-h incubation in blocking buffer containing 1% casein in maleate buffer [100 m*M* maleic acid (Sigma-Aldrich), and 150 mM NaCl, pH 7.5]. Hybrids were detected by exposing the membranes to a 1:50,000 dilution of antidigoxigenin antibody conjugated to alkaline phosphatase (Roche Diagnostics) for 30 min. Signals were detected by chemiluminescence using a chemiluminescent agent (CDP-Star, Roche Diagnostics) for 30 min on the membranes at room temperature and exposed to films in autoradiographic cassettes for 30 min. Films were developed and then photographed using a digital photodocumentation system (DigiDoc, BioRad Laboratories, Hercules, CA). Signals were detected with specialized software (Quantity One, BioRad Laboratories), adjusted by subtracting the average plus two standard deviations of the noise and background detected in the empty lanes, and converted to absolute counts by comparison with the standards on the membrane. Failure to detect a signal was recorded as zero.

TABLE II. Surface Characteristics of the Films

Surface	Ra Profilometer (Scan 1 mm) (μm)	R_{RMS} AFM (Scan 5 × 5 μm) (nm)	Effective surface AREA AFM (μm^2)	Film Thickness (nm)	Contact Angle (°)
a-C film on stainless steel medical grade 316L sandblasted substrate	1.83 ± 0.3	240 ± 60	35.9	66.2 ± 3.7	82 ± 3 
a-C film on silicon substrate	0.023 ± 0.0081	1.01 ± 0.33	25.03		50 ± 2 
Ti film on stainless steel medical grade 316L sandblasted substrate	1.89 ± 0.5	140 ± 73	38.4	61.9 ± 2.5	79 ± 5 
Ti film on silicon substrate	0.028 ± 0.0033	2.72 ± 0.37	25.2		52 ± 1 

Biofilm Morphology

To observe biofilm morphology on the different test surfaces, an extra sample for each surface was prepared for SEM following standard procedures. Specimens were fixed in 2.0% glutaraldehyde 24 h at room temperature. Then washed three times with phosphate buffer solution (pH 7.4) and dehydrated through a series of graded ethanol solutions of 20, 40, 60, 80, and 100%. Samples were subsequently vacuum dried, sputter coated with Au, and observed using a scanning electron microscope Cambridge – Leica, Stereoscan 440 at 20 kV.

Data Analysis

The number of CFUs was normalized to the sample size. Data are presented as mean ± standard error of the mean (SEM) of bacterial counts $\times 10^5$. Significant differences in the number of CFUs on each surface were determined using Student's *t* test and Bonferroni's modification of Student's *t* test. The proportion of each strain in the biofilms was analyzed using the Mann-Whitney test and significant differences were determined after adjusting for multiple comparisons.[28] The roughness parameter and the effective area were obtained from the AFM images using the WSxM 4.0 SPMAGE07 software.[29]

RESULTS

Table II shows the thickness, contact angle, and roughness parameters of the four samples: rough films (a-C r, Ti r) and smooth films (a-C s Ti s). The roughness of the surfaces measured using both profilometer and AFM are shown in this table. By profilometer, not clear variation in the roughness between a-C and Ti was observed, and the difference between rough and smooth surfaces was of two orders of magnitude. However, a more precise measurement of the local roughness was attained by AFM analysis, which showed differences among the four surfaces. From this table, it might be seen that the rough surfaces seem to be more hydrophobic than smooth surfaces, contact angle of a-C r and Ti r was 82 and 79° respectively, whereas contact angle of a-C s and Ti s was 50 and 52°, respectively.

AFM images of all samples are presented in Figure 1(a–d), these images showed that not only the roughness values were modified but also the topographical features of the samples were different. The samples deposited on silicon showed a spiky homogeneous topography but the maximum height of the peaks is in the nanometer scale. Although for the rough surfaces, the topography is like a series of non-homogeneous hills and valleys, reaching heights in the micrometer scale.

Bacterial adhesion on the test samples varied depending on the media used, the surface roughness and the surface chemistry, data are presented in Figure 2 as the number of CFUs/cm^2 × 10^5. There were consistently more bacteria on the rough surfaces and in the surfaces cultivated with MM. The number of CFU's was reduced on the Ti surfaces compared with the a-C surfaces. Significant differences were observed between Ti s and Ti r ($p < 0.05$) and Ti s and a-C s ($p < 0.05$). When HS was used, lower bacterial counts were detected on all surfaces compared to the MM. Indeed, the number of CFU's was highly reduced on the a-C s surfaces, and statistical differences were found comparing a-C s vs. a-C r and a-C s vs. Ti s ($p < 0.05$).

Figure 3 shows the proportion of each bacterial strain on the initial biofilm for the different surfaces and two culture media. The differences observed in the figure suggested that the biofilm composition was sensitive to the substrate properties and the incubation media. The statistical analysis is presented in the table at the bottom of Figure 3. This analysis was divided according to the influence of (a) roughness (rough, r vs. smooth, s) for similar surface

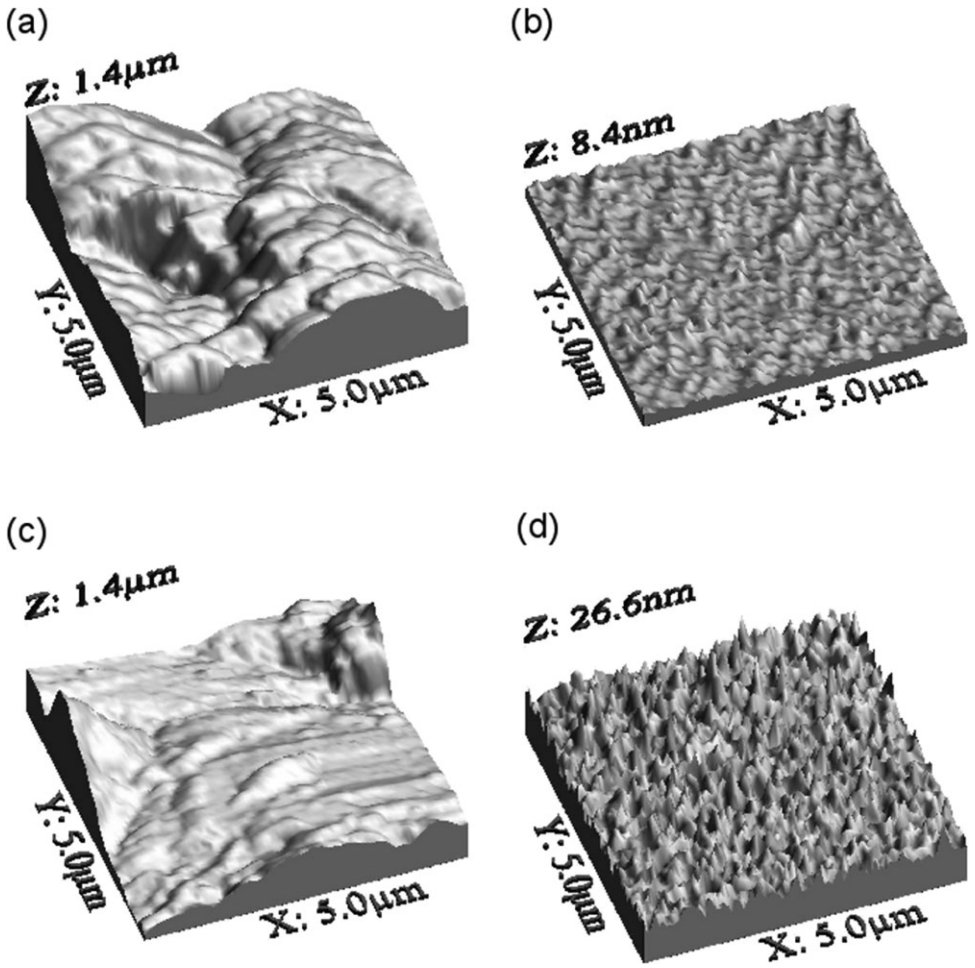


Figure 1. AFM images of the test substrates. Vertical scale has been normalized, *Z* value indicates maximum height in each film. (a) a-C film deposited on the stainless steel sandblasted substrate (a-C r). (b) a-C film deposited on silicon substrate (a-C s). (c) Ti film deposited on the stainless steel sandblasted substrate (Ti r). (d) Ti film deposited on silicon substrate (Ti s).

chemistry, (b) roughness independently of the surface chemistry, (c) surface chemistry (a-C vs. Ti) independently of the roughness, and (d) the influence of the culture media (saliva or mycoplasma) on samples with different roughness (r vs. s) and chemistry (a-C vs. Ti).

The differences in bacterial colonization on the test surfaces as a function of the roughness were also observed in the scanning electron micrographs (Figure 4). Figure 4 shows the SEM images of bacteria attached to the different surfaces for both culture media. When bacteria were cultivated on the smooth surfaces, we observed reduced quantities of attached bacteria. Meanwhile, bacteria growing on rough surfaces are larger in number and appeared to conform to sheet-like regions, which are difficult to distinguish as individuals (more easily observed by AFM image in Figure 5). Figure 5 shows that the bacteria are able to colonize the hills and valleys of the rough samples, and this was correlated to the higher number of CFU's founded on the rough samples. Greater amounts of biofilm were found on the samples cultured in MM compared to HS as observed in Figure 4. Similarly, these SEM images showed that coccal forms were more abundant when MM was used and more bacilli-shaped bacteria tended to congregate in focal regions on the smooth surfaces cultivated with HS.

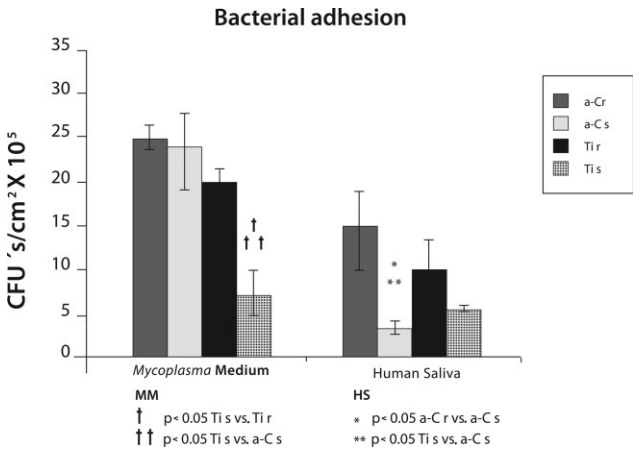
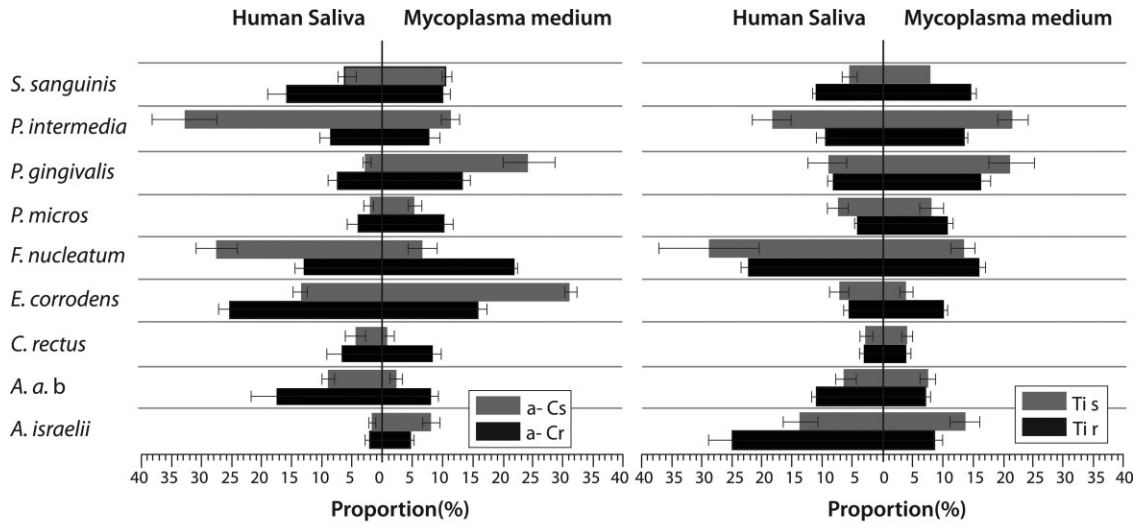


Figure 2. Bacterial adhesion (CFUs $\times$ 10^5) on rough (r) and smooth (s) a-C and Ti films, after 24 h of anaerobic incubation with mycoplasma medium (MM) or human saliva (HS).



p> 0.05
NS= no significant

a-C r vs. a- Cs		Ti r vs. Ti s	
Saliva	*Mycoplasma*	Saliva	*Mycoplasma*
E. corredens *S. sanguinis* *F. nucleatum*	*E. corredens* *F. nucleatum* *C. rectus*	*S. sanguinis*	*E. corredens* *S. sanguinis*

Roughness (r vs. s)		Surface chemistry (a-C VS. ti)		Incubation Media (Saliva vs. Mycoplasma)			
Saliva	*Mycoplasma*	Saliva	*Mycoplasma*	r	s	a-C	Ti
P. intermedia *S. sanguinis*	*NS*	*E. corrodens* *A. israelii*	*E. corrodens*	*P. micros* *P. gingivalis*	*NS*	*P. gingivalis* *A. israelii*	*NS*

Figure 3. Proportion of the nine bacterial strains in the biofilms formed on rough (r) and smooth (s) a-C and Ti films, after 24 h of anaerobic incubation with mycoplasma medium (MM) or human saliva (HS). Table at the bottom shows the statistical analysis divided according to the factors that influence the bacterial colonization. Where the bacteria name is shown, it indicates a significance of $p < 0.05$ for that strain and NS means no statistical difference.

DISCUSSION

This article presented the bacterial adhesion of oral species representative of the normal subgingival dental plaque[7] on amorphous carbon and titanium films with different roughness and using two different incubation media. These factors clearly modified the number of CFU's and the proportion of the attached species. Considering only the data where statistically significance was obtained, as shown in the bottom table of Figure 3, we presented in the following paragraphs an analysis of the effect of roughness, surface chemistry, and incubation media.

Roughness

The influence of roughness was clearly observed in the number of bacteria attached to the rough surfaces (number of CFU's). Similarly, a positive correlation between surface roughness and bacterial attachment *in vitro* has been shown,[30] indicating that surface roughness has an important influence in oral bacterial colonization, which indeed was stronger than the wettability effect. Usually, bacterial adhesion is enhanced on hydrophilic surfaces[4] and the smooth a-C and Ti samples were more hydrophilic than the rough samples. We explained the increment on the number of bacteria as a consequence of the larger effective surface area (estimated from the AFM analysis) on the rough surfaces, which was approximately 1.5 times larger than the smooth surfaces. This was also observed in the SEM and AFM images, where the hills and valleys of the rough surface are completely covered by bacteria, whereas in the smooth surfaces empty spaces are usually observed. Nevertheless, other studies have suggested that regarding to bacterial adhesion or initial biofilm formation; roughness appears to be a minor factor.[31]

Concerning the bacterial strain proportions, surface roughness seems to be an important factor in the colonization ability of certain species. This effect seems to be more pronounced when saliva was used as culture media, since for mycoplasma no statistical differences were obtained (see table in Figure 3). The proportion of *Streptococcus sanguinis* was significantly higher ($p < 0.05$) on rough surfaces for both a-C and Ti, that is independently of the surface chemistry. Meanwhile, *Prevotella intermedia* showed a higher proportion on the smooth surfaces ($p < 0.05$).

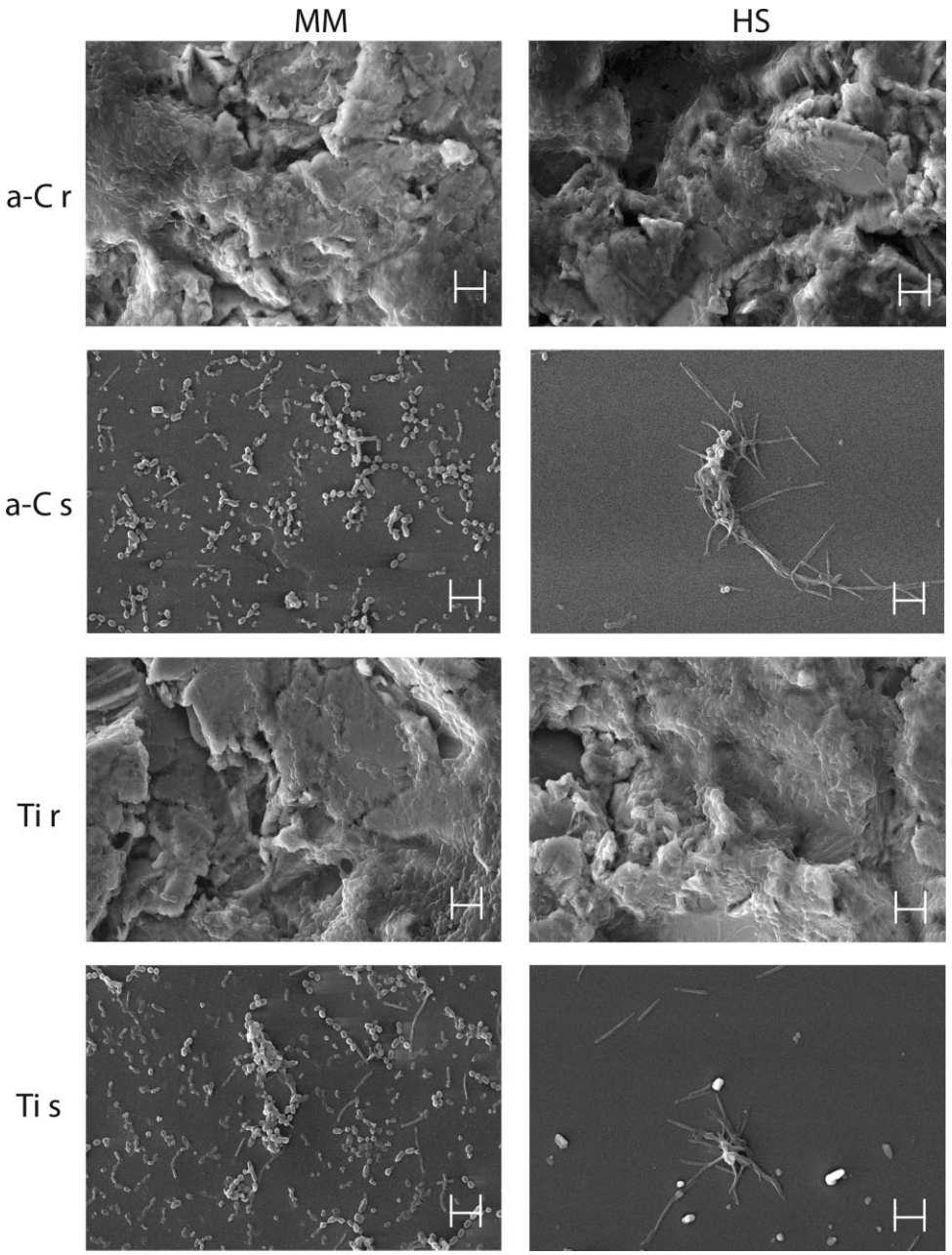


Figure 4. SEM images of the biofilms formed on the a-C (r and s) and Ti (r and s) film surfaces after 24 h of anaerobic incubation with mycoplasma medium (MM) or human saliva (HS).

Surface Chemistry

Higher numbers of attached bacteria (CFU's) were detected on amorphous carbon than on the Ti samples, except for the smooth a-C surface when saliva was used. These results differ with other published articles that have reported that carbon-based films can inhibit bacterial adhesion.[22–24] Nevertheless, in most of these studies no more than three bacterial species were tested and according to our results, the antibacterial properties are also highly dependent on strain used to test the surface, as seen in Figure 3. Regarding Ti bacterial adhesion, it has been suggested that Ti has some antibacterial properties explained due to the formation of peroxides at the surface.[32] Although another study suggested that pure Ti was more colonized by two oral bacteria strains in comparison to other surfaces like TiN, ZrN, or TiO_2.[8]

An interesting finding was the proportion of *Eikenella corrodens* on the biofilms formed on the a-C surfaces. This strain was found in higher proportions on the a-C samples on both, rough and smooth surfaces, for both media; mycoplasma or saliva. This suggested that *E. corrodens* was more sensitive to surface chemistry than to roughness or the cultured media used. This finding supported the notion that chemical surface is directly affecting the colonization of the oral bacteria.[33] *E. corrodens* posses an specific lectin-like substance that mediates its adherence to various host tissue cell surfaces,[34]

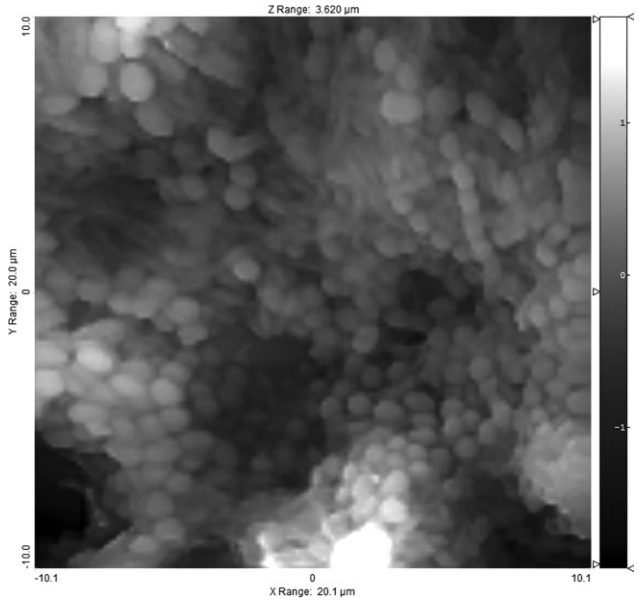


Figure 5. AFM image of the biofilm formed on the a-C r film surface after 24 h of anaerobic incubation with mycoplasma medium (MM).

so it is possible that the specificity that this microorganism show to the a-C surfaces has to be with some specific adhesion properties of this strain. Conversely, *Actinomyces israelii* was found on higher proportions on the Ti surfaces ($p < 0.05$) regardless the culture media and roughness.

Incubation Media

We found higher numbers of bacteria on all surfaces when mycoplasma culture media was used. A possible explanation could be the differences between the components of both media, saliva contains an important presence of some antimicrobial substances, such as lysozyme, lactoferrin, lactoperoxidase, and secretory IgA.[35] Meanwhile, the MM contains only nutrients and some proteins. The saliva is a more biologically significant media for the bacterial adhesion test. However, the components of saliva cannot be controlled and this can lead to a larger dispersion. Many studies indicate that the saliva is critical for the colonization of certain taxa,[36,37] and it is determinant for the type and amount of bacteria that will attach on a surface.[36,38] In addition, culture media influence the colonization of specific bacteria, on the smooth surfaces (a-C s and Ti s) more bacilli-shaped microorganisms were observed by SEM when saliva was used for incubation.

The proportion of the species on the biofilms was both substrate dependent and media dependent. Some species were more sensitive to the incubation media, whereas some were more affected by surface roughness. For example, *Aggregatibacter actinomycetemcomitans* considered a periodontal pathogen[39] was found in higher proportions on the a-C and Ti surfaces in the presence of HS. This is interesting because there are no reports regarding the colonization of this microorganism on amorphous carbon surfaces and moreover, it has been reported that *A. actinomycetemcomitans* is capable to colonize in low rates titanium implant surfaces both by *in vivo* and *in vitro* studies.[13,40,41] *Porphyromonas gingivalis* that is a very important periodontal pathogen[42] was found in higher proportions on the smooth surfaces of a-C and Ti when incubated with mycoplasma medium. This finding is consistent with those previously reported showing that *P. gingivalis* is capable of colonizing Ti surfaces at very high rate.[13,43]

CONCLUSIONS

In conclusion, our results support the notion that there is a strong influence of the physical and chemical properties of the substrate in the colonization of oral bacteria. More bacteria were found on the a-C surfaces, however, when using HS, significantly reduced levels of bacteria were found on the a-C smooth surfaces. When the proportion of each individual strain on the biofilms was analyzed, we observed that the chemical composition of the surface was a major determinant in the colonization of certain taxa like *E. corrodens* that was capable to colonize in very high rates the a-C surfaces despite of their roughness or the culture media used. In contrast, other species like *A. israelii* or *P. gingivalis* were more sensitive to surface roughness than to surface chemistry. The incubation media were also an important factor on bacterial adhesion; there were significantly lower counts of bacteria on the saliva incubated samples, in both a-C and Ti surfaces. However, the error associated to the use of saliva was also larger, suggesting that to study the antibacterial properties of the biomaterials surface, it is more reliable and convenient to use a well-controlled culture media.

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