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Oral bacterial adhesion on amorphous carbon films

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ABSTRACT

It is now well established that all the different forms of amorphous carbon films are biocompatible and suitable for specific biomedical applications. On the other hand, bacterial adhesion on implant surfaces has also a strong influence on the healing and long-term outcome of biomedical devices and this has not been thoroughly studied for the carbon films. The purpose of this study was to evaluate the bacterial adhesion on graphite-like amorphous carbon (a-C) films in comparison to titanium (Ti) films and stainless steel (SS) substrates using different bacteria strains from the normal oral microbiota. Medical grade stainless steel discs of 15 mm in diameter were coated by either Ti or a-C films using magnetron sputtering. The bacterial adhesion of single species and a mixture of nine different microorganisms was tested on the three surfaces. The bacteria were anaerobically incubated on the surfaces for 24 h, then colony forming units (CFUs) were counted. The total amount of CFUs was found higher on the a-C and SS surfaces in comparison to Ti films when the nine strains were mixed together, suggesting that Ti surfaces are better than the a-C and SS to avoid bacterial adhesion. However, when single species were analyzed the individual strains showed different adhesion profiles. Some species like *Aggregatibacter actinomycetemcomitans*, *Eikenella corrodens*, *Campylobacter rectus*, and *Fusobacterium nucleatum* were found in higher counts on the a-C surfaces, while other species like *Actinomyces israelii*, *Porphyromonas gingivalis*, *Prevotella intermedia* and *Streptococcus sanguinis* were found in lower counts comparing to the Ti films. These results suggested that the determination of antibacterial properties of a surface by studying the bacterial adhesion of individual strains, as usually done, might not be representative of the *in vivo* response, where more than one strain are surely present.
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1. Introduction

Bacterial adhesion on implant surfaces has a strong influence on healing and long-term outcome of biomedical devices like dental implants. Prosthetics infections are due to the formation of biofilms, which are defined as communities of microorganisms embedded in an extracellular polymeric slime attached to a solid surface (tooth or implant surface) [1–3]. Once the biofilm is formed, bacterial cells become highly resistant to antibiotics, since the biofilm microenvironment protects them from host defences and antibiotics [4]. Clinical experience has shown that biofilms must be removed physically before the infection can be resolved. Therefore, the best solution to avoid microbial infections of implants is to inhibit the colonization of the surface by the oral bacteria. Bacterial adhesion to surfaces is an extremely complicated process that is affected by many factors including environment, bacterial properties and material surface characteristics, such as chemical composition, surface charge, hydrophobicity and topography-roughness [5,6]. Bacterial adhesion to a human tissue or surface is often the first discernible event in the process of colonizing a host. Moreover, studies of colonizing bacteria in the mouth were among the first to provide knowledge that microorganisms attach to different tissues and surfaces in a remarkably selective manner [7].

It has been shown that all the different forms of amorphous carbon can be considered as biocompatible [8–11] and might be adequate as a surface modification for biomedical applications, such as, dental and orthopaedic implants [12,13]. 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), graphite-like carbon (GLC) or when highly hydrogenated as polymer-like carbon (PLC). The amorphous carbon films studied in this work are graphite-

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like carbon films. The fundamental difference between graphite and diamond-like is the amount of sp^3 hybridized carbon atoms, which is very low in the first group and above 40–50% for DLC. This leads to strong differences in many of the physical properties, such as, optical gap, conductivity, surface energy, etc.

During the last years, we have been investigating different aspects of the interaction between human cells (osteoblasts) and graphite-like carbon films as possible candidates for coating dental implants. We decided to study GLC films instead of DLC, because the main interest was on the osseointegration and not on the tribological properties and graphite itself has been established as a good osteoinductor material [14,15]. Previous reports have shown that the GLC films presented in this work have also good osteoconductive properties [16,17].

Nevertheless, another important factor for the success of dental implants is to avoid the formation of biofilms that might lead to implant failure or strong inflammatory processes. The number of papers regarding bacterial adhesion on the different carbon films is not very large, as shown in Table 1. These works included mainly DLC or modified-DLC films and concluded that the carbon surface has great biocompatibility properties and good resistance to microbial adhesion [18–27]. However, these results could not be extrapolated to the GLC films due to the strong differences between DLC and GLC physical properties, which might affect the bacterial adhesion. Moreover, since none of the studies in Table 1 include the oral microbiota and in any case no more than three bacterial strains were used, we decided to study the oral bacterial adhesion on the GLC films evaluating a larger number of bacteria strains representative of the dental plaque, trying to simulate as close as possible the *in-vivo* situation.

The bacterial adhesion of microorganisms belonging to the normal oral microbiota was evaluated on amorphous carbon (a-C) films in comparison to titanium (Ti) and stainless steel (SS) control surfaces. Titanium was produced as a thin film deposited on the stainless steel substrate by magnetron sputtering and it was used as both a control surface and a buffer layer to promote the adhesion between the a-C film and the stainless steel substrate [28–30]. We tested in the first place; the adhesion profile from a mixture of nine different microorganisms in order to simulate an oral micro-environment, but later the individual adhesion profiles of the same nine species was evaluated. The information from the two studies was used to evaluate the bacterial colonization profiles over the same surface by different strains, i.e., the surface affinity to each of the strains.

Table 2
Reference strains employed for the adhesion assays.

Species	Strain*
Aggregatibacter actinomycetemcomitans serotipe b	43718
Actinomyces israelii	12102
Campylobacter rectus	33238
Eikenella corrodens	23834
Fusobacterium nucleatum ss nucleatum	25586
Parvimonas micros	33270
Porphyromonas gingivalis	33277
Prevotella intermedia	25611
Streptococcus sanguinis	10556

* American Type Culture Collection, Rockville, MD.

2. Materials and methods

2.1. Films

All the experiments were done using three samples of each one: a-C films, Ti films and stainless steel (AISI 316L) substrates.

The substrates were sandblasted using SiO_2 particles to obtain a uniform surface roughness of approximately 2 μm. Before deposition, the SS 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 deposition conditions were 20 sccm of argon (purity 99.999%), 4 Pa of deposition pressure and 0.4 A of DC current for 5 min, leading to a film thickness around 150 nm.

The Ti films were deposited in a pulse DC magnetron sputtering system (250 kHz, 250 W), using a high purity Ti target and argon plasma (10 sccm) at 0.2 A and 0.4 Pa leading to a film thickness around 170 nm. X-ray diffraction spectra and X-ray photoelectron spectroscopy showed that the films were mainly metallic titanium. Similar Ti films (but thinner) were used as a buffer layer between the substrate

Table 1
Bacterial adhesion studies on carbon surfaces.

Strain	Surface	Result	Reference
S. epidermidis	PVC and DLC silver/fluorinated coatings	Bacterial adhesion from minor to higher: Ag thicker (less) Ag thin Ag/DLC DLC Fluorinated surfaces (higher)	[18]
E. coli	DLC with Ag	Ag-doped DLC films show good antimicrobial characteristics.	[19]
S. aureus *S. epidermidis*	Amorphous carbon on PET	Bacterial adhesion is energetically unfavorable on the PLC deposited on PET.	[20]
E. coli	DLC coatings	DLC coatings exhibited great resistance to microbial adherence.	[21]
P. aeruginosa	DLC and F-DLC	The eradication of *E. coli* attached to F-DLC films was 15% higher than to DLC films.	[22]
P. aeruginosa *S. epidermidis* *S. aureus*	Si-doped DLC coatings	*P. aeruginosa* shows the lower initial bacterial adhesion, compared with *S. epidermidis* and *S. aureus*. Si-doped DLC films with 3.8% Si performed best in reducing bacterial adhesion.	[23]
S. aureus *S. epidermidis* *P. aeruginosa*	Pyrolytic carbon manufactured with Silicon	*S. aureus* was the most adherent specie. *P. aeruginosa* was intermediately adherent and *S. epidermidis* was the least adherent specie.	[24]
S. warneri	DLC–Ag DLC–Pt DLC–AgPt	DLC silver films demonstrated 50% lower colonization and DLC silver-platinum films demonstrated a 90% lower colonization compared with the uncoated silicon substrate.	[25]
E. coli	C:H films H free amorphous Carbon films	Bacterial adhesion was higher on C:H films than in H free amorphous carbon films.	[26]
S. aureus *S. epidermidis*	DLC DLC-PTFE-h	The DLC-PTFE-h coating showed better antimicrobial properties than DLC alone.	[27]

and the a-C film, since it has been reported that the film adhesion is improved [28–30].

The carbon film composition, morphology and bonding characteristics were investigated by different techniques: X-ray photoelectron spectroscopy (XPS), scanning electron microscopy (SEM-Cambridge–Leica, Stereoscan 440 at 20 kV.), atomic force microscopy (JSPM-4210), Raman spectroscopy (Renishaw spectrometer of 514.5 nm), and spectroscopy ellipsometry (Jovin Yvon Uvisel DH10), water contact angle was measured using double-distilled water with the

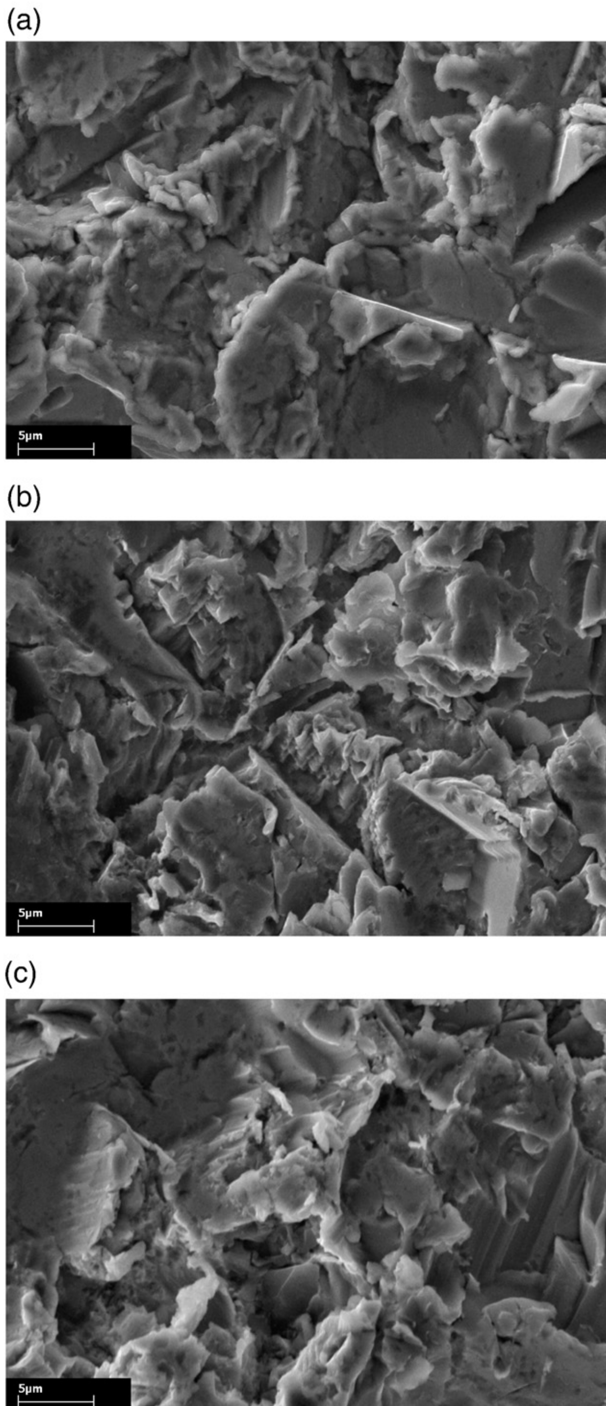

Fig. 1. SEM images of the surfaces. (a) a-C film deposited on the stainless steel sandblasted substrate. (b) Ti film deposited on the stainless steel sandblasted substrate. (c) Stainless steel sandblasted substrate.

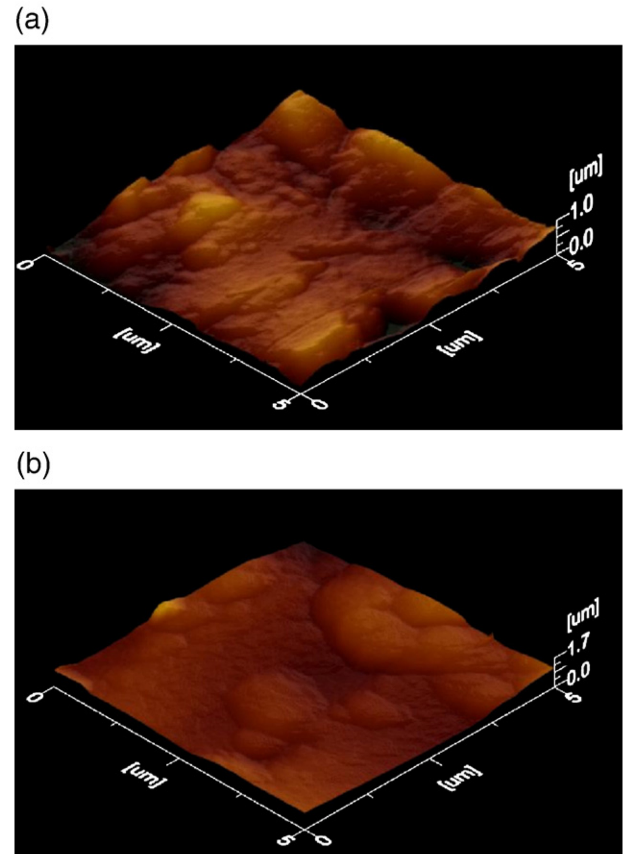

Fig. 2. AFM images of the surfaces. (a) a-C film deposited on the stainless steel sandblasted substrate. (b) Ti film deposited on the stainless steel sandblasted substrate.

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.

2.2. Bacterial strains

Nine reference strains representative of the normal subgingival dental plaque were used (Table 2). Lyophilized bacterial stocks (American Type Culture Collection, Rockville, MD) were rehydrated in *Mycoplasma* broth base (BBL, Becton-Dickinson and Co., Sparks, MD). All strains were grown on *Mycoplasma* agar base (BBL, Becton-Dickinson and Co., Sparks, MD) supplemented with 5% defibrinated sheep blood, 5 μg/ml hemin (Sigma-Aldrich) and 0.3 μg/ml menadione (Sigma-Aldrich) under anaerobic conditions (80% N_2, 10% CO_2 and 10% H_2).

2.3. Bacterial adhesion test

Samples previously sterilized (autoclave), were placed individually in 12 well culture plates. 10^6 cells/ml suspension of each strain were added to obtain a mixed culture in a total volume of 1 ml. In addition, 10^6 cells/ml suspension of each strain were placed individually on another set of samples. Plates with the samples were incubated for 24 h at 35 °C under anaerobic conditions. After anaerobic incubation, each sample was washed twice with *Mycoplasma* broth. After washing, 1 ml of enriched *Mycoplasma* broth base (5 μg/ml hemin and 0.3 μg/ml menadione) was added, and samples were sonicated for 5 periods of 10 s in order to de-attach the bacteria that were present in each sample. After sonication, five-fold dilutions of this suspension

Table 3
Surface roughness and water contact angle measurements of the surfaces.

Surface	Ra (μm) (scan length, 5 mm)	Contact angle (mean ± SD)
Stainless steel medical grade 316L	2.01	97° ± 8°
a-C film on stainless steel substrate	1.83	71° ± 2°
Ti film on stainless steel substrate	1.89	101° ± 5°

were plated on enriched agar plates and after 7 days of anaerobic incubation, colony-forming units (CFUs) were visually counted in order to obtain the number of viable bacteria per ml that were present in each sample. After incubation time, colonies in the agar plates can be visualized and counted. Calculations are made, based in the dilutions that were done and a log transformation was calculated.

In order to observe biofilm morphology in each of the test substrates, samples were prepared for Scanning Electron Microscopy (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 and sputter-coated with Au before observation.

2.4. Statistical analysis

Microbiological data are presented as mean ± standard error of the mean (SEM) of bacterial counts (CFU's) × 10^5. Significance of differences of the number of CFUs between surfaces was determined using the Student's *t*-test and significant differences determined using Bonferroni's modification of Student's *t*-test.

3. Results

3.1. Surface characterization

The surface roughness and topography of the substrates were not significantly modified by the film deposition as can be qualitatively observed by SEM and AFM images, shown in Figs. 1 and 2, respectively. The average surface roughness was confirmed by profilometer measurements as shown in Table 3, which also include the water-contact angle measured for the three surfaces; it might be seen that all of the samples were hydrophobic (contact angle >65°). Fig. 3a shows the XPS spectra of the a-C films; it might be seen that only carbon and oxygen were found. The oxygen could be removed after a few minutes under moderate argon cleaning, suggesting that it was only surface-adsorbed oxygen. These results were confirmed by infrared spectroscopy (data not shown), in which C–O or O–H bands were not detected. Similar results were obtained for the Ti films, where only the presence of Ti atoms remained after argon cleaning (data not shown). Fig. 3b shows typical Raman spectra of the amorphous carbon films, where two bands around 1358 and 1586 cm^{-1} can be observed (Gaussian function) and correspond to the presence of aromatic six-membered sp^2 clusters and the sp^2 CC bonds, respectively [31]. Raman spectroscopy allows us to classify the carbon films as highly sp^2 bonded films having large sp^2 clusters or aromatic nanodomains, which are known to control the optoelectronic properties of the films. The opto-electronic properties were determined by ellipsometry showing that the films have semiconductor characteristics with a low band-gap. Fig. 3c shows the position of the optical Tauc gap, which is a

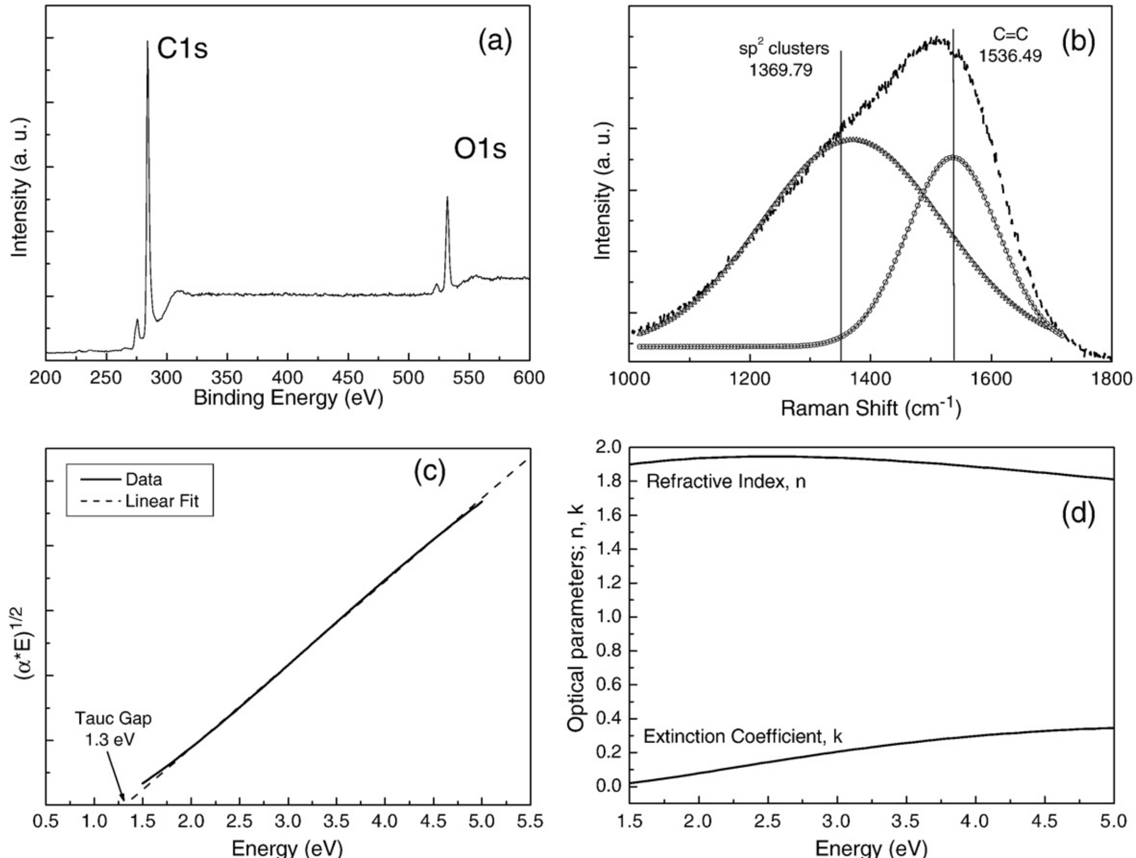

Fig. 3. (a) XPS spectra from an amorphous carbon film. (b) Raman spectra typical of an sp^2 bonded carbon film. (c) Optical gap estimated using the Tauc method. (d) Complex refractive index vs. photon energy of the a-C film.

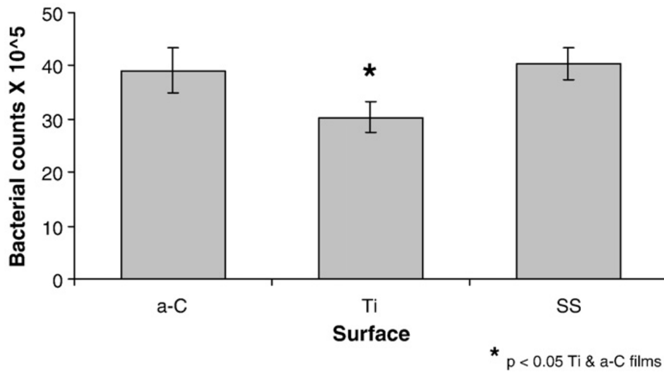

Fig. 4. Bacterial counts $\times 10^5$ when all bacterial strains (nine species) were tested together on a-C, Ti and SS surfaces after 24 h of anaerobic incubation.

measure of the forbidden electronic gap in amorphous semiconductors [32]. The optical gap was close to 1.3 eV, lower than the values usually obtained for DLC and PLC films, which exceed 2 eV [33]. Finally, Fig. 3d shows the complex refractive index $n = n + \mathrm{i}\ k$, as a function of the photon energy. The real part of the refractive index was around 2, lower than the 2.4 reported for DLC [33]. These results demonstrated the graphite-like character of the a-C films and also were used to compare the film properties with those previously reported [14,17] confirming the reproducibility of the deposition process.

3.2. Bacterial adhesion test

Fig. 4 shows the total amount of bacteria that were capable of colonizing the tested surfaces when the bacterial strains were mixed together. Higher bacterial counts were detected on the stainless steel surfaces (SS) (40.4×10^5) and on the a-C films (39.1×10^5) in comparison to the Ti films (30.3×10^5). Statistical differences were found only between the a-C and the Ti films, $p < 0.05$, suggesting a conclusion that the Ti surfaces are better than the a-C and SS to avoid bacterial adhesion.

When single species were analyzed in order to determine if the bacterial adhesion on the surfaces was independent of the identity of the strain, we found different adhesion patterns, as observed in Fig. 5. Considering only the results where the differences were statistically significant, it might be seen that on the a-C surfaces, the number of *Eikenella corrodens* and *Fusobacterium nucleatum* were always higher than on the Ti surfaces. Meanwhile, *Actinomyces israelii* and *Porphyromonas gingivalis* were found in lower counts on a-C, when compared to both Ti and SS surfaces. Similarly, when the adhesion pattern of Ti was compared to the other surfaces; there was a larger adhesion of *Prevotella intermedia*, disclaiming the result obtained from the experiment using the mixed strains (Fig. 4), that Ti was better than a-C and SS to avoid bacterial adhesion. Since for the same strain, we found reduced bacterial adhesion on the a-C surface.

Fig. 6 shows the SEM images of the bacteria attached to the surfaces. These images are representative of the bacterial counts founded in each surface when all strains were used. More bacteria were observed on the a-C films and the SS surfaces compared to the Ti surface.

4. Discussion

The oral cavity is capable to be colonized for more than 500 species of microorganisms [34,35], and for any dental implant the response of the material's surface to this complex microenvironment, might determine the long-term outcome of the implant. Any artificial surface located at a site where dental plaque usually forms, like natural teeth, will also be susceptible of biofilm formation [36], with the subsequent complications that might lead to implant failure. Nevertheless, by recognizing the differences in the bacterial adhesion profiles on natural oral hard tissues, like enamel, compared to the implant surface [37,38], it could be possible to design specific surface modifications searching for a reduced bacterial adhesion. Factors influencing bacterial adhesion to a biomaterial surface are complex and dependent on both surface and bacteria properties

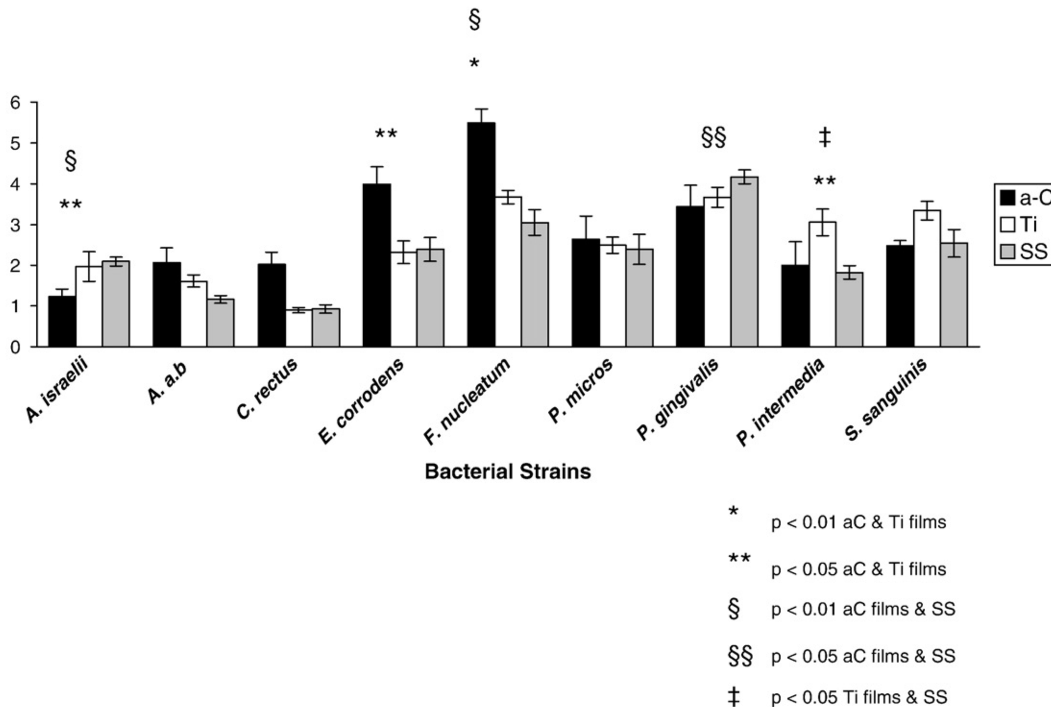

Fig. 5. Bacterial counts $\times 10^5$ of the individual bacterial strains tested on a-C, Ti and SS surfaces after 24 h of anaerobic incubation.

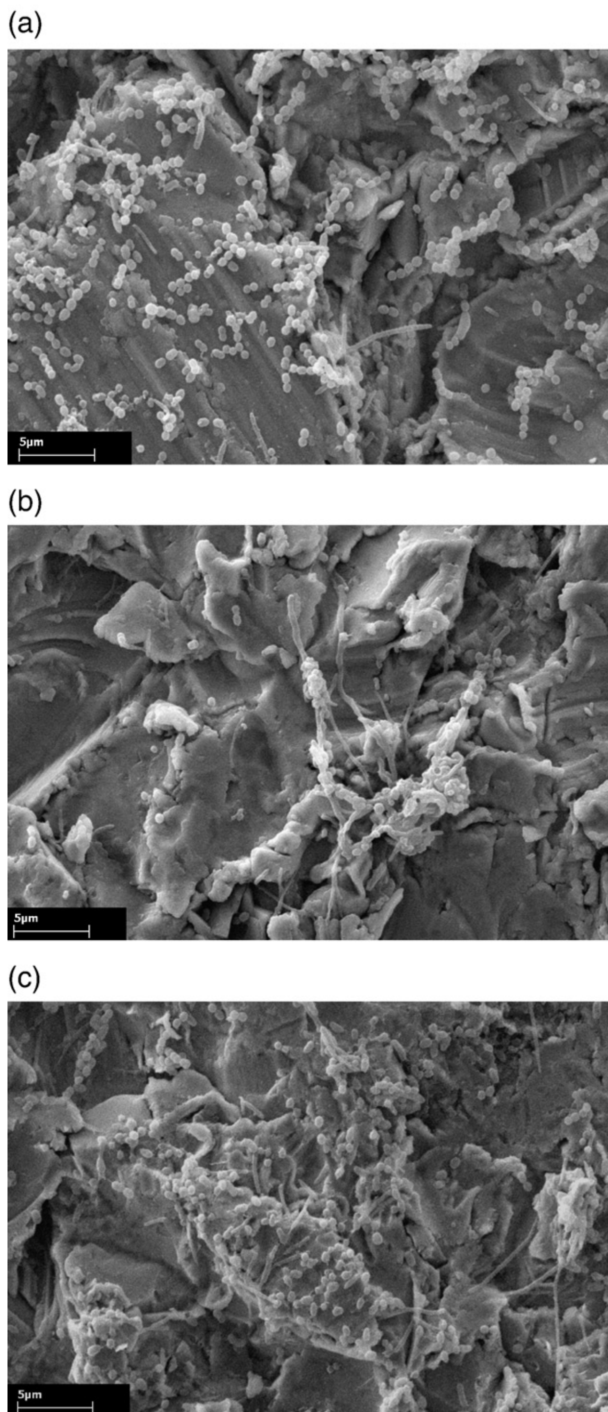

Fig. 6. SEM images of the bacteria attached on the substrates. (a) Biofilm on a-C films. (b) Biofilm on Ti films. (c) Biofilm on SS surfaces.

[5,6,37,39–42] and no recipes have been found so far to control the amount of bacterial adhesion on the implant surfaces. In this work, we report the interactions between three different surfaces with nine different bacteria strains belonging to the normal oral microbiota, trying to determine if the a-C surface compared to Ti and SS might or not be considered as an oral bacterial repellent surface, but also trying to find correlations between the surface properties and the bacterial adhesion profiles.

Our results showed that indeed bacterial adhesion was sensitive to surface chemistry; we found that a-C and SS surfaces were more colonized by bacteria than titanium, when the nine bacteria strains were mixed (Fig. 4). It is interesting to remark that statistically significant differences were found for both the number of CFU's and the contact angle between a-C and Ti (101° for Ti compared to 71° for a-C), which might be an indication of the role played by the wettability on the bacterial adhesion. The present results are consistent with the notion that there is a significant correlation between surface free energy and bacterial adhesion [18,43,44]. However, in our case, more evidence is required before reaching any conclusion.

The effect of the bacteria's properties can be observed when adhesion from single strains was analyzed. Fig. 5 shows the intricate relationship between bacteria's properties and surface's properties. In the oral cavity, biofilm formation occurs by the sequential colonization of certain bacterial strains [45]. Some strains have been identified as early colonizers of the dental surface, attaching and proliferating at an early stage (i.e. *A. israelii* and *Streptococcus sanguinis*). A second group of bacteria functions as a bridge between the early and late colonizers like *F. nucleatum*, *E. corrodens*, *Campylobacter rectus* and *P. intermedia*. Finally, the third group of species appears at late stages in biofilm development and is considered true periodontal pathogens like *P. gingivalis* [46]. From Fig. 5 we can observe that *F. nucleatum* and *E. corrodens*, which are from the group of second colonizers, had a strong affinity to a-C in comparison to the other surfaces. Meanwhile, *A. israelii* (early colonizer) and *P. gingivalis* (late colonizer) had a larger affinity to SS, and *P. intermedia* had a clear affinity for Ti.

Previous studies have shown different adhesion patterns of some oral microorganisms; for example, it has been reported that *P. gingivalis*, a very important periodontal pathogen [47], is capable of colonizing Ti surfaces at very high rates [48]; in our study this strain was found in high numbers on all surfaces tested, although it was significantly higher on SS. Another study showed that *S. sanguinis*, was found in higher counts in metal surfaces like titanium and gold compared to enamel and composite materials [49]. Our results were consistent with this finding, since we found a higher adhesion of this microorganism to Ti and SS surfaces than to a-C.

The relevance on biomedical applications between the different profiles and the sequence of bacterial adhesion is still difficult to evaluate, but it clearly demonstrates that the use of one single strain is not enough to catalogue the surface as having anti-bacterial properties.

As resumed in Table 1, studies on a-C and DLC films have shown that these films can inhibited bacterial adhesion, specially with the addition of N, Si [50], Ag [19] or in comparison to uncoated polyethylene terephthalate [20]. However, the results were obtained from the analysis of no more than three bacterial species and for different studies, the same strain was used. Comparing these data to our results, in which differences in the bacterial adhesion were observed depending on the specific strain used, it seems that the previous results describe only partially the surface-bacteria response. Therefore, the surface should be considered as repellent to that specific strain, but it cannot be concluded that the surface has anti-bacterial properties as a general rule.

5. Conclusions

Based on the results obtained using the mixed nine strains (Fig. 4), which is closer to the *in-vivo* process, we could conclude that graphite-like amorphous carbon is not a suitable surface to prevent adhesion from the oral media. Meanwhile by using a single strain assay, a-C was effective to repel bacteria, such as, *A. israelii*, *P. gingivalis* and *P. intermedia*.

The use of different bacterial strains from the oral cavity to study the bacteria adhesion profile on amorphous carbon have shown that it is not straightforward to reach conclusions about the anti-bacterial properties of the surface. When bacterial adhesion was examined with the mixture of bacteria strains, the differences between the three surfaces, suggested a better response of Ti to inhibit bacterial adhesion. However, when

individual species were used, the adhesion profiles varied on the same surface depending of the bacterial strain. Therefore, our conclusion is that the determination of bacterial adhesion properties on biomaterials using only one or two bacterial strains is not accurate and cannot lead to general conclusions about the anti-bacterial properties of the biomaterial, at least when strains from the oral cavity are used.

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