

# Oral Bacterial Adhesion and Biocompatibility of Silver-Amorphous Carbon Films: A Surface Modification for Dental Implants

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## 1. Introduction

Bacterial adhesion and the subsequent biofilm formation on dental implants is a persistent problem that can cause implant failure. Once biofilm is formed, bacterial cells become highly resistant to antibiotics and host defences (Costerton et al., 1999, Patel, 2005), and clinical experience has shown that biofilms must be removed physically before the infection can be resolved (Costerton, 2005).

There is an apparent clinical and microbiological similarity between peri-implantitis and periodontitis (Listgarten and Lai, 1999, Papaioannou et al., 1996). The first indication of the specific role of bacteria in peri-implant infections was originated from microscopic analysis of samples taken from failing implants that shown an abundance of motile rods, fusiform bacteria and spirochetes, whereas samples from successful implants contained only a small number of coccoid cells and very few rods (Mombelli, 2002, Mombelli et al., 1987, Rams and Link, 1983). These findings revealed a site-specific disease process with microorganisms associated in patterns known from chronic periodontitis of natural teeth. The term peri-implantitis introduced in the 1980s, describe a destructive inflammatory process affecting the soft and hard tissues around osseointegrated implants, leading to the formation of a peri-implant pocket and loss of supporting bone (Mombelli et al., 1987).

Adhesion to a surface is the essential first step in the development of a biofilm and the sequential colonization and formation of the dental plaque is highly orchestrated (Xie et al., 2000). The association of bacteria within mixed biofilms is not random; it has been shown that there are specific associations (complexes) among bacteria in dental biofilms (Socransky and Haffajee, 2005, Socransky et al., 1998, Kolenbrander et al., 2006). In addition, these microbial complexes, can be used to describe the sequential colonization of the subgingival plaque. Some bacterial strains, mainly belonging to the genus *Actinomyces* (blue complex) and *Streptococcus* (yellow complex) have been identified as early colonizers of the dental surface, attaching and proliferating at an early stage. A second group of bacteria that functions as bridge between the early and late colonizers are formed by species belonging to the green, purple and orange complexes (i.e. *Fusobacterium nucleatum*, *Capnocytophaga sputigena*, *Eikenella corrodens*). Finally, the third group of species that appears at late stages in

biofilm development and that are considered true periodontal pathogens are species of the red complex (*Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*).

This biomaterial-related infection is characterized by chronicity, persistence and lack of susceptibility to antimicrobial agents. This information suggests that the first steps on the biofilm formation on implant surfaces such as titanium (Ti) or stainless steel (SS) can be similar to the process on teeth. In fact, the experimental gingivitis model described by L oe et al. that demonstrated the cause and effect relationship between biofilm formation on teeth and gingivitis (Loe et al., 1965), has been also used to explain the implant and peri-implant mucositis (Pontoriero et al., 1994, Zitzmann et al., 2001). Studies about the peri-implant microbiota *in vivo* have examined the influence of oral health status on the presence of specific bacterial species. Some of these studies reported similar supra and subgingival microbiota on teeth and Ti implants (Shibli et al., 2008, Furst et al., 2007, Groessner-Schreiber et al., 2004). In contrast, other studies found an absence of periodontal pathogens like *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans* and *Porphyromonas gingivalis* (Heuer et al., 2007) or sporadic high numbers of *Parvimonas micra* (formerly *Peptostreptococcus micros*), *Staphylococcus aureus* and *Staphylococcus epidermidis* (Furst et al., 2007, Salvi et al., 2008).

The biofilm formation process is extremely complicated and this is particularly true when multiple species are present in the biofilm as in dental plaque. This process is affected by many factors including environment, bacterial properties and material surface characteristics, such as chemical composition, surface energy, hydrophilicity and topography (Katsikogianni and Missirlis, 2004, An and Friedman, 1998, Merritt and Chang, 1991). In addition, for *in vitro* studies, the media play an important role as well, as was shown in a previous work comparing the influence of two different media on the bacterial adhesion and the initial biofilm formation (*Mycoplasma* media, a standard bacterial culture media, and sterilized human saliva); revealing different patterns of adhesion on the same surface when bacteria were cultured with different media (Almaguer-Flores et al., 2010).

## **2. Surface modifications to prevent bacterial adhesion and biofilm formation on dental implants**

Surfaces are critical in the field of biomaterials; the nature of an implant surface determines their interaction with the biological environment. Surface modifications can be classified as physical modifications (sandblasting, patterning, etching, lithography, etc) or chemical modifications (deposition, thin films, polymer coating, etc). Often these techniques are applied in combination to alter both topography and surface energy. Surface modifications can be classified on basically three classes: (a) topographic modifications; such as size and porous distribution and roughness, (b) chemical modifications of the surface; which involve controlled cleaning and oxidation by glow discharge plasma techniques, thin film growth, and deposition of organic overlayers such as polymers, proteins and antimicrobial substances and (c) micromechanical or viscoelastic modifications of the surface, which can affect (enforce or reduce) the mechanical stress-strain fields at the interface (Bagno and Di Bello, 2004, Kasemo and Gold, 1999). These modifications offers the possibility of combining ideal bulk properties with desired properties such as increased bioactivity or prevent bacterial adhesion.

Antibacterial surface modifications have been created in order to prevent bacterial contamination and resulting infections, which can lead to the loss of the implant. These surface modifications must be designed to retard bacterial colonization or can function as

antimicrobial agents without affecting the cells and tissues adjacent to the implant; in other words, they need to be biocompatible.

Some studies have explored the *in vitro* biofilm formation on modified titanium implant surfaces; unfortunately, most of these biofilm models have only included one or two oral bacterial strains (Table 1). In the oral cavity however, the microbial ecology is complex and consists of hundreds of species (Socransky and Haffajee, 2005). For this reason, these models are good tools in order to study species-specific infections or mono-infections, but not very useful to study mixed anaerobic infections such as peri-implantitis.

Strain	Surface	Ref.
<i>S. sanguis</i> <i>S. mutans</i>	Ti, and TiN, ZrN, TiO <sub>2</sub>	(Grossner-Schreiber et al., 2001)
<i>P. gingivalis</i>	Ti with ion implantation (Ca <sup>+</sup> , N <sup>+</sup> , F <sup>+</sup> ), oxidation (anode oxidation, titania spraying), ion plating (TiN, alumina) and ion beam mixing (Ag, Sn, Zn, Pt)	(Yoshinari et al., 2001)
<i>E. coli</i> <i>P. aeruginosa</i> <i>B. cepacia</i> <i>B. subtilis</i>	11 different glass and metal oxide-coated glass surfaces	(Li and Logan, 2004)
<i>S. sanguis</i>	Ti with different roughness	(Pereira da Silva et al., 2005)
<i>S. aureus</i> <i>P. aeruginosa</i>	Ti coated with albumin	(Kinnari et al., 2005)
<i>P. gingivalis</i>	Ti and TiN thin films	(Jeyachandran et al., 2007)
<i>S. aureus</i> <i>S. epidermidis</i> <i>S. mutans</i> <i>P. aeruginosa</i>	Ti with bioactive polymers layers	[Maddikeri, 2008 #56]
<i>S. sanguinis</i>	Ti, Au, and ceramic and composite dental materials	(Hauser-Gerspach et al., 2007)
<i>S. aureus</i>	Ti with polyelectrolyte multilayers	(Chua et al., 2008)
<i>S. aureus</i> <i>S. epidermidis</i>	Ti with (P(MAA)) followed by immobilization of silk sericin	(Zhang et al., 2008)
<i>S. aureus</i>	Ti, Ta, Cr and DLC surfaces	(Levon et al., 2009)
<i>S. sanguinis</i>	Ti with two modified different roughness	(Burgers et al., 2010)
<i>S. aureus</i> <i>P. aeruginosa</i>	Ti with micro and nano scale surface roughness	(Truong et al., 2010)
<i>S. epidermidis</i>	Ti with four modified different roughness	(Wu et al., 2011)

Table 1. *In vitro* bacterial adhesion studies on Ti modified implant surfaces.

### 2.1 Amorphous carbon films

It has been shown that all the different forms of amorphous carbon can be considered as biocompatible (Das et al., 2007, Hauert, 2003, Lettington, 1998, Du et al., 1998) and might be

adequate as a surface modification for biomedical applications, such as, dental and orthopaedic implants (Santavirta, 2003, Affatato et al., 2000). 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 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. (Robertson, 2002).

### 2.1.1 Biocompatibility

Biomaterials must satisfy certain criteria in order to be used as implants or medical devices. The most important requirement for biomaterials is that they need to be biocompatible. Several definitions of biocompatibility have been established, but in general, biocompatibility can be defined as the ability of the material, intentionally in contact or implanted into the body tissues, to perform as designed without inducing any local effect in the cells or tissue or a systemic response that elicits an immunological reaction. In addition, the biomaterial should not cause denaturalization of the proteins adsorbed on the surface or leach any substance that can induce toxicity to the cells or tissues adjacent to the implant.

In dental and orthopaedic implants, *de novo* bone formation in direct contact with the bone, also known as osseointegration, is desired. In order to achieve successful osseointegration, progenitor cells and osteoblasts must attach to the implant surface, differentiate into mature osteoblasts, produce an organized extracellular matrix, and finally mineralize the extracellular matrix. Several biomaterials have been applied with a broad range of success in orthopaedic and dental implants. Metallic biomaterials generally have been used for dental and orthopaedic application due to their mechanical properties. Several publications have addressed *in vitro*, *in vivo*, and clinically that metallic implant surface modifications improve osseointegration, increase bone to implant contact, decrease healing time, and are clinically successful (Schwarz et al., 2010, Stanford, Li et al., 2010, Dohan Ehrenfest et al., 2010, Karabuda et al., 2010, Schatzle et al., 2009).

During the last years, we have been investigating different aspects of the interaction between human cells (osteoblasts) and graphite-like carbon films (GLC) as possible candidates for coating dental implants. Contrary to many other research groups, we choose GLC instead of DLC films 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 (Rodil et al., 2003, Rodil et al., 2005). Graphite-like amorphous carbon films were produced by a hollow cathode DC magnetron sputtering system attached to a high vacuum chamber. We have obtained good results concerning the interaction to human osteoblasts and also good osteoconductive properties (Rodil et al., 2006, Olivares et al., 2004).

### 2.1.2 Bacterial adhesion

Nevertheless, another important factor for the success of implants (included dental implants) is to avoid formation of biofilms that might lead to implant failure or strong inflammatory process (infection). Limited studies regarding bacterial adhesion on the different carbon films have been published before (Wang et al., 2004, Ishihara et al., 2006,

Jones et al., 2006, Katsikogianni et al., 2006, Morrison et al., 2006, Kwok et al., 2007, Zhao et al., 2007, Zhou et al., 2008, Kinnari et al., 2008). 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. However, these results could not be extrapolated to the GLC films due to the strong differences between DLC and GLC physical properties, which are known to affect the bacterial adhesion. Moreover, none of these studies include oral bacteria and in any case no more than three bacterial strains were used. We have developed a biofilm model using nine selected species representative of all the complexes of the subgingival dental plaque, described by Socransky et al. (Socransky et al., 1998) (Table 2). We used *A. israelii* and *S. sanguinis* as early colonizers. A second group of bacteria included *F. nucleatum*, *C. rectus*, *E. corrodens*, *P. micra* and *P. intermedia*, these species are known because functions as a bridge between the early and late colonizers. *P. gingivalis* was used as a representative of the third group of species that appears at late stages of biofilm development and *A. actinomycetemcomitans* was used due to the role that seems to have in periodontal infections (Wilson and Henderson, 1995). All strains were grown under anaerobic conditions (80% N<sub>2</sub>, 10% CO<sub>2</sub> and 10% H<sub>2</sub>).

In a first study, the bacterial adhesion of these microorganisms was evaluated on amorphous carbon (a-C) films in comparison to titanium (Ti) and stainless steel (SS) control surfaces. The results showed that the oral bacterial adhesion on these GLC films was relative high in comparison to standard surfaces (Ti and SS) (Almaguer-Flores et al., 2009).

In a second experiment, (Almaguer-Flores et al., 2010), the influence of the surface roughness and culture media was investigated comparing carbon and titanium films. The surface roughness was modified by deposition of films on both rough stainless steel and silicon substrates, the roughness of the stainless steel was significantly larger than the silicon ( $1.89 \pm 0.5 \mu\text{m}$  and  $0.028 \pm 0.003 \mu\text{m}$ , respectively) therefore two different roughness were compared. In addition, the study was done comparing two different media; *Mycoplasma media* (MM), which is an standard bacterial culture media, and sterilized human saliva (HS) because is the major bulk fluid in the oral cavity.

Specie	ATCC*
<i>Aggregatibacter actinomycetemcomitans</i> serotipe b	43718
<i>Actinomyces israelii</i>	12102
<i>Campylobacter rectus</i>	33238
<i>Eikenella corrodens</i>	23834
<i>Fusobacterium nucleatum</i> subsp. <i>nucleatum</i>	25586
<i>Paroimonas micra</i>	33270
<i>Porphyromonas gingivalis</i>	33277
<i>Prevotella intermedia</i>	25611
<i>Streptococcus sanguinis</i>	10556

\* American Type Culture Collection, Rockville, MD

Table 2. Reference strains employed for the adhesion and biofilm formation assays.

AFM images of the samples presented in Figure 1 (a,b,c and d), 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. While for the rough surfaces, the topography is like a series of non-homogeneous hills and valleys, reaching heights in the micrometer scale.

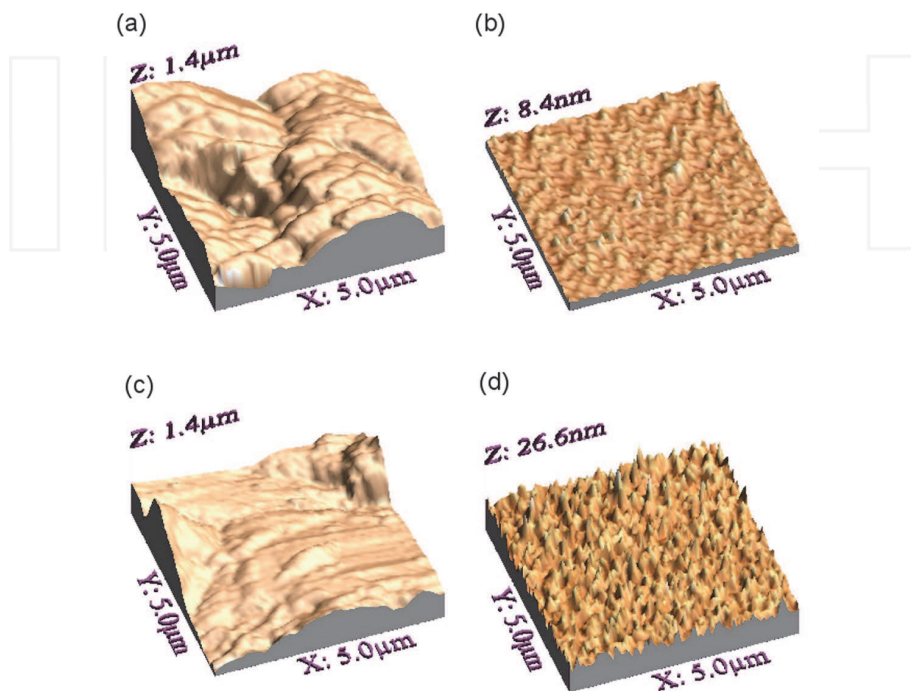
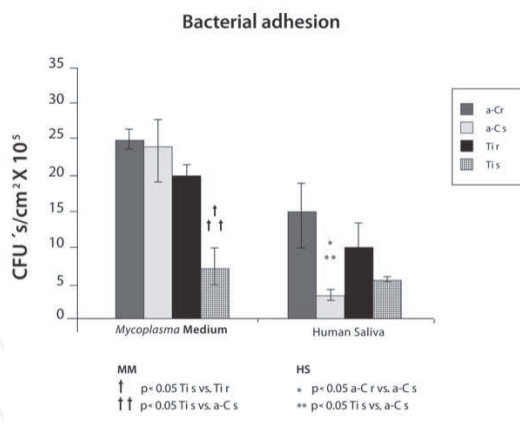


Fig. 1. AFM images of the test substrates. Vertical scale has been normalized, Z value indicate maximum height in each film. (a) a-C film deposited on the stainless steel sandblasted substrate (a-C r). (b) a-C film deposited on silicon substrate (a-C s). (c) Ti film deposited on the stainless steel sandblasted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

Bacterial adhesion on the test samples varied depending on the media used, the surface roughness and the surface chemistry, data are presented in Figure 2A as the number of CFUs/cm<sup>2</sup> × 10<sup>5</sup>. There were consistently more bacteria on the rough surfaces and in the surfaces cultivated with *Mycoplasma* media. 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 human saliva was used, lower bacterial counts were detected on all surfaces compared to the *Mycoplasma* media. 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 ( $p < 0.05$ ).



A

Fig. 2A. Bacterial adhesion (CFU's x 10<sup>5</sup>) on rough (r) and smooth (s) a-C and Ti films, after 24h of anaerobic incubation with *Mycoplasma* medium (MM) or Human Saliva (HS).

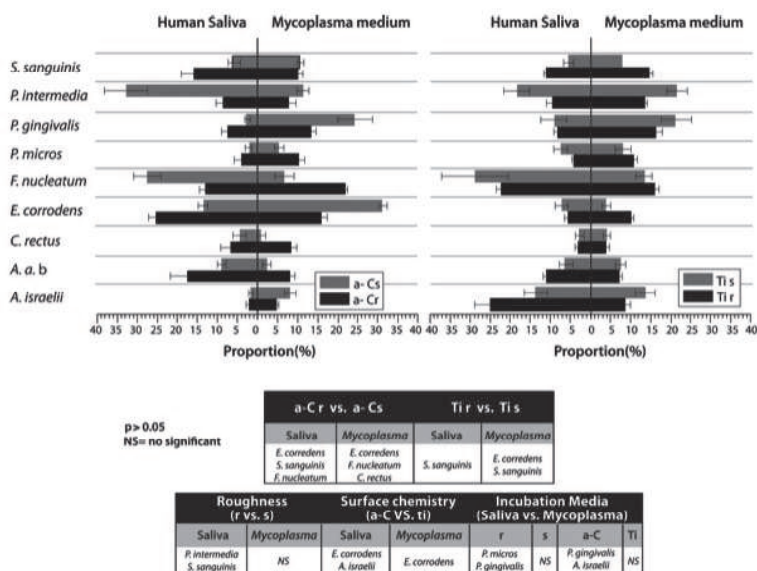


Fig. 2B. Proportion of the nine bacterial strains in the biofilms formed on rough (r) and smooth (s) a-C and Ti films, after 24h of anaerobic incubation with *Mycoplasma* medium (MM) or Human Saliva (HS). Table at the bottom shows the statistical analysis divided according to the factors that influence 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.

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 (Quirynen et al., 1996). The proportions of the bacterial strains were also affected by the surface roughness and this effect seems to be more pronounced when saliva was used. The proportion of *S. sanguinis* was significantly higher ( $p < 0.05$ ) on rough surfaces for both a-C and Ti, i.e. independently of the surface chemistry. Meanwhile, *P. intermedia* showed a higher proportion on the smooth surfaces ( $p < 0.05$ ). Nevertheless, other studies have suggested that regarding to bacterial adhesion or initial biofilm formation; roughness appears to be a minor factor (Bos et al., 1999).

Regarding the surface chemistry, higher numbers of attached bacteria (CFU's) were detected on amorphous carbon than on the Ti samples, confirming the results from the initial study that indicate large affinity of oral bacteria for the carbon surface (Almaguer-Flores et al., 2009). These results differ with other published papers that have reported that carbon-based films can inhibit bacterial adhesion (Wang et al., 2004, Liu et al., 2008, Zhou et al., 2008). In addition, it has been suggested that Ti has some antibacterial properties explained due to the formation of peroxides at the surface (Jeyachandran et al., 2007). 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<sub>2</sub>, (Grossner-Schreiber et al., 2001).

An interesting finding was the proportion of *E. 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 MM or HS, suggesting 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 (Grossner-Schreiber et al., 2004). *E. corrodens* possesses a specific lectin-like substance that mediates its adherence to various host tissue cell surfaces (Yamazaki et al., 1988), 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.

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 lysozyme, lactoferrin, lactoperoxidase, and secretory IgA (Tenovuo, 1998). Meanwhile, the *Mycoplasma* media contains only nutrients and some proteins. The saliva is a more biologically significant media for the bacterial adhesion test and many studies indicate that the saliva is critical for the colonization of certain taxa (Gibbons, 1996, De Jong and Van der Hoeven, 1987), and it is determinant for the type and amount of bacteria that will attach on a surface (Gibbons, 1996, Sela et al., 2007). However, human saliva is a very complex and non-homogeneous media in comparison to the MM, and actually changes in the composition can be found from donor to donor. So, in order to study the surface-bacteria interactions, a more homogeneous media could be more convenient.

## 2.2 Silver – amorphous carbon films (a-C:Ag)

Searching for reducing implant infections different modifications have been proposed such as functionalization of the surface with bactericidal polycationic groups (Tiller et al., 2001, Cen et al., 2004), developing delivery systems to coating the surface with polymers loaded with antibiotic or antimicrobial substances (Shi et al., 2006, Schmidmaier et al., 2006) or covering the implant surface with quaternary ammonium compounds or silver and iodine ions (Yorganci et al., 2002, Nohr and Macdonald, 1994, Tyagi and Singh, 1997, Ewald et al., 2006).

Silver antimicrobial properties have been recognized since historic times (Klasen, 2000, Burrell, 2003), and the coating of medical devices with silver coatings (Ewald et al., 2006,



Bosetti et al., 2002, Darouiche, 1999, Schierholz et al., 1998) or the addition of silver nanoparticles (Chen et al., 2006, Kwok et al., 2007, Jung et al., 2009, Rai et al., 2009) into the material's surface might be a good method to prevent device-associated infections by physical routes instead of the chemical routes mentioned above. Silver exhibits a rather broad-spectrum antimicrobial activity *in vitro* by binding both to microbial DNA, preventing bacterial replication, and to the sulfhydryl groups of the metabolic enzymes of the bacterial electron transport chain, causing their inactivation (Darouiche, 1999). Considering the well-known silver antibacterial activity, amorphous carbon films with silver nanoparticles inclusions were produced and the biocompatibility and antibacterial properties of such films was evaluated. Details concerning the deposition conditions and properties of the films can be found somewhere else (Garcia-Zarco et al., 2009).

### 2.2.1 Biocompatibility

Several assays are used to test biocompatibility; however, due to the scope of this chapter focus on common assays that we perform to test the biocompatibility of our surface modifications.

To test biocompatibility of a-C:Ag surfaces, we perform the following assays.

#### *MTT Assay*

The MTT is a colorimetric assay that measures the reduction of a tetrazolium component (MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into a insoluble purple formazan product by the cell mitochondria. This reaction only occurs in viable cells, which have metabolic activity are capable to reduce the MTT. This assay is commonly used to determine the cytotoxicity of potential medical agents and biomaterials since released molecules can cause metabolic dysfunction that decreases or abolishes MTT reduction in the mitochondria and result in cell toxicity (Mosmann, 1983, Denizot and Lang, 1986). Thus, MTT reduction is proportional to cellular metabolic activity, and decreased MTT reduction infers a possible toxicity of the biomaterial or drug tested (Chen et al., 2011, Niu et al., 2011, Sahithi et al., 2010, Bispo et al., 2010).

#### *Cell culture*

Human MG63 osteoblast-like cells are commonly used in testing metallic biomaterials. MG63 cells present an immature osteoblast phenotype, which gives them the potential to be studied as a model of osteoblastic differentiation (Bachle and Kohal, 2004) (Schwartz et al., 1999). MG63 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM, cellgro®, Manassas, VA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA) and 1% penicillin-streptomycin (Gibco) at 37°C in 5% CO<sub>2</sub> and 100% humidity.

#### *Cell viability*

MG63 cells were plated at a density of 10,000 cells/cm<sup>2</sup> on tissue culture polystyrene (TCPS) or substrates coated with amorphous carbon (a-C) or amorphous carbon/silver (a-C:Ag). Cell viability was measured using Methylthiazolyldiphenyl-tetrazolium bromide (MTT) dye after 1, 3, or 7 days in culture. MTT dye was dissolved in water to yield a 5 mg/mL solution. MTT dye was then added to culture media of each well to a final concentration of 1 mg/mL and incubated for 4 hours. The media was then removed, the monolayer rinsed twice with PBS, and formazan crystals dissolved in 500 µL DMSO. 200 µL of the resulting solution was

aliquoted into a 96 well microplate and read in an absorbance plate reader using a test wavelength of 570 nm and a reference wavelength of 630 nm.

Viability of MG63 cells cultured on a-C or a-C:Ag thin films was measured using MTT. One day after plating, cells cultured on both a-C and a-C:Ag had similar MTT activity to cells cultured on TCPS (Fig. 4). Viability at day 3 and day 7 was also similar in cells cultured on thin films than cells on TCPS. These results indicate that thin film coatings analyzed in this experiment did not affect cellular metabolism, indicating that they are not cytotoxic.

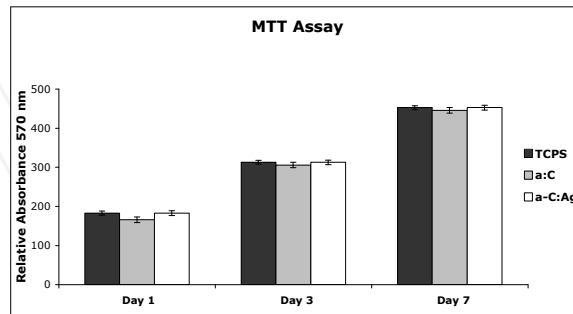


Fig. 4. Effect of thin films on MG63 cell viability. MG63 cells were cultured on tissue culture polystyrene (TCPS) or amorphous carbon (a-C) or amorphous carbon-silver (a-C:Ag) thin films. MTT activity was measured after 1 day, 3 days, or 7 days in culture. No significant differences were detected between the surfaces.

#### *Osteoblast phenotype*

MG63 cells were plated at a density of 10,000 cells/cm<sup>2</sup> on tissue culture polystyrene (TCPS) or substrates coated with amorphous carbon (a-C) or amorphous carbon/silver (a-C:Ag). At confluence, cells were incubated with fresh media for 24h. Conditioned media was harvested and levels of secreted osteocalcin measured using a commercially available radioimmunoassay (Biomedical Technologies, Inc., Stoughton, MA). Cells were detached from the surface using two sequential incubations in 0.25% trypsin-EDTA and cell number determined using a Z2 Particle Counter (Beckman Coulter, Hiialeah, FL). Cells were lysed in 0.05% Triton X-100 and homogenized by sonicating each sample for 10 s. Alkaline phosphatase specific activity was measured in cell lysates as a function of the release of p-nitrophenol from p-nitrophenylphosphate at pH 10.2 (Martin et al., 1996, Bretauiere, 1984) and normalized to the total protein concentration (BCA Protein Assay, Pierce Chemical Co., Rockford, IL).

Whether surface modifications enhance osteoblast maturation can be assessed using three specific outcomes: cell number, alkaline phosphatase specific activity, and osteocalcin levels. Cells attach to biomaterials through the proteins adsorbed in the surface of the material. After this event, cell undergo proliferation and extracellular matrix production. It has been demonstrated that proliferation is reduced when cells undergo differentiation (Stein et al., 1990). In our experiment, cell number was lower on a-C and a-C:Ag thin films than on TCPS (Fig. 5A). Alkaline phosphatase specific activity, an early marker of osteoblast differentiation, is commonly used as a marker of bone formation. Alkaline phosphatase is an enzyme that acts on the phosphate groups of various molecules and generates a microenvironment rich in phosphate ions, which, in concert with calcium, mineralize the extracellular matrix to form bone. In osteoblasts, alkaline phosphatase increases when

proliferation is inhibited during differentiation. Our results show that alkaline phosphatase was higher in cells grown on a-C and a-C:Ag than on TCPS (Fig. 5B). However, alkaline phosphatase specific activity increases in early differentiation, reaches a maximum, and begins to decrease as mineralization is initiated (Stein et al., 1990). To establish the specific stage of osteoblast maturation, osteocalcin was measured in the conditioned media. Osteocalcin is considered a later marker of osteoblast maturation, is present in all mineralized tissues in our body, and increases in relation to total mineralization during bone formation. In our experiments, cells cultured on a-C and a-C:Ag secreted more osteocalcin than cells on TCPS (Fig. 5C). The combination of these factors allows us to gauge more precisely the stage of osteoblast maturation, and the ability of surface modifications to enhance this, than any one factor alone. Taken together, our results establish that both a-C and a-C:Ag are not toxic, and promote osteoblast maturation increasing two main factors needed for bone formation, alkaline phosphatase activity and osteocalcin.

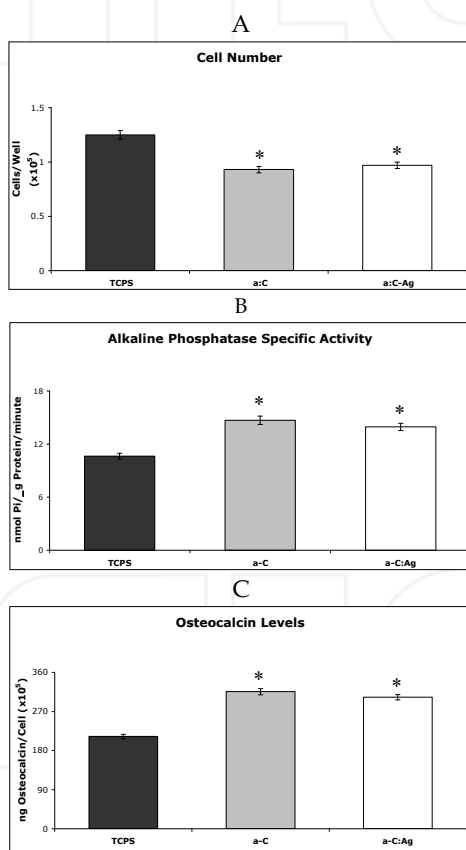


Fig. 5. Osteoblast phenotype in response to culture on thin films. MG63 cells were cultured on tissue culture polystyrene (TCPS) or amorphous carbon (a-C) or amorphous carbon-silver (a-C:Ag) thin films. At confluence, cell number (A), alkaline phosphatase specific activity in cell lysates (B), and secreted osteocalcin (C) were measured. \* $p < 0.05$ , vs. TCPS.

### 2.2.2 Antibacterial effect

The oral bacterial adhesion and the initial biofilm formation on the amorphous carbon films modified with the Ag nanoparticles, was evaluated using our standard protocol for the oral bacteria. All surfaces (Table 3) were incubated for 24 hours, 3 and 7 days with a mixture of the nine bacterial strains. One set of surfaces was used for determining the total counts of bacteria attached to each surface, by counting the colony forming units (CFU's) from each sample. In order to observe biofilm morphology and the surface coverage by bacteria, an additional set of samples was prepared for Scanning Electron Microscopy (SEM) following standard procedures.

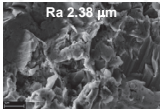

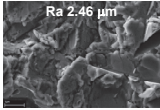
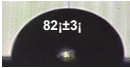
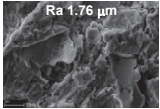

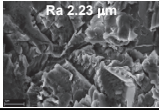

Surface	SEM image (scale 5 $\mu$ m) and Ra profilometer (Scan 5 mm)	Film thickness	Contact angle
Stainless steel medical grade 316L sandblasted substrate		NA	
a-C film stainless steel medical grade 316L sandblasted substrate		53.8 nm	
a-C:Ag film stainless steel medical grade 316L sandblasted substrate		82.5 nm	
Ti film on stainless steel medical grade 316L sandblasted substrate		190 nm	

Table 3. Surface characterization.

The morphology of the four surfaces was very similar even after the film deposition. This was also confirmed by measuring the roughness before and after deposition, which remained close to 2  $\mu$ m. The combined effect of the roughness value and the chemical composition lead to water contact angles, which cannot be directly related to the surface energy, but reflects the wettability of the surfaces. The more hydrophobic surface was the silver modified carbon film. The silver atomic percentage in these samples was around 6 at% and the average particle size as calculated using the Image J software (Collins, 2007) was 63.5 nm corresponding to ~10% of the surface area.

Figure 6 shows the BE images of the bacteria colonies as a function of the incubation time and for the four surfaces, using low magnification in order to observe the bacterial distribution among a large area of the surfaces (1.76 mm<sup>2</sup>). At the first day, mainly isolated attached bacteria were observed, as time went on, the formation of the biofilm was clearly observed and represents the large dark areas in the image, where the thick glycoprotein matrix was developing.

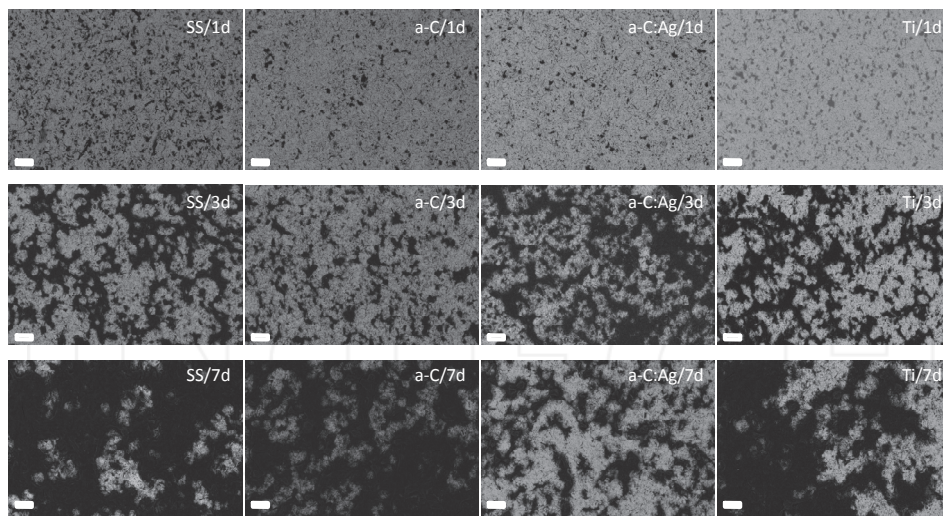


Fig. 6. Backscattering SEM images of the biofilms formed on the SS, a-C, a-C:Ag and Ti surfaces after 1, 3 and 7 days of anaerobic incubation. The scale bar corresponds to 100  $\mu$ m and the magnification used was 250X.

The increment in the percentage of the surface covered by bacteria (or bacteria surface growth, BSG) as a function of time is clearly observed in figure 7, which also included the statistical analysis. Six different zones were analyzed and the area quantification was done at least three times for each zone, therefore it is possible to describe statistically the variations in the BSG. At the first day, less than 10 % of the total area was covered; actually, the bacteria were observed forming small groups (figure 6). However, at this time, a large amount of bacteria were found in the a-C:Ag films and less number was observed on the metallic surfaces. At three days, the larger number was found on the a-C films, while the other surfaces present similar coverage (~20 %). At 7 days, the amount of bacteria was highly reduced in the a-C:Ag films compared to the other surfaces.

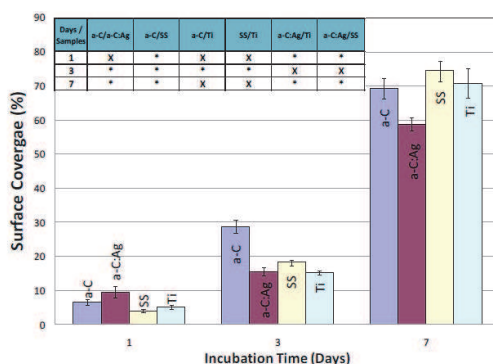


Fig. 7. Surface coverage estimated as the percentage of area covered by bacteria in the images shown in figure 6, using the ImageJ particle analyzer function. Statistically significant data are included in the table (\*  $p < 0.05$ ).

The reduction in surface coverage obtained for the a-C:Ag films after 7 days of incubation was significant lower than in the other surfaces. This is in agreement with the antimicrobial mechanism of the silver, where the active agents are really silver ions or radicals that in our samples will be produced via an erosion/corrosion process, taking place as the sample is immersed in the medium.

The addition of silver nanoparticles into the amorphous carbon matrix reduced the bacterial surface growth approximately 10% in comparison to the pure carbon matrix. The results indicated that the action of silver ions occurs in time, therefore not immediate response was observed for the bacterial adhesion.

### 3. Conclusions

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 any surface. When bacterial adhesion was tested using individual species, the adhesion profiles varied on the same surface depending of the bacterial strain.

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, moreover when using human saliva, significantly reduced levels of bacteria were found on the a-C smooth surfaces.

In summary, it seems that Graphite-like amorphous carbon is not a suitable surface to prevent adhesion from the oral media but a-C:Ag films seems to inhibit bacteria adhesion after seven days of incubation. However, a-C films seem to be good to repel bacteria from certain oral strains, such as, *A. israelii*, *P. gingivalis* and *P. intermedia*. Although *E. corrodens* was capable to colonize in very high rates the a-C surfaces despite of their roughness or the culture media used.

Therefore, 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 use.

Further studies are required in order to evaluate other physical, chemical and biological properties of the a-C:Ag films in order to understand the observed differences and also to analyze the sequential formation of a bacterial biofilm over these and other implant surfaces.

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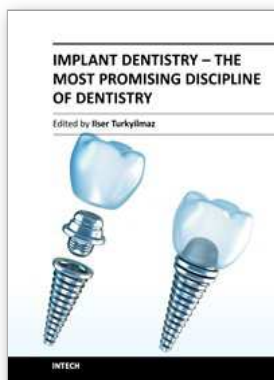


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## **Implant Dentistry - The Most Promising Discipline of Dentistry**

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Since Dr. Branemark presented the osseointegration concept with dental implants, implant dentistry has changed and improved dramatically. The use of dental implants has skyrocketed in the past thirty years. As the benefits of therapy became apparent, implant treatment earned a widespread acceptance. The need for dental implants has resulted in a rapid expansion of the market worldwide. To date, general dentists and a variety of specialists offer implants as a solution to partial and complete edentulism. Implant dentistry continues to advance with the development of new surgical and prosthodontic techniques. The purpose of *Implant Dentistry - The Most Promising Discipline of Dentistry* is to present a contemporary resource for dentists who want to replace missing teeth with dental implants. It is a text that integrates common threads among basic science, clinical experience and future concepts. This book consists of twenty-one chapters divided into four sections.

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