



Biomaterialized diamond-like carbon films with incorporated titanium dioxide nanoparticles improved bioactivity properties and reduced biofilm formation

F.S. Lopes^{a,b}, J.R. Oliveira^c, J. Milani^b, L.D. Oliveira^c, J.P.B. Machado^d, V.J. Trava-Airoldi^d, A.O. Lobo^{a,b,e,f,g}, F.R. Marciano^{a,b,e,f,*}

^a Laboratory of Biomedical Nanotechnology, Universidade Brasil, 08230-030 Itaquera, São Paulo, Brazil

^b Laboratory of Biomedical Nanotechnology, Universidade do Vale do Paraíba, São José dos Campos, 12244-000, São Paulo, Brazil

^c Department of Biosciences and Oral Diagnosis, Institute of Science and Technology, UNESP-Univ Estadual Paulista, São José dos Campos, SP, Brazil

^d Associated Laboratory of Sensors and Materials, National Institute for Space Research, São José dos Campos, SP, Brazil

^e Biomaterials Innovation Research Center, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Cambridge, MA 02139, USA

^f Nanomedicine Lab, Department of Chemical Engineering, Northeastern University, Boston, MA 02115, USA

^g Interdisciplinary Laboratory for Advanced Materials, PPGCM, Technology Center, Federal University of Piauí, 64049-550 Teresina, PI, Brazil

ARTICLE INFO

Keywords:

Diamond-like carbon
Titanium dioxide
Nanoparticles
Biomaterialization
Hydroxyapatite
Antibacterial activity

ABSTRACT

Recently, the development of coatings to protect biomedical alloys from oxidation, passivation and to reduce the ability for a bacterial biofilm to form after implantation has emerged. Diamond-like carbon films are commonly used for implanted medical due to their physical and chemical characteristics, showing good interactions with the biological environment. However, these properties can be significantly improved when titanium dioxide nanoparticles are included, especially to enhance the bactericidal properties of the films. So far, the deposition of hydroxyapatite on the film surface has been studied in order to improve biocompatibility and bioactive behavior. Herein, we developed a new route to obtain a homogeneous and crystalline apatite coating on diamond-like carbon films grown on 304 biomedical stainless steel and evaluated its antibacterial effect. For this purpose, films containing two different concentrations of titanium dioxide (0.1 and 0.3 g/L) were obtained by chemical vapor deposition. To obtain the apatite layer, the samples were soaked in simulated body fluid solution for up to 21 days. The antibacterial activity of the films was evaluated by bacterial eradication tests using *Staphylococcus aureus* biofilm. Scanning electron microscopy, X-ray diffraction, Raman scattering spectroscopy, and goniometry showed that homogeneous, crystalline, and hydrophilic apatite films were formed independently of the titanium dioxide concentration. Interestingly, the diamond-like films containing titanium dioxide and hydroxyapatite reduced the biofilm formation compared to controls. A synergism between hydroxyapatite and titanium dioxide that provided an antimicrobial effect against opportunistic pathogens was clearly observed.

1. Introduction

Currently, alternatives are being developed to improve the biological properties of biomedical metal alloys and to reduce the need to administer antibiotics during and after their implantation by surgical procedures. It's known that metal alloys have been used for many years as substitutes for bones and/or to promote bone regeneration after fracture. To solve this, the use of stainless steel for biomedical applications is cheaper and has been extensively used [1–3]. However, several problems have been encountered when stainless steel is implanted at human body [4,5]. To solve these problems, several alternatives have been developed to modify the surface of metal alloys to

improve their biocompatibility [6,7]. Contributing to this, the deposition of thin films can be interesting due to the feasibility and reproducibility of their direct deposition on metal alloys.

Diamond-like carbon (DLC) coatings can impart wear resistance, hardness, and corrosion resistance to the surface of a medical device [8–11]. DLC films are mostly obtained by plasma decomposition of a hydrocarbon-rich atmosphere using chemical vapor deposition [8–11]. Commonly, when DLC films are obtained by methane decomposition, a typical sp²-hybridized cluster interconnected with sp³-hybridized carbon atoms is produced. Then, the mechanical properties (e.g. hardness, Young's modulus, adhesion to the substrate, internal stresses) as well as important electronic properties (e.g. optical gap,

* Corresponding author at: Laboratory of Biomedical Nanotechnology, Universidade Brasil, 08230-030 Itaquera, São Paulo, Brazil.
E-mail address: frmarciano@pq.cnpq.br (F.R. Marciano).

photoluminescence, and conduction behavior) may be pre-determined to a certain extent by varying the sp^3/sp^2 bonding ratio [8–11]. These coatings consist of dense amorphous carbon or hydrocarbon and their mechanical properties fall between those of graphite and diamond [8,9]. However, the addition of different elements to the films can easily tune their surface chemical behavior [12].

Titanium is a reactive metal that in air, water, or any electrolyte spontaneously forms a thin native oxide film, which is responsible for the biocompatibility of the titanium [13]. This oxide layer is responsible for the bone-bonding characteristics of titanium implants [14]. Its photo-semiconductor properties enable TiO_2 use as an antibacterial agent for decomposition of organisms [15–18]. However, these properties are strongly dependent on the crystalline structure, morphology, and crystallite size [15].

Many alternatives have been developed to obtain nanoparticles, nanocarriers and thin films for biological applications. In this context, hybrid materials and inorganic materials are upcoming materials for delivery, therapy, sensors, and so on [19–23]. However, the application of TiO_2 -DLC films has become of interest and was already reported [24]. Antibacterial tests against *Escherichia coli* showed an increase of DLC bactericidal activity when the amount of TiO_2 was increased. Thorwarth et al. [25] showed that a-C:H layers deposited on TiAl6V4 promoted cell proliferation and differentiation. Amin et al. [26] showed that TiO_2 -DLC film presented biomimetic properties. However, the antibacterial properties of biomineralized- TiO_2 -DLC coatings have not yet been studied.

Herein, for the first time, we obtained a new thin film using a simple approach to produce biomineralized- TiO_2 -DLC and its bactericidal properties were also further explored. Interestingly, the apatite- TiO_2 -DLC films avoided and reduced biofilm formation by a very common pathogenic agent in a hospital environment. Our alternatives open perspectives for the application of such developed films to improve bone regeneration and to control biofilm formation, allowing the administration of antibiotics to be avoided.

2. Materials and methods

2.1. Deposition and characterization of biomineralized diamond-like carbon films containing titanium dioxide nanoparticles and hydroxyapatite

Discs of 304 stainless steel (diameter of 6 mm and thickness of 1 mm) were used as substrates. DLC and TiO_2 -DLC films (0.1 and 0.3 g/L) were produced using plasma-enhanced chemical vapor deposition as already reported [24].

The structural characteristics were analyzed by Raman spectroscopy (Renishaw 2000 system with Ar^+ -ions, $\lambda = 514$ nm, spot-size = 2.5 μ m, and power = ~ 0.6 mW) with backscattering geometry.

To obtain apatite formation, the different samples were soaked in simulated body fluid (SBF, 5 $\times$, Table 1) [27]. The pH of the solution was adjusted to 6.10 using HCl (0.1% v/v) and the media was changed every three days. After the biomineralization period, samples were removed, washed in hot deionized water, dried in an oven at 50 $^\circ$ C for 24 h, and sterilized by autoclave (121 $^\circ$ C/15 min).

Scanning electron microscopy (SEM, EVO MA 10, Zeiss) was used to analyze the apatite produced. Energy dispersive X-ray spectroscopy

(EDX) (Inca Penta FET $\times 3$, Oxford Instruments) was used to identify the Ca and P elements after biomineralization. The structural analysis of biological apatites on stainless steel, DLC, and TiO_2 -DLC films was performed at room temperature by X-ray diffractometry (XRD) using an X-Pert Philips instrument with Cu $K\alpha$ radiation ($\lambda = 0.154056$ nm) with a 2θ angle of 10 $^\circ$ to 50 $^\circ$ under the following conditions: voltage of 40 kV, current of 30 mA, step size of 0.02 $^\circ$, and counting time of 2 s per step. The diffraction peaks were indexed according to the Joint Committee on Powder Diffraction Standards (JCPDS) using X'pert HighScore software (www.panalytical.com).

The sessile drop method (Kruss EasyDrop DSA 100) was used to measure the contact angle (θ). Two different liquids (distilled water and diiodomethane) were used for surface energy calculations, following the Owens method [28]. The liquid was dropped automatically by a computer-controlled system. These values were used to calculate the thermodynamic work of adhesion (W_{Ad}) using the standard Young-Dupré relationship [29,30]. All measurements were carried out at room temperature.

2.2. Bactericidal evaluation of biomineralized diamond-like carbon films containing titanium dioxide nanoparticles and hydroxyapatite

Standard strains of *Staphylococcus aureus* (ATCC 6538) were used to evaluate the antibacterial activity of the biofilm. HAp/stainless steel, HAp/DLC, HAp/ TiO_2 -DLC (0.1 g/L), and HAp/ TiO_2 -DLC (0.3 g/L) were used. Firstly, *S. aureus* was cultured in Brain Heart Infusion agar (BHI, Himedia, Mumbai, India) for 24 h at 37 $^\circ$ C. Subsequently, the suspension was centrifuged (2000 rpm/10 min) and the pellet was suspended in sterile saline solution (0.9% NaCl). Then, the turbidity of the solution was adjusted with a spectrophotometer to obtain a concentration of 10⁶ CFU/mL (colony forming units per milliliter). Next, we added 1000 μ L of BHI, different samples ($n = 5$), and 100 μ L of the standardized suspension of *S. aureus* (using a 24-well plate, 37 $^\circ$ C) and incubated for 24 h. The medium was changed after 24 h. Thus, the biofilm formed on the discs was disaggregated by ultrasonic homogenizer (Sonoplus HD 2200, 50 W, Bandelin Electronic, Berlin, Germany) for 30 s at 25% power. Then, the discs were transferred to plastic tubes containing 10 mL of sterile saline solution and after disaggregation of the biofilm, the microbial suspension was diluted (1:10) and 100 μ L of each dilution was seeded on BHI agar in duplicate. After 48 h of incubation, the CFUs were counted and the values were converted to log₁₀. The statistical differences were analyzed by one-way ANOVA (Graph Pad PRISM 6[®]). The bacterial populations on stainless steel, DLC, and TiO_2 -DLC films after biomineralization were obtained with a normal distribution and independently to each experiment. *P*-values of < 0.05 were considered to indicate statistical differences.

3. Results and discussion

Raman spectra were composed of two broad bands, centered at approximately ~ 1330 cm^{-1} (*D* band) and ~ 1550 cm^{-1} (*G* band), as already reported by Robertson and Ferrari [31]. The *D* and *G* band positions were determined by subtracting a linear background and fitting a Gaussian function to the peak of the Raman spectrum (Fig. 1). Table 2 shows the main parameters obtained through the spectra. The *G* band is due to the bond stretching of all pairs of sp^2 atoms in both rings and chains. The *D* band is assigned to breathing modes of sp^2 atoms in rings and appears only in the presence of defects. The TiO_2 -DLC films presented a shift in the *D* and *G* band positions toward higher wave-numbers due to the presence of defects. Using the full width at half maximum (FWHM) of the *G* band, it was possible to determine the structural disorder that arises from bond length distortions. The FWHM is small irrespective of whether the clusters are defect free, unstrained, or “molecular”. For a given cluster size, a higher bond length and bond angle disorder lead to a higher FWHM (*G*). This implies that the FWHM (*G*) is mainly a probe of structural disorder. Higher excitation energies

Table 1
Quantity of reagents used to prepare SBF.

Reagents	Concentration (g/L)
NaCl	40.0
MgCl ₂	1.52
CaCl ₂ ·2H ₂ O	1.84
Na ₂ HPO ₄ ·2H ₂ O	0.89
NaHCO ₃	1.76

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