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# Cell viability and adhesion on diamond-like carbon films containing titanium dioxide nanoparticles

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ABSTRACT

The combination of low friction, wear resistance, high hardness, biocompatibility and chemical inertness makes diamond-like carbon (DLC) films suitable in a numerous applications in biomedical engineering. The cell viability and adhesion of L929 mouse fibroblasts was investigated using two different colorimetric assays: (i) 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide (MTT), and (ii) lactate dehydrogenase (LDH). The films were growth on 316L stainless steel substrates using plasma enhanced chemical vapor deposition technique from a dispersion of $TiO_2$ nanopowder in hexane. The increasing concentration of $TiO_2$ nanoparticles in DLC films enhanced the mitochondrial activity and decreases the LDH activity on these samples. Fluorescence and scanning electron microscopy corroborate the results. These experiments show the potential use of DLC and $TiO_2$-DLC films in biomedical applications.
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## 1. Introduction

Surfaces play a vital role in biology and medicine with most biological reactions occurring at surfaces and interfaces [1]. The successful incorporation of an implant into the body depends on tissue integration and infection resistance, which is influenced by the adherence of autologous cells and bacteria to the surfaces [1,2]. Cell adhesion and spreading is fundamentally essential for biomaterials that are frequently used in biomedical devices [3]. In most cases, a surface modification of these biomaterials is considered to be a prerequisite for improving biocompatibility, since this kind of material should also be hard, wear resistant, with a low friction coefficient and corrosion resistant for certain applications [4].

Diamond-like carbon (DLC) coatings have been actively studied over the last decade in the field of material engineering. Consisting of dense amorphous carbon or hydrocarbon, DLC mechanical properties fall between those of graphite and diamond [5–8]. These coatings can also impart wear resistance, hardness, and corrosion resistance to a medical device surface, and have been considered for using in a variety of cardiovascular, orthopedic, biosensor, and implantable microelectromechanical system devices [6,9,10]. Recent studies have reported modified-DLC films improved biocompatibility, lubricity, stability and cell adhesion [11–14]. Nanoparticle-dispersed composite films are expected to have the potential of changing their performances according to the individual properties of nanoparticles [15]. According to Yun et al. (2008) [8], these characteristics are related to structural bonds [16,17], surface roughness [18,19] and whether the film is hydrophobic or hydrophilic [20,21].

Titanium is a reactive metal that forms, spontaneously, in the air, water or any other electrolyte, a thin native oxide film, which is responsible for titanium biocompatibility [22]. This oxide layer is responsible for the bone-bonding characteristics of titanium implants [23]. Titanium dioxide ($TiO_2$) has been widely studied as regards various electronic applications, utilizing the photocatalytic nature and transparent conductivity, which strongly depend on the crystalline structure, morphology and crystallite size [24]. Due to $TiO_2$ photo-semiconductor properties, it may find an application as antibacterial agent for the decomposition of organisms [25,26].

In the last recent years, various authors have been reported the production and characterization of $TiO_2$-DLC films for biological applications [2,15,27–29]. Our previous publication reported for the first time the production and characterization of $TiO_2$-DLC films using plasma enhanced chemical vapor deposition (PECVD) [30]. The used method permits the incorporation of $TiO_2$ nanoparticles in the film structure during the deposition process from a dispersion of $TiO_2$ in hexane. The results demonstrated these films are

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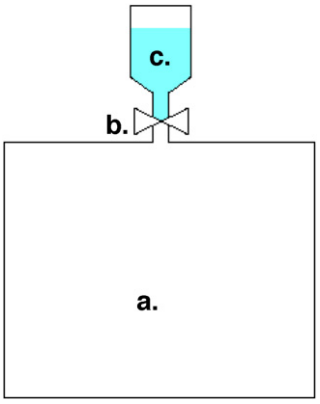


**Fig. 1.** Schematic drawing of the deposition setup: (a) is the deposition chamber; (b) is the valve and (c) is the compartment where hexane or hexane dispersion of $TiO_2$ nanoparticles were inserted into the chamber.

potential antibactericial agents with the increasing concentration of $TiO_2$ nanoparticles. After, it was investigated the cytotoxicity and cell adhesion on $TiO_2$-DLC films [31]. The cell spreading behavior was evaluated using physico-chemical properties. In the current paper, cell viability and adhesion of L929 mouse fibroblasts was investigated using two different colorimetric assays: (i) 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide (MTT), and (ii) lactate dehydrogenase (LDH). It is also compared the observation with Scanning Electron Microscopy (SEM) and Fluorescence Microscopy (FM).

## 2. Experimental procedures

The 316L stainless steel (SS) substrates ($1 \times 1\,cm^2$) were mechanically polished to a mirror-like finish surface, cleaned ultrasonically in an acetone bath for 15 min and dried in nitrogen atmosphere. The clean samples were mounted on a water-cooled, 10-cm diameter cathode powered by a pulsed directly current plasma enhanced chemical vapor deposition power supply, with variable pulse voltage from 0 to −1000 V, at a frequency of 20 kHz and duty-cycle of 50%.

Into the chamber (vacuum base pressure of 1.3 mPa) the substrates were additionally cleaned by argon discharge with 1 sccm gas flow at 11.3 Pa working pressure and a discharge voltage of −700 V for 10 min prior to deposition. In order to enhance the DLC film adhesion to metallic surfaces, a thin amorphous silicon interlayer (thickness around 200 nm) were deposited using silane as the precursor gas (1 sccm gas flow at 11.3 Pa for 12 min and a discharge voltage of −700 V) [32]. The DLC films were deposited using hexane as the feed gas to a thickness of around 2.0 μm (at 18.0 Pa for 60 min and a discharge voltage of −700 V).

In order to produce $TiO_2$-DLC films, $TiO_2$ nanoparticles (Aeroxide® $TiO_2$ P25 from Evonik), in anatase crystalline form with average particle size of 21 nm, were dispersed in hexane at 0.1 and 0.5 g/L. These dispersions replaced the pure hexane during the DLC deposition. Fig. 1 shows the schematic drawing of the deposition setup, where (a) is the deposition chamber, (b) is the valve and (c) is the compartment where hexane or hexane dispersion of $TiO_2$ nanoparticles were inserted into the chamber. In order to perform the deposition of $TiO_2$-DLC films, the valve (b) opens to hexane entering into the chamber.

The film roughness values were characterized by atomic force microscopy (AFM), VEECO Multimode V, operating in dynamic mode, with 0.01–0.025 Ω-cm Antimony ($n$) doped Si tip (model TESPW).

The contact angle ($\theta$) of the samples was measured by using the sessile drop method with a Kruss EasyDrop contact angle instrument (EasyDrop DSA 100). Two different test liquids (distilled water and diiodomethane) were used for surface energy calculations, according to the Owens method [33]. The liquid was dropped automatically by a computer-controlled system. All measurements were carried out at room temperature.

L-929 mouse fibroblasts cells were provided by Cell Line Bank at Rio de Janeiro (CR019). The cells were maintained as sub-confluent monolayer's in minimum essential medium with 1.5 mM l-glutamine adjusted to contain 2.2 g/L sodium bicarbonate 85%; fetal bovine serum 10% (Gibco, BRL), 100 units/mL penicillin–streptomycin (SIGMA), and 25 μg/mL L-ascorbic acid (SIGMA). The incubation occurred within a $CO_2$ (5%) atmosphere at 37 °C.

The cytotoxicity assay was evaluated according to ISO 10993-5 "Biological evaluation of medical devices – Test for cytotoxicity: in vitro methods" (or EN 30993-5), using direct contact. The cytotoxicity assay was evaluated by two different colorimetric assays: (i) MTT [34] and (ii) LDH [35]. Latex fragments were used as positive control [36]. Fragments of filter paper to prove the nontoxic nature were used as negative control. The dimensions of these fragments were the same of the substrates with DLC and $TiO_2$-DLC films.

All the samples (1 $cm^2$) were sterilized in humid vapor (121 °C, 1 atm) and placed in individual wells of 24-well culture plates. The cells were seeded in each well at a concentration of $5 \times 10^5$ cells/mL, supplemented with 10% fetal bovine serum (Gibco, BRL). The incubation was performed under a $CO_2$ (5%) atmosphere, at 37 °C during 24 h. After the incubation, the substrates with DLC and $TiO_2$-DLC films, and the positive and negative control fragments were removed from the respective wells. Only the cells adhered to the well walls were assayed with (i) MTT solution and (ii) LDH. The absorbance of the content of each well was measured at 570 nm (MTT) and 490 nm (LDH) with a 24-well microplate reader on a spectrophotometer Spectra Count (Packard). The blank reference was taken from wells without cells, also incubated with MTT and LDH solution. The background was taken from wells without cells. The optical density (OD) were normalized by the cell culture, and expressed in percentage: $[OD_{sample} - OD_{background}/OD_{cells} - OD_{background}] \times 100$. The experiments were carried out in quintuplicate in order to confirm the reproducibility.

In order to analyze the surface and adhesion morphology of the cells on DLC films, the cell culture ($5 \times 10^5$ cells/mL) were seeded on the samples with DLC and $TiO_2$-DLC films and incubated under a $CO_2$ (5%) atmosphere, at 37 °C during 24 h. After this, the medium was removed and the samples were fixed with a 3% glutaraldehyde (0.1 M) sodium cacodylate buffer for 1 h and dehydrated in a graded acetone solution series (50, 70, 90, 100%) for 10 min each. The drying stage used a 1:1 solution of ethanol with hexamethyldisilazane (HMDS) and the samples were dry with pure HMDS at room temperature. After deposition of a thin gold layer, the cell spreading on the samples were examined by Scanning Electron Microscopy (SEM - ZEISS EVO MA10).

The actin filaments and nucleous morphology were assayed. For this, the cell culture ($5 \times 10^5$ cells/mL) was seeded on the samples with DLC and $TiO_2$-DLC films and incubated under a $CO_2$ (5%) atmosphere, at 37 °C during 24 h. After this, the medium was removed and were rinsed with PHEM buffer ($MgCl_2$ 50 mM, KCl 70 mM, EGTA 10 mM, HEPES 20 mM, PIPES 60 mM pH 6.8) fixed with a 4% paraformaldehyde, 0.1% glutaraldehyde and 0.1% Triton X100 in 0.1 M fosfate buffer (pH 7.2) for 30 min. Then the cells were rinsed with PHEM buffer and incubated with PHEM buffer added 1% bovine serum albumin for 10 min, treated with ammonium chloride (50 mM) for 30 min and incubated with rhodamine–phalloidin (1:100-PHEM) for 60 min and rinsed again with PHEM buffer. The same material were incubated with 0.03 μM of 4,6-diamidino-2-phenylindole, dihidrocloruro (DAPI) (Molecular Probes, Eugene,

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