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Cytotoxicity evaluation of nanocrystalline diamond coatings by fibroblast cell cultures

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Abstract

The cytotoxicity profile of nanocrystalline diamond (NCD) coatings on a Si_3N_4 ceramic was investigated. This material is envisaged to have biomedical dental applications such as burrs and surgical instruments. Two fibroblast cell culture systems were used to address the cytotoxicity of NCD-coated samples: L929 cells (a mouse permanent cell line) and human gingival fibroblasts. Cell behavior was evaluated in terms of cell adhesion, cell viability/proliferation (mitochondrial function, MTT assay) and the pattern of cell growth. Fibroblast cell behavior on standard polystyrene culture plates was used as control, as Si_3N_4 substrates have previously been shown to be biocompatible. NCD coatings provided a suitable surface for cell attachment, spreading and proliferation. Human gingival cells showed a homogeneous cytoplasm spreading, a flattened elongated morphology and a typical parallel alignment on confluent cultures. In comparison, L929 cells denoted a lower cytoplasm expansion, a heterogeneous spreading but a higher proliferation rate. For both cells, after few days, the NCD coating was completely covered with continuous cell layers. As compared to standard polystyrene culture plates, no deleterious or cytotoxic responses were observed with L929 and human fibroblast cell cultures, and in both a slight enhancement in cell proliferation was observed. In addition, the seeded NCD film allowed reproduction of the typical features of the two cell culture systems tested, further suggesting the lack of cytotoxicity of this coating.

Keywords: Nanocrystalline diamond coating; Cytotoxicity; Fibroblast cell cultures; Silicon nitride; Biomedical dental applications

1. Introduction

Nanocrystalline diamond (NCD) films have attracted the attention of researchers from different areas, and have therefore been the object of several studies in recent years. NCD is a unique material presenting the exceptional properties of diamond combined with smoother surfaces, higher toughness, lower friction coefficient, wide band gap and higher electron emission efficiency [1]. Thus, a wide range of applications is anticipated for NCD, particularly in the biomedical field. Implants and surgical instruments for dentistry, cardiology, orthopedics, ophthalmology, arterial and venous disorder repair, as well as biosensors and scaffolds for tissue engineering, are just some examples where the use of NCD is particularly innovative [2–9]. In addition, NCD films exhibit the highest resistance to bacterial colonization when compared to medical steel and titanium [10], a relevant issue since bacterial infection associated with the use of biomaterials is still a significant clinical problem.

Dental applications may take advantage of NCD films. In particular, NCD coating of surgical and dental instruments, such as scalpels and dental burrs, may improve the performance and lifetime of tools, as well as their

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biocompatibility. Diamond-coated dental burrs are an excellent option to substitute the conventional ones [11,12], which are made by embedding synthetic diamond particles into the working surfaces using a binder matrix material containing metallic ions. Those ions are responsible for contaminating of oral tissues, inflammatory responses, tissue disturbance and metal artifacts on magnetic resonance images [5,11–13]. The deposition of a diamond coating without metallic binder between the crystals would be extremely helpful in terms of reducing contamination-related problems. Furthermore, diamond-coated burrs can work with no signs of deterioration in more than 1000 operations, while the conventional burrs are ineffective after 30–60 operations [12]. To date, only chemical vapor deposition of microcrystalline diamond (MCD) has been reported in the literature [11,12], and, to the best of our knowledge, there are no references about the use of NCD on dental burrs. Clinical application of MCD-coated dental burrs, mounted on an ultrasonic handpiece, allow for a more precise cavity preparation in hard tissue ablation, resulting in a greater conservation of sound tooth structure [14]. Further, this system allows a higher angulation during tissue ablation and this makes interproximal preparation and finishing safer, reducing the chances of hitting adjacent structures [14]. In addition, patient discomfort, which is usually associated with mechanical vibrations, is greatly reduced [14]. Even with the early satisfactory clinical results with MCD diamond burrs, NCD may additionally improve the burr performance due to its lower surface roughness and higher toughness when compared to MCD [1].

NCD films can be deposited in several types of substrates used in biomedical applications, such as silicon [7,8], stainless steel [10,15], cobalt–chromium alloys [16], titanium alloys [6,17,18] and WC–Co cement carbide substrates [19]. An advantageous substrate is the silicon nitride (Si_3N_4) ceramic because it presents a thermal expansion coefficient very close to that of diamond [20], ensuring good film adhesion, a crucial requirement for biomedical applications. In addition, Si_3N_4 substrates possess the mechanical resistance necessary to work as a substrate for coated materials for biomedical applications [21] and were previously shown to elicit positive responses on cultured cells [22,23].

This work aims to evaluate the cytotoxicity profile of a NCD-coated Si_3N_4 ceramic, as part of ongoing projects regarding the use of this material combination in biomedical dental applications, namely burrs and surgical tools. The few reported publications on the biological behavior of NCD coatings are very specific regarding the cell type/application [3,24–26]; in the present work, two fibroblast cell culture systems were used to address the cytotoxicity of NCD-coated Si_3N_4 samples: L929 cells (a mouse permanent cell line) and human gingival fibroblasts. Cell behavior was evaluated in terms of cell adhesion on the NCD films, cell viability/proliferation (mitochondrial function, MTT assay) and pattern of cell growth.

2. Materials and methods

2.1. Materials preparation and characterization

Disc-shaped Si_3N_4 substrates (diameter = 10 mm, thickness = 3 mm) were manufactured according to a processing route described in detail in a previous work [20]. Before deposition, the 15 μm lapped substrates were scratched in an ultrasonic bath for 1 h in a 1 μm diamond powder suspension in *n*-hexane and then ultrasonically cleaned in ethanol for 10 min. The Si_3N_4 discs were coated by NCD using the hot filament chemical vapor deposition (HFCVD) method. A Ar–CH_4–H_2 gas mixture was used with volume ratios of $Ar/H_2 = 0.1$ and $CH_4/H_2 = 0.04$. Other deposition parameters were as follows: $P = 5$ kPa (total gas pressure); $F = 50$ ml min^{-1} (total gas flow); $T_s = 750$ °C (substrate temperature); $T_f = 2300$ °C (filament temperature); $t_d = 2$ h (deposition time).

The as-grown NCD films were observed by atomic force microscopy (AFM, Digital Instrument Multimode IIIa) and micro-Raman spectroscopy (Jobin-Yvon T64000, Ar^+ 514.5 nm line).

Before being seeded with the fibroblast cells, the NCD-coated Si_3N_4 samples were washed with ethanol in an ultrasonic cleaner and sterilized by autoclaving.

2.2. Cell cultures

2.2.1. Fibroblast cell line L929

The fibroblast cell line L929 was cultured in α-minimal essential medium (α-MEM) containing 10% fetal bovine serum, 50 μg ml^{-1} ascorbic acid, 50 μg ml^{-1} gentamicin and 2.5 μg ml^{-1} fungizone, at 37 °C, in a humidified atmosphere of 5% CO_2 in air. For subculture, the cell monolayer was washed twice with phosphate-buffered saline (PBS) and incubated with trypsin–EDTA solution (0.05% trypsin, 0.25% EDTA) for 5 min at 37 °C to detach the cells. Cells were resuspended in culture medium and cultured (10^4 cells cm^{-2}) for 8 days in standard plastic culture plates and on the surface of the NCD films in the "as-prepared" condition. The medium was changed every 2–3 days. Cultures were evaluated for cell viability/proliferation at days 1, 4 and 8 (MTT assay) and observed by scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM), to assess cell morphology during cell adhesion to the substrate (1, 6, 12 and 24 h) and throughout the culture period.

2.2.2. Human gingival fibroblast cells

Gingiva was collected from a patient undergoing a third molar extraction for orthodontic reasons. Informed consent to use this biological tissue, which would be otherwise discarded, was obtained. Primary cultures were obtained by culturing explants of gingiva, following established procedures [27–29]. Briefly, the tissue was washed in PBS, cut into small pieces and cultured in the same experimental conditions as those used in the culture of L929 cells. Cell

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