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Bactericide and bacterial anti-adhesive properties of the nanocrystalline diamond surface

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A B S T R A C T

We performed a systematic study of the bactericide and bacterial anti-adhesive properties of nanocrystalline diamond (NCD) and microcrystalline diamond (MCD) in comparison to other important industrial materials, such as copper, silver, polyethylene (Poly), and stainless steel (SS). The data show that NCD has better bactericide and bacterial anti-adhesive activity than Ag, but not as good as Cu. MCD, on the other hand, does not appear to have significant anti-bacterial activity. The superlative properties of NCD, such as mechanical hardness, resistance to oxidation and corrosion, and biological compatibility with blood and tissue, enable its use as antibacterial coating for medical implants. This is an application that cannot be achieved by Cu, which is known to be a highly effective antibacterial material but is not biocompatible. We also discuss possible underlying mechanisms to help understand the bactericide and bacterial anti-adhesive properties of the NCD surface.

1. Introduction

The National Institutes of Health USA reported that over 60% of all microbial infections are caused by biofilms. *Pseudomonas aeruginosa* is a gram-negative bacterium potentially pathogenic for humans, which adheres to surfaces, producing a biofilm. The formation of these clusters of bacteria and extracellular material represents a major health problem in hospitals [1]. These bacteria contaminate devices that are implanted within the body, such as, prostheses, intrauterine devices, catheters, and central line or heart valves [2]. The biofilms formed by these bacteria cause high resistance to antibiotics and disinfectants.

The population most vulnerable to infections by *P. aeruginosa*, a well-known nosocomial bacteria, is that of patients suffering from cystic fibrosis, which eventually destroys their lung function, accelerating the individual's death [3]. The prevention of this type of biofilm has motivated the scientific community to search for materials that are biocompatible, resistant to oxidation and corrosion in the biological environment, and having physical and chemical properties compatible with their specific function in the human body. Studies [4-8] that evaluate biomaterials for their potential to be applied as implants in humans focus mostly on the physico-chemical parameters and compatibility with human tissue, leaving outside the biomaterial susceptibility for bacteria adhesion, colonization, formation of biofilms and possible infection. The materials' ability to inhibit bacteria and reduce their capacity to make biofilms is the focus of this study.

In recent years, a great deal of research has examined the exceptional characteristics of the different allotropic forms of carbon in the field of medicine. For example, NCD has been considered a potential candidate for implants [4,9-11]. A study, using NCD as a coating on stainless steel (AISI 316 L) and titanium (Ti6A14V), materials commonly used in medical implants, shows high resistance to colonization of NCD by *E. coli* bacteria, even in the presence of blood plasma proteins [11,12]. Mitura et al. [5], by testing *in vivo*, showed that NCD is biocompatible in cells and tissues around the implant. They also found that the surface of NCD in histogram analyses showed to be free of microbes. Moreover, coatings made with diamond like carbon (DLC) improved the surface properties without increasing the risk of infection by *Staphylococcus epidermidis, Staphylococcus aureus, Staphylococcus salivarius* and *Pseudomonas aeruginosa*, compared with materials commonly used in implants [13].

In this study, we evaluated the ability of *Pseudomonas aeruginosa* to survive on and colonize the surfaces of NCD and MCD, in comparison to Poly, SS, Ag and Cu. We also examined the harming role played by the hydrophobic and hydrophilic property of the materials' surface, and the relation between surface roughness and bacterial survival and adhesion.

2. Experimental

The films were grown using hot filament chemical vapor deposition (HFCVD) system, described in detail elsewhere [14]. Hereby, we provide

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a summary of the parameters employed for the synthesis of MCD and NCD samples: filament made of rhenium, mirror-polished molybdenum disks 1.0 cm in diameter as substrates, filament-substrate distance of 8 mm, 20 Torr deposition and filament temperature of 2400 °C. The as-received Mo substrates were first hand polished with fine and ultrafine sandpapers of three different grits: 600, 1000, 1500, 2000 and 2500 (Norton Company, Worcester, MA). They were subsequently polished with < 1 μm synthetic diamond powder (Alfa Aesar, Ward Hill, MA). After polishing, all the substrates were ultrasonically cleaned thoroughly in methanol for 15 min. They were then dried in inert gas and placed immediately on the substrate holder integrated with a graphite heater. A gas mixture of 0.3% CH_4 in H_2 with 500 ppm of H_2S was used to grow MCD samples at 500 °C; and 2% CH_4 in H_2 with 500 ppm of H_2S was used to grow NCD samples at 800 °C. The Ag, Poly, Cu and SS substrates were acquired from Goodfellow.

All materials were fully characterized before performing the antibacterial experiments by high resolution scanning electron microscopy (HRSEM), atomic force microscopy (AFM), contact angle measurements, and Raman spectroscopy. The HRSEM images were taken with a JEOL JSM 7500 F and acceleration voltage of 15 kV. The Raman spectra were recorded using a triple monochromator ISA Jobin-Yvon model T64000 and the 514.53 nm line of an Ar-ion laser for excitation. The root mean square (RMS) roughness of the surfaces was measured with a scan size of $5 \times 5 \mu m^2$ using a AFM, Model MMAFM-2, Veeco, operated in tapping mode in ambient air. The contact angle was measured using the static sessile drop method with a Fire Wire goniometric model 120, at room temperature and using deionized water as liquid. Additional details are provided elsewhere [15]. All materials were cleaned in ultrasonic bath in isopropanol for 10 min, rinsed with deionized water, dried with N_2, and finally isolated in petri dishes, ready to be exposed to bacteria.

The antibacterial properties of the materials were tested against the gram-negative *Pseudomonas aeruginosa* bacteria (0353E3, MicroBiologics, MN, USA). The initial concentration of bacteria strain was of 5.4×10^3 colony-forming units (CFUs)/mL diluted in 5 mL of nutrient broth and incubated at 35 °C during 48 hours. The growth curve was obtained by adding the inoculated *P. aeruginosa* into 150 mL of nutrient broth. When the curve reached the logarithmic stage, in approximately two hours, the samples were immersed in 4 mL of bacterial strain for five minutes, then drained and transferred into another dish to begin the transferring of the surface adhered bacteria to a nutrient agar medium and finally incubated at 37 °C. The viable cells on each of the plates were counted by quantifying the CFUs. This process was carried out every 24 h for 72 h in the initial set of experiments. Then, the process was done every hour for 24 h for the surfaces showing bactericide properties in less than 24 h.

The bacterial anti-adhesive activity of the materials' surface was quantitatively evaluated by analysis of the SEM images. For this, the samples were dried and covered with a gold film of 30 nm using an Auto Sputter Coater Pelo SC-7. The SEM images were taken with a JEOL 5800 LV and acceleration voltage of 5 kV. The data reported were obtained from six independent bacteriological samples that were analyzed fifteen times each on areas of $5x5 \mu m^2$. The results are presented using descriptive statistics: mean standard deviation and one-way analysis of variance (ANOVA) with Turkey's post-test comparisons for multiple datasets. The statistical analysis started with $\alpha = 0.05$ and lower P values were considered to be statistically significant. The colonization factor (CF) was determined by the relation: CF = (area occupied by the bacteria × 100)/(total area studied), having considered the average size of the bacteria over each sample.

3. Results and discussion

Fig. 1 shows the high resolution SEM and AFM images of MCD and NCD films, respectively. It can be readily seen from these images differences in crystalline quality and grain size, with typical diameters lower than 20 nm for NCD films and larger 500 nm for MCD films.

The Raman spectra of the MCD and NCD films are shown in Fig. 2. The Raman spectrum of MCD films shows peaks at 1332 cm^{-1} and 1480 cm^{-1}. The first peak is the signature of high quality crystalline diamond [6,16]. The band centered at 1480 cm^{-1} is often attributed to the presence of *trans*-polyacetylene (TPA) segments that typically accompany nanodiamond and presumably formed during the initial growth phase [6-18]. The Raman spectrum of the NCD films shows a broad band centered at around 1140 cm^{-1}, corresponding to nanocrystalline diamond [6,19] and/or TPA [18]. A second band centered at 1338 cm^{-1} is attributable to diamond [6,16], and a third band centered at 1596 cm^{-1} indicates the presence of sp^2 C [20].

The roughness and wettability of the NCD, MCD, SS, Ag, Poly and Cu substrate surfaces were studied prior to exposure to the *P. aeruginosa* bacteria (see Table 1). The roughness of the substrates' surface was measured by AFM image analysis. The roughness values vary significantly from rough (109 nm) to smooth (23 nm) for the substrates employed in the antibacterial properties study. Water contact angle was used as a quantitative measure of the hydrophobicity and hydrophilicity of the substrates' surface. The threshold of hydrophobicity is a surface with a contact angle greater than 65°; below this value the surface is considered to be hydrophilic [21]. The results show the super-hydrophobicity of the NCD films' surface and the hydrophobic property of MCD, SS and Cu surfaces, while the wettability of the Ag and Poly substrates is within the hydrophilic range. The hydrophobic nature of the NCD and MCD films' surface arises as a consequence of deposition process with the HFCVD method that leaves the films hydrogen terminated after the deposition [22].

In order to evaluate the bactericidal activity of the surfaces under study, we performed systematic temporal survival studies. The initial temporal studies were done at 24-hour intervals for 72 h (see Table 2). We repeatedly found that the NCD films exhibit bactericidal effects within 24 h. The NCD results are similar to those for Cu and Ag surfaces that are widely known to have antibacterial properties. In fact, the United State Environmental Protection Agency registered Cu as the first metal with highly effective bactericidal properties because it can inhibit bacterial growth within the first two hours of contact [23]. On the other end, the SS and MCD substrates' surface did not show any bactericide effect up to the 72 h of the study. Polyethylene substrates' surface showed bactericide effects within 48 h, better than SS and MCD, but not as good as Cu, Ag and NCD.

A second set of temporal survival studies was done for NCD, Cu and Ag, with a time resolution of one-hour at intervals of 24 h in order to establish the time period required for these materials to induce bactericide effects, (Table 3). NCD substrates' surface consistently showed bactericide effects within 13 h, exceeding the bactericide effect of Ag surfaces, which required 15 h to show bactericide effects. The results also confirmed the strong bactericide power of the Cu substrates' surface, which consistently showed bactericide effects in less than two hours of exposure to the bacteria, in agreement with other reports [23]. However, the main drawback of Cu is its physico-chemical reactions with human tissue and fluids [23,24]. Moreover, the weak mechanical properties of Cu make it unsuitable for medical implants that undergo friction and wear. For these reasons, a hard biocompatible coating material that is also bactericide is required for biomedical implants.

We also studied the anti-adhesive activity of NCD, MCD, SS, Poly and Ag surface against *P. aeruginosa* bacteria through the assessment of the SEM images (Fig. 3) taken after the temporal survival studies described above were completed. The quantitative data on percent bacterial colonization area are shown in Table 4. There are wide differences in bacterial anti-adhesive activity among the materials studied, with percent colonization area ranging from 0% to almost 100%. The Cu substrates' surface showed complete resistance to bacterial colonization with no significant bacteria surface coverage. The next lowest colonization density occurred for Poly and NCD substrates, with 28% and 25%, respectively. On the other hand, biofilm structures formed over the whole area of the SS substrates' surface and, to a lesser degree, on the Ag and MCD substrates' surface.