GROWTH OF ESCHERICHIA COLI ON NANOCRYSTALLINE DIAMOND

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Abstract

Bacteria attached to solid surfaces are able to form bacterial biofilms which present serious problems both in healthcare and in industrial applications. Naturally, the development of anti-adhesive coatings that prevent bacterial attachment is of great importance nowadays. Nanocrystalline diamond (NCD) is one of the promising materials thanks its favourable mechanical and chemical properties. NCD appeared to be highly compatible with mammalian tissue cells; however, the knowledge on interactions between NCD and bacteria is rather scarce.

In this contribution, we compared the attachment of gram-negative model bacterium *Escherichia coli* to uncoated glass and glass coated by NCD films. NCD films were grown by chemical vapour deposition on silica fused glass substrates. To achieve different wetting properties, the NCD were terminated by hydrogen or oxygen plasma. The NCD films were characterized by atomic force microscopy, scanning electron microscopy, Raman spectroscopy, X-ray photoelectron spectroscopy and contact angle measurement. AFM confirmed differences in surface roughness between uncoated and NCD coated glass. Oxidized NCD films were hydrophilic compared to hydrogenated ones. Autoclaving was used for NCD samples sterilization; this method was shown to maintain original wetting properties of NCD films. For attachment experiments, continuous cultivation in commercially available CDC Bioreactor was used. Antibacterial tests indicated higher attachment of gram-negative model bacterium *Escherichia coli* to NCD surface compared to uncoated glass. We assign this effect to higher roughness of NCD surface compared to glass. Bacterial cells preferred the hydrophobic surface of hydrogenated NCD surface to hydrophilic oxidized NCD for their attachment.

Keywords: nanocrystalline diamond, bacteria, *Escherichia coli*, anti-adhesive, CDC Bioreactor

1. INTRODUCTION

Attachment and growth on surfaces is a common living strategy for most of the bacterial species in natural conditions. On artificial surfaces, however, attachment of various bacteria frequently causes problems in many fields of industry and pathogenic bacteria attached on the implants, catheters and other medical tools complicate the treatment of hospitalised patients. Attached bacteria form biofilm that consists from one or, most frequently, from many different bacterial species. The adhered cells synthesize the extracellular matrix that forms the highest proportion of the biofilm. In medical applications, the biofilms on the surfaces of implants are often inaccessible and very difficult to eradicate. Moreover, the effect of the antibacterial agents on the cells in biofilms might be severely reduced; diffusion through the biofilm is often restricted and antibacterial compounds can even be degraded by some members of the microbial community. For the biofilm formation by different bacteria, the initial attachment is enhanced by different molecular structures. The best prevention of bacterial growth on an artificial surface is to reduce the initial adhesion of bacterial cells. Therefore, continuous need exists for antibacterial and anti-adhesive materials. Nanocrystalline diamond (NCD) is a material with exquisite properties – chemical inertness, mechanical durability, high hardness and good compatibility with eukaryotic tissue cells [1, 2]. However, its antibacterial properties have not been studied thoroughly so far. Recently, several authors have reported the antibacterial potential of nanodiamond in the form of nanoparticles [3-5]. In contrast, NCD in the form of thin films deposited on an

inert surface has been studied even less. A few works indicate that this material has the potential to lower the bacterial attachment to certain extent [6, 7].

The ability of bacteria to adhere to the solid surface often correlates to surface roughness, hydrophobicity and surface energy [8-12]. From the results of many studies it is evident that it is not a single parameter but their complex combination which is responsible for the initial attachment of bacterial cells. Consequently, the prediction of ideal anti-adhesive surface for different groups of bacteria is a complicated and still very challenging task. In this work, we present deposition of nanocrystalline diamond films on glass substrates and their testing against gram-negative model bacterium *Escherichia coli* in the perfusion cultivation system.

2. MATERIAL AND METHODS

2.1. Preparation and characterization of nanocrystalline diamond films

For the purpose of this study the NCD films were deposited on silica fused glass substrates (1.7 × 7.7 cm, Menzel). Prior the deposition, glass substrates were cleaned in ultrasonic bath (Transsonic T570/H, Elma GmbH) at 100 kHz for 10 minutes. The cleaned substrates were seeded by ultrasound agitation in a water-based diamond nanoparticles suspension (mean size 5 nm, NanoAmando, New Metals and Chemicals Corp. Ltd.). The growth of NCD films was performed in two steps using large area linear antenna microwave plasma system (modified AK 400 system from Roth&Rau MicroSystems, GmbH). In the first step the deposition lasted 5 hours, the gas flow was 100 /30/5 in sccm of $H_2/CO_2/CH_4$ mixture. In the second step the deposition lasted 20 h and the gas composition was 200/20/5 in sccm of H_2/CO_2 /CH_4. Both procedures were performed under the following conditions: microwave power 2000 W with on/off pulse cycle 6/3 ms, pressure 0.1 mbar and substrate temperature around 430 °C. As-grown NCD films were hydrogen terminated as a result of deposition process with applied deposition parameters. Part of as-grown (hydrogenated) NCD films was oxidized by RF plasma in Femto PCCE system reactor (Diener, 100% O_2, 4 min, 1.1 mbar, 100 W).

The surface morphology of NCD films was investigated by scanning electron microscope SmartSEM V05.02.02 (Zeiss) and atomic force microscope ICON in a tapping mode (50 mV amp, scan area was 1 × 1 μm^2). The quality of grown NCD films was characterized by Raman spectroscopy (Renishaw inVia Reflex Raman microscope) with excitation wavelength 442 nm. The NCD surface chemical composition was analysed by X-ray photoelectron spectroscopy (XPS, Phoibos 150, Specs) using an Al Kα X-ray source (1486.6 eV, Specs). Survey spectra were measured with pass energy of 40 eV and high resolution with pass energy of 10 eV at constant take-off angle 90 °. The recorded spectra were then referenced to the peak at 285.1 eV which corresponds to sp^3 hybridized carbon [13]. The deconvolution of C 1s peaks was made as published previously [14]. For curve fitting, CasaXPS software was used. The hydrophobicity of NCD films was characterized by contact angle measurements with a reflection goniometer (Surface Energy Evaluation (SEE) System) equipped with a CCD. The contact angle was measured and calculated by multipoint fitting of the drop (3 µl of deionized water) profile using the SEE software.

Prior to bacteria attachment studies, the NCD samples were sterilized. The three different sterilization methods were tested: autoclaving (121 °C, 200 kPa, 20 min), dry heat (160 °C, 3 h) and UV radiation (253.7 nm, 20 min). The contact angle was measured before and after the sterilization step to assess the influence of respective method on the properties of NCD sample. Among all these methods, the sterilization by autoclaving was chosen due to a negligible effect on the contact angle (see chapter 3.2).

2.2. Bacterial strain and cultivation of bacteria

The bacterium *Escherichia coli*, strain K-12 (laboratory stock) was cultivated in CDC Bioreactor (BioSurface Technologies Corporation) according to method described by Hadi [15]. The bacterial batch culture from mid-exponential phase of growth was used for inoculation of CDC bioreactor to final OD_{450} = 0.025. During bacterial growth, sterile material samples (uncoated glass or NCD-coated glass) were immersed in the

bacterial suspension. The bacteria were cultivated for 22 hours at 37 °C in the batch mode (without medium influx) and 24 hours under perfusion conditions with mixing (200 rpm). During the continuous cultivation, the medium inflow speed was 6.25 ml·min^{-1}. The LB medium diluted with distilled water (1:2) was used for continuous cultivation.

2.3. Bacterial biofilm quantification

At the end of cultivation, the material samples with grown biofilm were removed from CDC Bioreactor and washed with 5 ml of sterile saline solution (0.8 % (w/v) NaCl) from both sides to remove loosely adhering bacteria. For microscopic observation the samples were stained with fluorescent dye Hoechst 33342 (1 g·l^{-1}, Invitrogen, Promega) and observed with fluorescent microscope Olympus Cell-R. To determine the biomass present at the sample surface, the bacteria were detached from the sample surface by sonication (ultrasonic processor UP50H, Hielscher, with Sonotrody MS2 probe, at room temperature) in 40 ml of fresh saline twice from both sides for 30 s (100 % amplitude, cycle 1). The saline solutions with detached bacterial cells were then frozen (-20 °C) and analysed the next day with use of BacTiter-Glo™ Microbial Cell Viability Assay kit (Promega). The determination of the overall biomass was based on the determination of ATP content in the sonicated samples.

3. RESULTS AND DISCUSSION

3.1. The properties of NCD films

Scanning electron microscopy (SEM) image of NCD film is shown in **Fig. 1A**. It indicates a typical surface morphology of nanocrystalline diamond film. The mean grain size was about 100 nm. The NCD films were also continuous and uniform within the whole surface. The AFM topography images of NCD film and uncoated glass are shown in **Figs. 1B** and **1C**, respectively. The NCD films were rougher

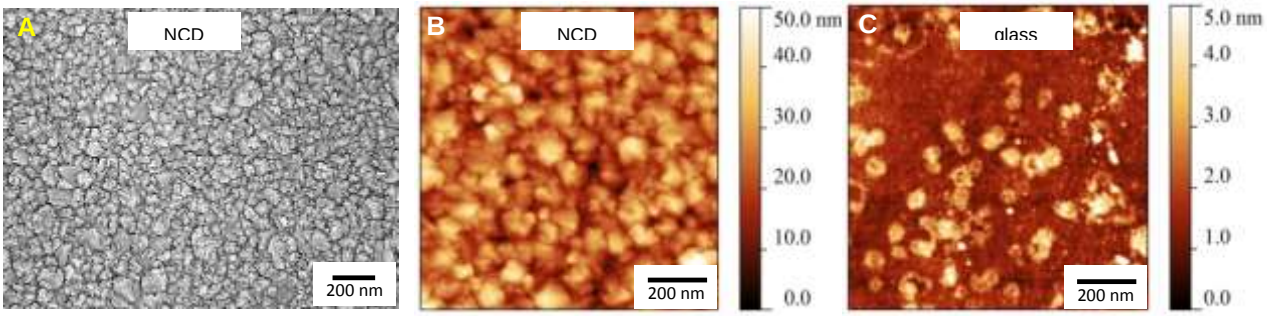

Fig.1 A SEM image of NCD film. **B,C** AFM topography images of NCD film and uncoated glass, respectively

(RMS = 6.9 nm) than the untreated glass (RMS = 0.9 nm). **Fig. 2** shows the representative Raman spectrum of NCD film with three discernible features. The band at 1100 cm^{-1} represents trans-polyacetylene present at the crystal boundaries [16, 17]. The most prominent peak at 1331 cm^{-1} corresponds to sp^3 hybridized carbon atoms confirming the diamond character of grown films. The broad band at 1520 cm^{-1} reflects sp^2 hybridization of carbon phase. The dominance of the 1331 cm^{-1} diamond peak indicates a relatively high proportion of diamond phase. The chemical composition of the NCD surfaces, determined by XPS, is presented in **Table 1**. As expected, the hydrogenated NCD contained less oxygen than oxidized NCD film. The content of 13 % oxygen on the oxidized NCD film surface and presence of the carbonyl groups (3 %) confirms the successful oxidation of the film [18]. The higher content of sp^2 hybridized carbon atoms on the surface of oxidized NCD film (23 %) in comparison with the hydrogenated one (7 %) is a consequence of partial graphitization of diamond surface due to O_2 plasma treatment [19]. Contact angle (CA) measurements confirmed that oxidized NCD surfaces are hydrophilic with CA typically below 10 °, in contrast to hydrophobic hydrogenated NCD surfaces with CA around 80 °. The contact angle of uncoated glass was around 24.5 °.

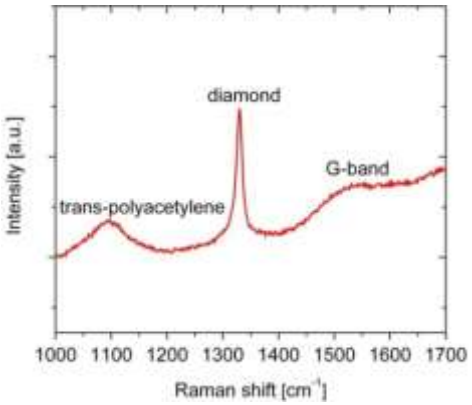

Fig. 2 Raman spectrum of NCD film.

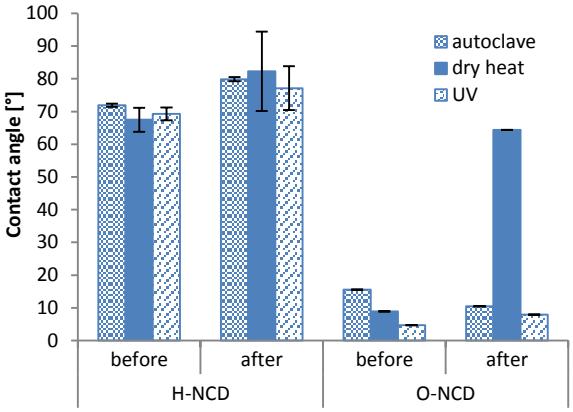

Fig. 3 CA of NCD before and after sterilization.

Table 1 The chemical composition of hydrogenated and oxidized NCD films evaluated from XPS

Material	O [at.%]	C [at.%]	sp^2 [%]	sp^3 [%]	C-O [%]	C=O [%]
hydrogenated NCD	3	97	7	77	16	-
oxidized NCD	13	87	23	59	15	3

3.2. Sterilization of NCD material

In order to find the sterilization method which does not change the surface properties of the tested material, three methods widely used for inactivation of bacterial contamination were compared. Neither the sterilization by autoclaving nor the sterilization by UV light changed significantly the contact angle of hydrogenated or oxidized NCD films (**Fig. 3**). In contrast, the dry heat sterilization substantially changed the surface of oxidized NCD films. Therefore, for further experiments the autoclaving was used as the most reliable method for routine sterilization of our NCD films.

3.3. Adhesion of glass and NCD films

Bacterial adhesion to uncoated glass and to hydrogenated or oxidized NCD was tested by biofilm growth and its consequent quantification. The samples were cultivated in CDC bioreactor for 46 hours (see chapter 2.2). The attachment of bacteria was assessed according the ATP level released from the biofilm after sonication. Grown biofilms stained by Hoechst 33342 were also observed by fluorescence microscope. Results are presented in **Fig. 4**. The comparison of the total ATP levels clearly shows that the NCD coating did not prevent bacterial attachment. On the contrary, we observed a slightly higher adhesion of bacteria to both NCD surfaces. This is in contradiction with the report of Medina *et al*. [6] who observed reduced adhesion of bacteria on the NCD surface compared to substrate stainless steel. However, Li and Logan observed in their experiments that bacteria adhered less to glass than to other, rougher materials [20]. Also other authors showed that higher roughness enhances the attachment of the bacteria on the surface [21-24]. Therefore we assume that higher colonization of NCD surface by bacteria could be the consequence of the higher roughness of NCD coating (compared with the uncoated glass). The highest yield of *E. coli* cells was observed for the hydrogenated NCD. This effect we assign to the hydrophobic character of hydrogenated NCD surface. Previous findings of other authors also affirm that different species of bacteria adhere preferentially to hydrophobic surfaces [21, 25-29]. This is probably due to increased hydrophobic interactions between bacterial cell and the substrate surface which play important role in bacterial attachment [8]. Also fimbriae and pili, hydrophobic appendages normally present on the *E.coli* surface, have been shown to promote adhesion on hydrophobic substrates [30].

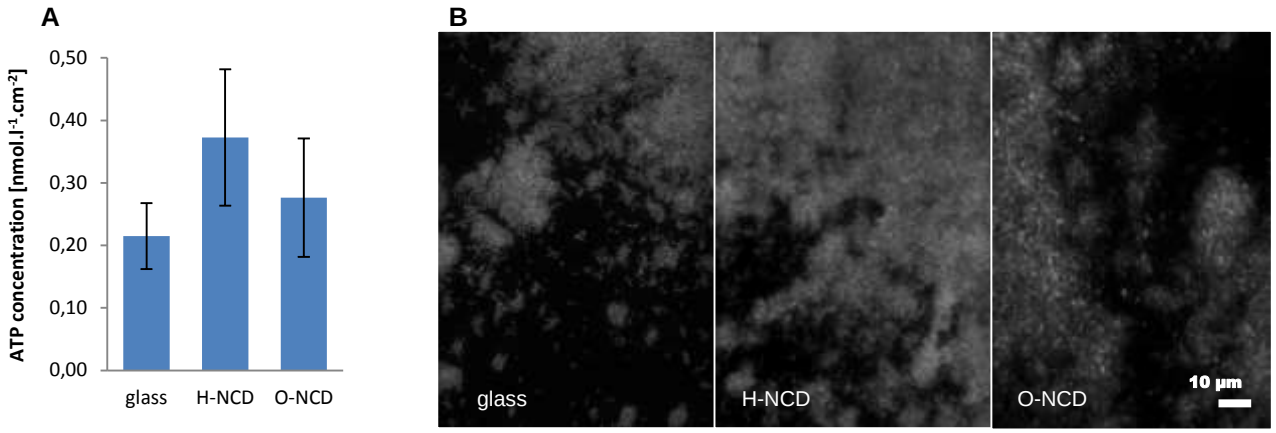

Fig. 4 Quantification of total biomass from *E. coli* biofilms grown on glass, hydrogenated NCD (H-NCD) and oxidized NCD (O-NCD) after 46 h of cultivation (**A**). Corresponding microscopic images are shown in (**B**).

4. CONCLUSION

The ability of NCD films to prevent adhesion and biofilm growth of model bacterium *Escherichia coli* was investigated in the perfusion environment of CDC Bioreactor. The antibacterial properties of hydrogenated and oxidized NCD films were compared with those of uncoated glass as the reference. We found that the NCD films were not capable to prevent the attachment of *E. coli*. Compared to uncoated glass slightly higher adhesion of bacteria to both NCD surfaces was observed. In agreement with other studies, we assigned this to the higher roughness of NCD (compared with the uncoated glass). A higher bacterial colonization observed for hydrogenated NCD films might be caused by their hydrophobic character which is generally more favourable for colonization by bacteria than hydrophilic one due to increased hydrophobic interactions between the bacterial cell and surface. In order to better understand interaction of bacteria with the diamond surface, additional studies are in progress.

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