Nanodiamond as promising material for bone tissue engineering

Lubica Grausova, Lucie Bacakova, Alexander Kromka, Stepan Potocky, Milan Vanecek, Milos Nesladek, Vera Lisa

1Institute of Physiology, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague 4, Czech Republic
Phone: +420 2 9644 2521, +420 2 9644 2523, Fax: +420 2 9644 2488, +420 2 4106 2488
E-mail: grausova@biomed.cas.cz; lucy@biomed.cas.cz

2Institute of Physics, Academy of Sciences of the Czech Republic, Cukrovarnicka 10, 162 53 Prague 6, Czech Republic
Phone: +420 220318437, Fax: +420 233343184
E-mail: kromka@fzu.cz; potocky@fzu.cz; vanecek@fzu.cz

3CEA-LIST, Centre d’Etudes Saclay, Bat. 451, p.84, 91191 Gif Sur Yvette, France
Phone: +331 69088704, Fax: +331 69087679
E-mail: Milos.NESLADEK@cea.fr

Address for correspondence:
Lubica Grausova, MSc.
Institute of Physiology
Academy of Sciences of the Czech Republic
Videnska 1083
142 20 Prague 4
Czech Republic
Phone: +420 2 9644 2521
Fax: +420 2 9644 2488, +420 2 4106 2488
E-mail: grausova@biomed.cas.cz
ABSTRACT

The adhesion, growth and differentiation of human osteoblast-like MG 63 cells were investigated in cultures grown on nanostructured nanocrystalline diamond (NCD) films with either low surface roughness \((rms\) of 8.2 nm) or hierarchically organized surfaces made of low roughness NCD films deposited on Si surfaces with the original microroughness \((rms\) of 301.0 nm and 7.6 nm, respectively). The NCD films were grown using a microwave plasma-enhanced CVD method in an ellipsoidal cavity reactor. The films were treated in oxygen plasma to enhance the hydrophilic character of the diamond surface (water drop contact angle approx. 20°). The samples were then sterilized by 70% ethanol, inserted into 12-well polystyrene multidishes (diameter 2.2 cm), seeded with human osteoblast-like MG 63 cells (40 000 cells/dish, 10 530 cells/cm\(^2\)) and incubated in 2 ml of DMEM medium with 10% of fetal bovine serum. On day 3 after seeding, the cell numbers were significantly higher on the nanostructured NCD films (72 020 ± 6 540 cells/cm\(^2\)) and also on the hierarchically micro- and nanostructured films (60 200 ± 6 420 cells/cm\(^2\)) than on the control polystyrene culture dish (40 750 ± 2 530 cells/cm\(^2\)). The cells on hierarchically micro- and nanostructured diamond substrates also adhered over a significantly larger area (3 730 ± 180 µm\(^2\) compared to 2 740 ± 130 µm\(^2\) on polystyrene). The cell viability, measured by a LIVE/DEAD viability/cytotoxicity kit, reached 98 % to 100 % on both types of NCD films. The XTT test showed that the cells on both nanodiamond layers had significantly higher metabolic activity than those on the control polystyrene dish (approx. 2 to 3 times). Immunofluorescence staining of the cells on both NCD films revealed talin-containing focal adhesion plaques and beta-actin filaments, well apparent particularly at the cell periphery, as well as the presence of considerable amounts of osteocalcin, i.e. a marker of osteogenic cell differentiation. These results suggest that nanocrystalline diamond films give good support for adhesion, growth and
differentiation of osteogenic cells and could be used for surface modification of bone implants in order to improve their integration with the surrounding bone tissue.

**Key words:** carbon nanoparticles, nanotechnology, microroughness, nanoroughness, osteoblasts

Submitted July 4, 2007; accepted November 26, 2007

**INTRODUCTION**

In recent years, nanocrystalline diamond (NCD) has attracted important attention as a promising material for advanced biomedical applications. These NCD thin films exhibit a wide range of unique physicochemical and biological properties, such as mechanical hardness, chemical and thermal resistance, interesting optical and electrical properties. For applications in biology and bioelectronics, important properties are non-cytotoxicity, relative ease of surface functionalization and the possibility to immobilize various biomolecules (e.g., nucleic acids, enzymes, antibodies and other proteins) on an NCD surface with strong covalent bonding. All these advantageous features make NCD promising building blocks for constructing microchips, nanorobots or biosensors, carriers for controlled drug and gene delivery or tracers for novel imaging technologies. In addition, biocompatible NCD films could be a suitable coating for medical implants, particularly those designed for hard tissue surgery. Nanocrystalline diamond and diamond-like carbon have already been used for coating the heads and cups of artificial joint replacements, e.g. metallic and polymeric prostheses of hip or temporomandibular joints. In addition, NCD films could be applied in the bone-anchoring stems of articular prostheses or other permanent bone implants in order to improve their interaction with the surrounding bone tissue. This beneficial action of NCD
films can be anticipated not only due to their excellent mechanical and chemical resistance but also due to their surface nanoroughness (i.e. irregularities less than 100 nm). The surface nanotopography has been reported to stimulate adhesion, growth and maturation of osteoblasts, as well as differentiation of bone progenitor cells towards the osteoblastic phenotype\textsuperscript{8,10}. It has been shown that type IIa diamond, microcontact-printed with laminin, supports adhesion of mouse cortical neurons and neurite outgrowth\textsuperscript{11}. On the other hand, nanostructured materials are believed to promote preferential adhesion of osteoblasts over other cell types (e.g., fibroblasts, chondrocytes, smooth muscle cells), which could prevent fibrous encapsulation of the bone implant\textsuperscript{8,9}. In studies \textit{in vivo}, diamond layers deposited on metallic probes implanted into rabbit femur showed very high bonding strength to the metal base and also to the surrounding bone tissue, without any problems with corrosion\textsuperscript{11}.

Not only the nanoroughness of the material surface, but also its microstructure, is important for its colonization with osteoblasts and for regeneration of the well functioning bone tissue. Surface roughness on the submicron or micrometer scale (\(R_a\) parameter, i.e. departures of the roughness profile from the mean line, in the range approx. from hundreds of nanometers to several micrometers) has been repeatedly shown to enhance the strength of osteoblast adhesion, their spreading and differentiation\textsuperscript{13-17}, including the deposition of mineralized bone extracellular matrix\textsuperscript{18} and acquisition of osteoblast phenotype in mesenchymal and osteoprogenitor cells\textsuperscript{19-20}. At the same time, increasing surface microroughness reduced the activity of osteoclasts and thus bone resorption\textsuperscript{21}. Microstructured surfaces also modulated the number of attached cells, cell shape, presence and activity of cell adhesion receptors, assembly of focal adhesion plaques and cytoskeleton, locomotion, proliferation, reactivity to hormones, vitamins and growth factors as well as the production of various bioactive molecules in cells\textsuperscript{13-22}. 
Since it is evident that the nano- and micropatterning of the material surface strongly influence the cell physiology, NCD films of two different architectures were prepared on silicon substrates. The first was purely nanostructured, having root mean square roughness \((rms)\) 8.2 nm. The second is what we call a hierarchically arranged micro- and nanostructure \((rms\ 301\ \text{nm}\ \text{and} \ 7.6\ \text{nm}, \ \text{respectively})\) in order to mimic the hierarchical organization of natural tissues\(^{23}\). The adhesion, viability, metabolic activity, growth and differentiation of human osteoblast-like MG 63 were then investigated in cultures on both types of NCD films.

**MATERIAL AND METHODS**

**Preparation of nanocrystalline diamond (NCD) films**

NCD films were grown on (100) oriented silicon substrates (16 mm in diameter) by a microwave plasma enhanced chemical vapor deposition (PECVD) method in an ellipsoidal cavity reactor (AIXTRON - P6, Germany)\(^{24,25}\). The silicon substrates were either mechanically lapped to root mean square \((rms)\) roughness up to 300 nm or polished to atomic flatness \((rms\ \text{roughness about} \ 1\ \text{nm})\). Prior to the deposition process, the substrates were mechanically seeded in an ultrasonic bath, using a 5 nm diamond powder for 40 minutes. The nucleation procedure was followed by PECVD growth using \(\text{H}_2/\text{CH}_4\) gas mixtures. A constant methane concentration (1 % \(\text{CH}_4\) in \(\text{H}_2\)) at a total gas pressure of 30 mbar was used. The substrate temperature was 860° C, as measured by a two-color pyrometer working at wavelengths of 1.35 and 1.55 \(\mu\text{m}\). The silicon substrates were overcoated with an NCD film on both sides, i.e. on the top and bottom side, respectively. Thus, hermetic sealing of the Si substrate minimized any unwanted bio-chemical reaction, as an opened Si area could result in pollution and disturbances of the subsequent cell experiments. Finally, the deposited NCD
films were treated in oxygen plasma for 3 minutes at a total power of 300 W and gas pressure 1000 Pa to enhance the hydrophilic character of the diamond surface.

Therefore, the following groups of experimental samples were prepared and used for cell culture studies:

- Nanostructured (flat) silicon substrates polished to atomic flatness ($rms$ roughness about 1 nm)
- Microstructured (rough) silicon substrates lapped to $rms$ roughness up to 300 nm
- Nanostructured silicon substrates coated with nanocrystalline diamond
- Microstructured silicon substrates coated with nanocrystalline diamond with resulting hierarchically organized micro- and nanostructure of the surface
- As control samples, we used polystyrene culture dishes (TPP, Switzerland) and microscopic glass coverslips (Menzel Glaser, Germany), cleansed with ethanol.

**Evaluation of the physical and chemical properties of the NCD films**

The Raman spectra of the deposited NCD films were measured using a 514.5 nm excitation wavelength laser, which enabled us to determine the ratio of carbon in the $sp^3$ hybridization, characteristic for diamond, to the $sp^2$ C in the films. X-ray photoelectron spectroscopy (XPS) was additionally used to study the bonding structure of the NCD surfaces. The diamond film morphology was investigated by scanning electron microscopy (SEM) and atomic force microscopy (AFM). The film thickness and the refractive index were calculated from the optical reflection and absorption measurements$^{26}$. The surface wettability of the films was determined from the contact angle measured by a static method in a material-water droplet system using a reflection goniometer (SEE System, Masaryk University, Brno, Czech Republic).
Cells and culture conditions

The samples were sterilized with 70% ethanol for 1 hour, placed into 12-well polystyrene multidishes (TPP, Switzerland; diameter 22 mm) and seeded with human osteoblast-like MG 63 cells (European Collection of Cell Cultures, Salisbury, UK). Each dish contained 40 000 cells (i.e., 10 528 cells/cm$^2$) and 2 ml of Dulbecco’s modified Eagle’s Minimum Essential Medium (DME M; Sigma, U.S.A., Cat. No D5648) supplemented with 10% fetal bovine serum (FBS; Sebak GmbH, Aidenbach, Germany) and gentamicin (40 µg/ml, LEK, Ljubljana, Slovenia). The cells were cultured for 1, 3 and 5 days at 37° C in a humidified air atmosphere containing 5% of CO$_2$.

Evaluation of cell number and viability

On day 1, 3 and 5 after seeding, the cells were rinsed with phosphate-buffered saline (PBS; Sigma, U.S.A.) and their number and viability were determined by the LIVE/DEAD viability/cytotoxicity kit for mammalian cells (Invitrogen, Molecular Probes, U.S.A.) according the manufacturer’s protocol. Briefly, the cells were incubated in two probes detecting the esterase activity in living cells (calcein AM producing green fluorescence) or the membrane damage in dead cells (ethidium homodimer-1 emitting red fluorescence) for 5 to 10 min at room temperature. Live and dead cells were then counted on microphotographs taken under an epifluorescence microscope (Olympus IX-50, digital camera DP-70, Japan). For each experimental group and time interval, 3 samples were used, and from each sample, 10-20 microphotographs were taken in randomly selected fields homogeneously distributed on the sample surface.

Calculation of cell population doubling time

The doubling time (DT) was calculated using the following equation:
DT = \log_2 \left( \frac{t-t_o}{\log N_t - \log N_{t_0}} \right), \text{ where } t_o \text{ and } t \text{ represent earlier and later time intervals after seeding, respectively, and } N_{t_0} \text{ and } N_t \text{ the number of cells at these intervals.}

**Evaluation of cell metabolic activity by XTT test**

The cells were seeded on experimental samples, inserted in 12-well polystyrene plates, in a density of 40 000 cells/well (10 528 cells/cm²) and cultured for 3 days in the DMEM medium supplemented with FBS and gentamicin (see above). On day 3 after seeding, the medium was replaced with DMEM containing the same supplement but without phenol red and L-glutamine (Sigma, U.S.A., Cat. N° D 1145). The cell metabolic activity was estimated using a commercially available XTT kit (Roche Diagnostic GmbH, Mannheim, Germany, Cat. No. 11 465 015 001) according the manufacturer’s protocol. Briefly, the reagent solution, containing sodium 3’-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate, i.e. XTT, was added to the culture medium in the ratio of 1:2 and incubated with cells at 37° C in an atmosphere of air with 5% of CO₂. Metabolically active cells then converted the tetrazolium salt XTT into an orange formazan dye by their mitochondrial dehydrogenases. The resulting solution was then moved, either immediately or after 4 hours of incubation (37°C, 5% CO₂), into fresh 96-well polystyrene test plates (Nunc, Denmark, well diameter 6 mm, 200 µm of the solution per well), and the absorbance of the formazan dye was measured using a Multilabel Counter Wallac Victor 1420 (Perkin Elmer Life and Analytical Sciences, Inc., Wellesley, MA, U.S.A.) at a wavelength of 490 (reference value 690 nm). As the blank sample, a solution from polystyrene dishes containing a medium with XTT but not cells was used. For each experimental group, 3 samples were used, and the solution from each was divided into two parallel wells.
**Immunofluorescence staining of talin, beta-actin and osteocalcin**

The presence and arrangement of talin, an integrin-associated protein of focal adhesion plaques, beta-actin, an important component of the cytoplasmatic cytoskeleton, and osteocalcin, a non-collagenous calcium-binding extracellular matrix glycoprotein and marker of osteogenic cell differentiation, were evaluated in cells in 3-day-old cultures, seeded on the tested materials relatively sparsely (5 000 cells/well with a diameter of 22 mm, i.e. 1 316 cells/cm²) in order to reduce cell overlapping. The cells were rinsed twice in PSB and fixed with cold 70% ethanol (-20°C, 5 min), pre-treated with 1% bovine serum albumin in PBS containing 0.05% Triton X-100 (Sigma, St. Louis, MO, U.S.A.) for 20 min at room temperature and incubated with the following primary antibodies: mouse monoclonal anti-human talin (Chemicon International Inc., Temecula, CA, U.S.A.; Cat No. MAB3264), monoclonal anti-beta-actin (clone AC-15, Sigma, St. Louis, MO, U.S.A., Cat. No. A-5441, dilution 1:400) or rabbit polyclonal anti-human osteocalcin 1:200 (Chemicon International Inc., Temecula, CA, U.S.A.; Cat No. AB1857). The antibodies were diluted in PBS to concentrations of 1:200 to 1:400 and applied overnight at 4°C. After rinsing with PBS, the secondary antibodies, represented by goat anti-mouse F(ab')2 fragment of IgG (for samples stained with both monoclonal antibodies; dilution 1:1000) or goat anti-rabbit F(ab')2 fragment of IgG (for samples stained with the polyclonal antibody; dilution 1:5000) were added for 1 hour at room temperature. Both secondary antibodies were conjugated with Alexa Fluor® 488 and purchased from Molecular Probes, Eugene, OR, U.S.A. (Cat. No. A11017 and A11070, respectively). After incubation with secondary antibodies, the cells were rinsed twice in PBS, mounted under microscopic glass coverslips in a Gel/Mount permanent fluorescence-preserving aqueous mounting medium (Biomeda Corporation, Foster City, CA, U.S.A.) and evaluated under an epifluorescence microscope (IX-50, Olympus, Japan) equipped with a digital camera (DP-70, Olympus, Japan).
Measurement of cell adhesion area

The cells immunostained against beta-actin were used for evaluating the size of the cell spreading area, i.e. the cell-material contact area. The cells were photographed using a microscope IX 50 (obj. 20) equipped with a digital camera DP 70 (Olympus, Japan) in 20 randomly selected microscopic fields (size approx. 1.38*10^{-3} cm^2) for each experimental group. The size of the area projected on the material was measured using Atlas software (Tescan, Brno, Czech Republic). The cells developing intercellular contacts were excluded from the evaluation. For each experimental group, 85 to 146 cells were evaluated.

Statistical analysis

The quantitative results were expressed as means ± Standard Error of Means (S.E.M.). The statistical analyses were performed using SigmaStat (Jandel Corporation). Multiple comparison procedures were made by the One Way Analysis of Variance (ANOVA), Student-Newman-Keuls method. p values equal to or less than 0.05 were considered significant.

RESULTS

Physical and chemical properties of the NCD films

The Raman spectra of nanocrystalline diamond films deposited on silicon substrates displayed a dominant peak centred at 1333 cm^{-1} (zone centre phonon in diamond, Fig. 1), which confirms the diamond character of the deposited films. The peak centred at 1140 cm^{-1} has often been attributed to nanocrystalline diamond, though its relation to the trans-polyacetylene group at the boundary of diamond grains has also been proven. In addition, the Raman spectrum exhibited two broad bands centred at 1350 and 1590 cm^{-1}, known as band D and G, and attributed to disordered carbon or graphitic carbon. In spite of this, only
a low amount of non-diamond components can be expected in the films, because Raman spectroscopy is known to be much more (50 to 250 times) sensitive for detecting \( sp^2 \) than \( sp^3 \) bonds\(^{30,31} \). The high ratio of carbon in \( sp^3 \) hybridization at the NCD surface, characteristic for diamond, to the \( sp^2 \) C in our films was confirmed by XPS, and amounted to more than 95\%\(^{32} \). The relatively steep rise of the Raman spectrum from the left to the right side (Fig. 1) indicates a background due to the luminescence at the silicon-diamond interface.

SEM showed that the nanocrystalline diamond film deposited on the flat silicon substrate exhibited a relatively flat surface (Fig. 2). The crystal size varied from 20 to 30 nm. No significant crystal faceting was observed. As measured by X-Ray diffraction (XRD), the deposited NCD films were randomly oriented (data not shown).

AFM confirmed that two types of NCD layers were prepared. The first was nanostructured with a relatively flat surface (Fig. 3A). On a detailed AFM scan of 1 x 1 \( \mu m \), the development of crystal faceting was observable. This NCD film consisted of small crystals up to 30 nm in size. The calculated \( \text{rms} \) roughness was 8.21 nm. The surface features were similar and independent of the scanned area.

The second type of layer was hierarchically micro- and nanostructured (Fig. 3B). A scan over a large area (50 x 50 \( \mu m \)) exhibited features of relatively high, i.e. submicron roughness up to \( \sim \)300 nm. As shown by the detailed scan of 1 x 1 \( \mu m \), the surface of these irregularities contained nano-scale features with \( \text{rms} \) roughness of 7.6 nm. Both nanostructured and hierarchically micro-nanostructured films thus differ mainly in surface roughness on the large scale.

As calculated from optical measurements\(^{26} \), the film thickness was 330 nm on the atomically polished side. The water drop contact angle was approximately 30\°, which confirmed that the NCD films were relatively highly wettable.
Number and viability of cells in cultures on NCD films

On day 1 after seeding (Fig. 4 A), the numbers of viable MG 63 cells adhering to both the nanostructured and the micro-nano structured NCD layers were 22 260 ± 2160 cells/cm² and 17 600 ± 880 cells/cm², respectively, and thus they were similar to the values found on the control polystyrene culture dishes (16 350 ± 950 cells/cm²), the microscopic glass coverslips (15800 ± 830 cells/cm²) and also the nanostructured silicon substrates (15 260 ± 3330 cells/cm²). The number of viable cells on all these surfaces ranged from 88 ± 5% to 100 ± 5% of the total number of attached cells. However, on microstructured silicon substrates, the number of viable cells was markedly lower, reaching only 6300 ± 680 cells/cm², which represented 78 ± 9 % of the total number of adhered cells.

On day 3 after seeding (Fig. 4 B), the number of viable cells on both flat and rough silicon substrates decreased markedly to 3 160 ± 1450 cells/cm² and 290 ± 150 cells/cm² respectively. In contrast, the cell number on both types of diamond films rapidly increased to 60 200 ± 6 420 cells/cm² on micro-nanostructured NCD and even 72 020 ± 6 540 cells/cm² on nanostructured NCD, and thus reached significantly higher values than on polystyrene (40 750 ± 2 530 cells/cm²) and glass (42 840 ± 8 480 cells/cm²). From day 1 to 3, the cell population doubling time was 29.1 ± 1.7 h on nanostructured NCD and 27.7 ± 1.3 h on micro-nanostructured NCD, whereas on polystyrene and glass, it was 37.1 ± 1.7 h and 34.7 ± 5.7 h, respectively. Nevertheless, the viability of the cells on both types of diamond layers reached more than 99 % and was similar to that on the control polystyrene and glass (97 ± 6 % and 98 ± 19 %, respectively). On the other hand, the viability of the cells on silicon substrates decreased markedly in parallel with their number. On flat silicon substrates, the cell viability was only 70 ± 32 %, and on the rough substrates, it dropped even to 20 ± 11 % (Figs. 4 B and 5).
From day 3 to 5 after seeding, the proliferation activity of cells on both NCD layers, i.e. materials with the highest cell population densities on day 3, declined (doubling time $77.9 \pm 7.2$ h on nanostructured NCD and $67.6 \pm 4.6$ h on micro-nanostructured NCD). At the same time, the doubling time of cells on polystyrene dishes and microscopic glass coverslips remained still comparable with the values between days 1 to 3, amounting to $42.9 \pm 0.9$ h and $39.6 \pm 5.8$ h, respectively. As a result, on day 5 after seeding (Fig. 4 C), the cells on the two diamond films, polystyrene and glass reached similar high numbers of viable cells, ranging from $165,450 \pm 10,650$ cells/cm$^2$ to $192,370 \pm 870$ cells/cm$^2$. The viability of the cells on all these samples reached 98-100 %. On the other hand, the number of cells on the silicon substrates was extremely low and almost none of them were viable.

**Metabolic activity of cells on NCD films**

As revealed by the XTT test (Fig. 6), the absorbance of the formazan dye produced by the cells on nanostructured and hierarchically micro-nanostructured diamond films was significantly higher (by $260 \pm 4 \%$ and $102 \pm 2 \%$, respectively) than on the control polystyrene dishes. The absorbance was not detectable on the silicon substrates, probably due to the very low number of viable cells on these materials. In addition, the color of the XTT reagent solution was altered, which may have been due to some its non-specific reaction with the silicon substrates. The higher absorbance of formazane from cells cultivated on the two diamond layers indicates higher metabolic activity (i.e., higher activity of mitochondrial enzymes) in these cells than in those on the control cell culture surfaces, and is in good correlation with the higher cell number found on both types of NCD films.
Immunofluorescence of talin, beta-actin and osteocalcin in cells on NCD films

Immunofluorescence staining, performed on day 3 after seeding, revealed that the MG 63 cells on the two diamond films and the control microscopic glass coverslip were brightly stained for talin, beta-actin and osteocalcin (Fig. 7). On pure glass coverslips and substrates with a nanostructured NCD layer, all these molecules usually showed a homogeneous fine granular distribution throughout the cells and often accumulated in the perinuclear region (Fig. 7 A, B, D, E, G, H). On a hierarchically micro- and nanostructured NCD layer, the fluorescence of these molecules often showed brighter and less bright regions, which could be related to variations in cell thickness due to irregularities on the material surface (Fig. 7 C, F, I). In addition, the cells on the glass and especially on both NDC films developed streak-like talin-containing focal adhesion plaques, well–apparent especially at the cell periphery, and distinct beta-actin containing filaments (Fig. 7 A-F).

Size of the adhesion area in cells on NCD films

The size of the cell spreading area, measured in cells stained against beta-actin, was similar in cells growing on the nanodiamond substrate (2 744 ± 116 µm²) and on polystyrene dishes (2 742 ± 133 µm²) but it was significantly larger in cells on hierarchically micro- and nanostructured diamond substrates (3 733 ± 179 µm², Fig. 8). The cells on all these samples mostly had a polygonal morphology; some of them were also spindle-shaped.

DISCUSSION

As already mentioned above, in this study we used two types of nanocrystalline diamond films, i.e. having either nanostructured (rms 8.21 nm) or hierarchically organized micro- and nanostructured (rms 301 and 7.6 nm, respectively) surfaces. Such nanostructuring makes them suitable for use in various biomedical applications¹⁵, particularly in hard tissue
surgery. Both types of NCD films provided good support for the attachment, spreading, growth, viability, metabolic activity and production of osteocalcin in human osteoblast-like MG 63 cells in an extent similar to or even better than in cells on standard cell culture surfaces, represented by polystyrene dishes or microscopic glass coverslips. This favorable cell behavior was probably due to the nanostructure of the material surface, which has been reported to be highly supportive for colonization with cells, preferentially osteoblasts. Nanostructured surfaces improve the adsorption of cell adhesion-mediating extracellular matrix (ECM) molecules, e.g. fibronectin, vitronectin and collagen, from the serum of the culture medium or body fluids. These proteins are adsorbed in an appropriate amount and probably also in a favorable spatial conformation, enabling the accessibility of specific amino acid sequences by cell adhesion receptors, e.g. integrins. Preferential adhesion of osteoblasts over other cell types has been explained by preferential adsorption of vitronectin on nanostructured surfaces, due to its relatively small molecule, and its preferential recognition by osteoblasts.

The microstructure of the NCD layers also seemed to have a positive effect on cell adhesion in this study. On hierarchically micro- and nanostructured surfaces, the MG 63 cells were better spread, i.e. they adhered over a significantly larger area than on the other tested substrates. This result is in good correlation with an earlier study reporting a supportive effect of the surface microroughness of dental titanium implants on the spreading of rat osteoblasts in primary cultures as well as the development of a polygonal shape in osteogenic cells. On the other hand, in our earlier study performed on carbon fiber-reinforced carbon composites (CFRC), cell spreading correlated inversely with surface roughness ($R_a$ from 3.25 ± 0.35 µm to 0.35 ± 0.09 µm) and on the rougher surfaces, the cells were mostly spindle-shaped. The elongated spindle-shaped morphology in osteoblasts has been often considered as a more differentiated phenotype. In addition, cell proliferation has been repeatedly
found to be slower on rougher surfaces\textsuperscript{13,18,22,33}. In accordance with this, on day 3 after seeding, the cells on hierarchically micro-nanostructured diamond showed a tendency to attain a lower cell population density in comparison with the cells on nanostructured NCD, though their growth started from similar initial cell population densities on day 1, and their metabolic activity was significantly lower.

Termination with oxygen, resulting in hydrophilicity of our diamond layers, may also have contributed to its good colonization with MG 63 cells. In our earlier studies, performed on ion-implanted or UV light-irradiated polymers or carbon-titanium-coated CFRC, the presence of oxygen-containing chemical functional groups in the material surface and its wettability markedly increased the adhesion, growth and expression of differentiation markers in vascular endothelial and smooth muscle cells as well as MG 63 cells\textsuperscript{33-37}. Similarly, hydroxylated Ti surfaces of high surface energy and hydrophilia promoted the differentiated phenotype in MG 63 cells\textsuperscript{15}. The principle of this supportive action is similar as for nanostructured surfaces: improved adsorption of cell adhesion-mediating ECM molecules from the surrounding environments\textsuperscript{33-37}.

In comparison with other cell types, particularly vascular smooth muscle or endothelial cells, talin and beta-actin in osteoblast-like MG 63 cells in this study were distributed more diffusely and were less assembled into specific structures, i.e. talin-containing focal adhesion plaques and beta-actin cables. This may be because the MG 63 cells are of tumor origin\textsuperscript{38}. The formation of well-apparent and numerous focal adhesion plaques as well as a rich actin cytoskeleton has been often regarded as desirable behavior of cells on artificial materials\textsuperscript{10,34,35}. On the other hand, large focal adhesions and thick actin stress fibres are typical for the classical “two-dimensional” cell culture system, i.e. conditions less physiological for cells, and can also be associated with a lower expression of differentiation markers in cells\textsuperscript{39}. The relatively good maturation state of MG 63 cells in this study is
suggested by the bright fluorescence of osteocalcin, though its concentration, together with other markers of osteoblastic cell differentiation (e.g., production of osteopontin, calcification and activity of alkaline phosphatase), remains to be quantified by biochemical methods.  

In contrast to the two NCD layers, the silicon substrates proved to be cytotoxic for MG 63 cells. Similar results were obtained with silicon substrates implanted into rabbit eyes, where these materials behaved as highly corrosive. On the other hand, when covered with diamond, these implants behaved as highly biocompatible, similarly as in our present study. The proved high biocompatibility of our diamond-coated silicon substrates suggested that the diamond layers prepared were continuous with no pinholes, non-permeable and resistant to detachment. Non-cytotoxicity of nanodiamonds (2 to 10 nm in size) and their supportive effect on cell adhesion and growth has been demonstrated on a variety cell types, including neuroblastoma cells, macrophages, and keratinocytes. The MTT assay showed that mitochondria in cells exposed to nanodiamonds remained intact and fully functioning, and the cells were not exposed to any oxidative damage, which is seen in the case of other carbon nanoparticles, such as fullerenes and nanotubes.  

Even though the NCD layers used in this study contained a small amount of carbon in sp² hybridization (about 5%) at the surface, determined by XPS, the layers are highly biocompatible. This is probably because graphite also proved to be a mechanically resistant and biocompatible material supporting the adhesion of osteoblasts and promising for coating bone implants.  

It could be hypothesized that the idea of a hierarchically-organized micro- and nanoarchitecture of biomaterials, resembling the arrangement of natural tissues, may also be useful for constructing three-dimensional porous bioartificial implants. For example, nanodiamonds could be admixed into a polymeric matrix designed for production of scaffolds for bone tissue engineering. The nanodiamonds would then decorate the walls of the pores,
usually hundreds of micrometers in diameter, and the resulting nanostructure of the pore walls would facilitate the ingrowth of osteoblasts and bone tissue regeneration.

CONCLUSION

The nanocrystalline diamond films used in this study, showing different levels of surface organization, i.e., nanostructured (\textit{rms} 8.2 nm) and hierarchically micro-nanostructured (\textit{rms} 301 and 7.6 nm, respectively), provided good support for the adhesion, spreading, viability, growth and maturation of human osteoblast-like MG 63 cell in cultures on these materials. Thus, these NCD films could be used in tissue engineering, particularly for surface modification of bone implants (e.g., bone-anchoring parts of joint prostheses or bone replacements) in order to improve their integration with the surrounding bone tissue.

ACKNOWLEDGEMENTS

This study was supported by the Grant Agency of the Czech Republic (Grant No. 204/06/0225) and the Acad. Sci. CR (Grant No. KAN400480701 and No. KAN400100701). We also thank Mrs. Ivana Zajanova (Inst. Physiol., Acad. Sci. CR) for her excellent technical assistance in immunofluorescence staining. Mr. Robin Healey (Czech Technical University, Prague) is gratefully acknowledged for the language revision of the manuscript.

REFERENCES


LEGENDS TO THE FIGURES

Fig. 1. Raman spectroscopy of nanostructured (A) and hierarchically micro- and nanostructured (B) nanocrystalline diamond (NCD) films. The peak 1333 cm\(^{-1}\), characteristic for diamond, and 1140 cm\(^{-1}\), often attributed to NCD, are well visible in both types of films.

Fig. 2. Scanning electron microscopy of nanocrystalline diamond (NCD) films. Microscope FE SEM 1550. Bar 4 µm (gross view) and 200 nm (detailed view).

Fig. 3. Atomic force microscopy of nanostructured (A) and hierarchically micro-nanostructured (B) NCD films. Root mean square (\(\text{rms}\)) roughness is 8.2 nm (A) or 301 nm and 7.6 nm (B). AFM Microscope Dimension 3100, Veeco.

Fig. 4. Number of MG 63 cells on day 1 (A), 3 (B) and 5 (C) after seeding on polystyrene culture dishes (PS), microscopic glass coverslips (Glass), nanostructured silicon substrates (Nano_Si), microstructured silicon substrates (Micro_Si), nanostructured diamond films (Nano_NCD) and diamond films with hierarchically organized micro- and nanostructure (Micro-nano_NCD). The first column: total cell number, the second column: number of viable cells. Mean ± S.E.M. from 3 samples. ANOVA, Student-Newman-Keuls Method. Statistical significance: \(I, II, III, IV, V, VI\): \(p \leq 0.05\) in comparison with the sample of the same Roman number.

Fig. 5. Human osteoblast-like MG 63 cells stained with LIVE/DEAD viability/cytotoxicity kit on day 3 after seeding on polystyrene culture dishes (A), microscopic glass coverslips (B), nanostructured diamond films (C), diamond films with hierarchically organized micro- and
nanostructure (D), nanostructured silicon substrates (E) and microstructured silicon substrates (F). Olympus epifluorescence microscope IX 50, digital camera DP 70, obj. 4x, bar=1mm.

**Fig. 6.** Metabolic activity of human osteoblast-like MG 63 cells on day 3 after seeding on polystyrene culture dishes (PS), nanostructured diamond films (Nano_NCD) and diamond films with hierarchically organized micro- and nanostructure (Micro-nano_NCD). Measured by XTT test. Mean ± S.E.M. from 3 samples. ANOVA, Student-Newman-Keuls Method. Statistical significance: I, II, III: p≤0.05 in comparison with the sample of the same Roman number.

**Fig. 7.** Immunofluorescence staining of talin (A-C), beta-actin (D-F) and osteocalcin (G-I) in human osteoblast-like MG 63 cells on day 3 after seeding on microscopic glass coverslips (A, D, G), nanostructured diamond films (B, E, H) and diamond films with hierarchically organized micro- and nanostructure (C F, I). Arrows indicate beta-actin-containing filaments. Olympus epifluorescence microscope IX 50, digital camera DP 70, obj. 100x, bar=20 µm.

**Fig. 8.** The size of adhesion area of human osteoblast-like MG 63 cells on day 3 after seeding on microscopic glass coverslips (Glass), nanostructured diamond films (Nano_NCD) and diamond films with a hierarchically organized micro- and nanostructure (Micro-nano_NCD). Mean ± S.E.M. from 85 to 146 cells for each experimental group. ANOVA, Student-Newman-Keuls Method. Statistical significance: I, II, III: p≤0.05 in comparison with the sample of the same Roman number.
Fig. 1

A

B

Intensity [a.u.]

Wavenumber [cm$^{-1}$]

1333 cm$^{-1}$
diamond

1350 and 1600 cm$^{-1}$
D and G band (sp$^2$)

1140 cm$^{-1}$
nanocrystalline

Intensity [a.u.]

Wavenumber [cm$^{-1}$]

1333 cm$^{-1}$
diamond

1350 and 1600 cm$^{-1}$
D and G band (sp$^2$)

1140 cm$^{-1}$
nanocrystalline
Fig. 2

Surface morphology (large area scan)

Surface morphology (detailed view)
Fig. 3

A. Scan: 1 x 1 µm
\[ \text{rms} = 8.2 \text{ nm} \]

B. Large area scan:
50 x 50 µm
\[ \text{rms} = 301 \text{ nm} \]

Detailed scan: 1 x 1 µm
\[ \text{rms} = 7.6 \text{ nm} \]
Fig. 4

A. Cell number on day 1 after seeding

<table>
<thead>
<tr>
<th>PS</th>
<th>Glass</th>
<th>Nano_Si</th>
<th>Micro_Si</th>
<th>Nano_NCD</th>
<th>Micro-nano_NCD</th>
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B. Cell Number on day 3 after seeding

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<th>Micro_Si</th>
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C. Cell number on day 5 after seeding

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Fig. 5
Fig. 6

**XTT test**

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- I. PS
- II. Nano_NCD
- III. Micro-nano_NCD

Absorbance

- I, II
- I, III
- II, III
Fig. 8

Cell spreading area

<table>
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<th>III. Micro-nano_NCD</th>
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Cell area (µm²)