Plasma deposited composite coatings to control biological response of osteoblast-like MG-63 cells

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Abstract. The successful osseointegration of a bone implant is greatly dependent on its ability to support cellular adhesion and functions. Deposition of thin composite coatings onto the implant surface is a promising approach to improve interactions with cells without compromising implant bulk properties. In this work, we have developed composite coatings, based on hexamethyldisiloxane (HMDS) and detonation nanodiamond (DND) particles and have studied adhesion, growth and function of osteoblast-like MG-63 cells. PPHMDS/DND composites are of interest for orthopedics because they combine superior mechanical properties and good biocompatibility of DND with high adherence of HMDS to different substrata including glass, metals and plastics. We have used two approaches of the implementation of DND particles into a polymer matrix: pre-mixture of both components followed by plasma polymerization and layer-by-layer deposition of HMDS and DND particles and found that the deposition approach affects significantly the surface properties of the resulting layers and cell behaviour. The composite, prepared by subsequent deposition of monomer and DND particles was hydrophilic, with a rougher surface and MG-63 cells demonstrated better spreading, growth and function compared to the other composite which was hydrophobic with a smooth surface similarly to unmodified polymer. Thus, by varying the deposition approach, different PPHMDS/DND composite coatings, enhancing or inhibiting osteoblast adhesion and functions, can be obtained. In addition, the effect of fibronectin pre-adsorption was studied and was found to increase greatly MG-63 cell spreading.

1. Introduction

Biomaterials for bone regeneration should support cellular adhesion and growth, maintain cellular differentiation and functions and also should have mechanical properties similar to the native bone [1-4]. Since materials designed as bone implants differ in their capability to attach cells many surface modification techniques, aiming to enhance and accelerate anchorage of bone cells to the implant surface are under investigation. An approach to improve surface properties of the implants without
compromising the overall structure of the material is deposition of thin coatings onto the implant surface. Plasma deposition is a cost effective and economic method for the preparation of thin and regularly structured layers on bone implants that has been gaining importance for the last several years as a tool to modify a material surface. A major advantage of this technique is that by simple adjusting of the process parameters from one monomer different polymers could be obtained [5]. Hexamethyldisiloxane is a widely used monomer in plasma polymerization due to its volatility, cheap availability and relatively non-toxic nature. By implementation of nanodiamond particles into a HMDS matrix during plasma polymerization it is possible to obtain highly dense, pinhole-free and well-adherent films on a variety of substrates like conventional polymers, glass and metals. Nanodiamonds have received great attention in recent years because they exhibit many properties that are attractive for orthopedic implants, such as extremely high hardness, chemical and thermal resistance, high fracture toughness and low coefficient of friction [6]. Moreover, due to their non-toxicity, high biocompatibility and feasibility to be modified with different macromolecules nanodiamonds can be used to improve interactions with bone tissue cells [7, 8].

Here we have compared the ability of two DND/PPHMDS composite layers, obtained by different procedures to support cell adhesion, growth and function by MG-63 cells. This cell line is a commonly used model for osteoblast cells because they express many of the characteristics of normal osteoblasts. We have correlated biological response with the surface properties of composite materials to provide an insight toward controlling osteoblast behavior by manipulating diamond-containing composite surfaces. Although there are several investigations on adhesion of osteoblast cells and cytotoxicity of ND coatings the biological response of osteoblasts toward diamond coatings is still not clear and has not been sufficiently studied to date.

2. Materials and methods

2.1. Preparation of plasma polymer films

Three groups of plasma polymer films were prepared for the purposes of this study: plasma polymerized hexamethyldisiloxane (PPHMDS) and two polymer composites of PPHMDS and DND, named as DND1 and DND2. As control surfaces cover glasses (CG) were used.

ND particles used in PPHMDS/DND composites were synthesized by detonation of carbon-containing explosive mixtures as previously described in [9] and were characterized by FTIR-spectroscopy, oxidative titration, and pH-measurement as described in [10]. For preparation of DND1 films 0.03 g DND powder was added to 30 ml HMDS and the suspension was treated ultrasonically for 15 min, then continuously stirred on a magnetic stirrer at 20°C and 275 rpm immediately before deposition aiming to homogenize well DND particles in the monomer. The deposition of PPHMDS/DND films on the CG was performed at the following parameters: current density 0.04 mA/cm², monomer flow rate of 5 revolutions in the valve for 15 minutes.

DND2 films were prepared by consequent deposition of PPHMDS as a first layer on CG at a current density 0.08 mA/cm² and monomer flow rate 10 l/h for 15 min, followed by plasma deposition of ethanol suspension of DND particles on the PPHMDS film at a current density 0.16 mA/cm² and monomer flow rate 10 l/h for 10 min. DND suspension was prepared by dispersion of DND powder in ethanol (C₂H₅OH), followed by ultrasonic treatment for 15 min and intensive stirring on the magnet stir at 20°C and 275 rpm. After the deposition the samples were washed with deionized water and air dried. All films were deposited onto commercially available cover glasses (Menzel Glaesser) in a vacuum chamber with a system for glow discharge previously described [9].

2.2. Surface characterization of plasma polymer films

The morphologies of the films were observed under an atomic force microscope (AFM, Solver PRO, NT-MDT, Russia). To obtain information about the wettability of samples water contact angles were measured through the sessile drop method using the Easy Drop analyzer (FM40, Kruss, Germany).
2.3. Cell culture
A human osteosarcoma cell line, MG-63 (CRL-1427, American Type Culture Collection), passage 23 to 26, was used for cell experiments cultivated under the conditions previously described [11].

2.4. Initial cell adhesion assay
MG-63 cells with a concentration of $2 \times 10^4$ cells/ml were seeded on polymeric films, individually placed in 24-well cell culture plates (Nunc, Denmark). Before cell seeding half of the materials were pre-adsorbed with 20 μg/ml fibronectin (Sigma, Germany) for 30 min at RT. Cells were incubated for 2 hours at 37°C in humidified air in serum-free culture medium (DMEM, Sigma). At the end of incubation non-adherent cells were removed by washing with PBS and phase-contrast picture of adherent cells were taken. The pictures were further used for counting the cell number and measuring the cell area by Image J software.

2.5. Cell proliferation assay
Cell proliferation was determined at the 1st and the 7th day of incubation using a modified lactate dehydrogenase assay (LDH, Roche Diagnostics, Germany), following the manufacturer’s instructions.

2.6. Alkaline Phosphatase Activity assay
To measure ALP activity cells on polymer films were washed with TBS and lysated with 5 mM MgCl$_2$/0,5%Trton X100. The cell lysates were incubated with p-nitrophenylphosphate (Sigma, Germany) at alkaline conditions (pH=10) for 2 hours and the reaction was stopped with 0,5N NaOH. The amount of p-nitrophenol liberated was measured by microplate reader at 405 nm.

2.7. Statistical Analysis
Cell experiments were conducted in triplicate and the results are presented as mean values ± standard deviation (SD). For statistical evaluation the student’s test has been applied. The significance was determined at p ≤0.05.

3. Results and discussion

3.1. Characterization of plasma polymer films
Our results showed that modification of HMDS with DND particles by two different approaches of plasma polymerization resulted in different surface properties of the obtained composites which in turn affected cell behaviour. DND2 films prepared by subsequent deposition of monomer and particles had higher surface roughness and hydrophilicity compared to the other composite layers (DND1) obtained by pre-mixing of monomer with the nanodiamond. AFM scans of the sample surfaces, shown in figure 1, revealed that the surface of DND2 is much rougher compared to PPHMDS and DND2 films probably because DND particles aggregate during their deposition onto PPHMDS layer and thus making the surfaces rough while DND1 composite has similar to PPHMDS smooth surface.

![AFM 3-D images of polymeric and composite films deposited on cover glasses.](image1.png)

Figure 1. AFM 3-D images of polymeric and composite films deposited on cover glasses.
Water contact angle (WCA) measurements, summarized in table 1, show that both composite layers differed significantly in respect to their hydrophilicity. While DND1 is hydrophobic similarly to unmodified PPHMDS, DND2 is moderately hydrophilic which is most probably due to the fact that the nanodiamonds are in ethanol, rendering them hydrophilic and resulting in a hydrophilic surface.

| Sample – name     | WCA [°] |
|-------------------|---------|
| PPHMDS            | 81±2    |
| PPHMDS/DND1       | 84±2    |
| PPHMDS/DND2       | 58±2    |

Table 1. Water contact angle (WCA) of plasma films deposited on cover glass.

Based on the literature, we assumed that DND2 would be preferred by osteoblasts, because rougher surfaces have been shown to promote the attachment, growth and differentiation of bone cells [12, 13]. On the other hand, it is well known that mammalian cells interact better with hydrophilic, rather than hydrophobic surfaces. Therefore higher hydrophilicity of DND2 suggested better interactions with cells. It is also believed that the hydrophobicity does not affect directly cells, but by changing the biological activity of the adsorbed adhesive proteins [14]. Since FN has been isolated and identified as a main protein responsible for the adhesion of cells in vitro, such variations in the biological activity of a material can be explained by the different conformation of adsorbed FN. On hydrophobic surfaces cells cannot reorganize adsorbed FN, because FN is bound very strongly to the material while on hydrophilic materials FN is loosely bound, its cell binding domains are expressed resulting in more cell contacts.

3.2. Cell adhesion

We have focused mainly on the initial adhesion of cells, estimating cell morphology, the number of attached cells and cell spreading area. In addition, we have studied cell growth and function which are other indicators of biocompatibility of the material. Phase-contrast micrographs showed that at the 2nd hour of incubation the majority of MG-63 cells on plain films remained rounded and small in size which is indication of poor cell adhesion (figure 2). On DND2 osteoblasts seem to be more spread out compared to the other materials. When materials were pre-coated with FN cell adhesion improves significantly as can be concluded from the polygonal morphology of cells. The largest cells were observed on PPHMDS.

![Cell adhesion images](image-url)

Figure 2. Overall morphology of MG63 cells cultured for 2 hours on plain (upper panel) and FN pre-coated (low panel) cover glass (A), PPHMDS (B), PPHMDS/DND1 (C) and PPHMDS/DND2 (D), bar 50 μm.
Results from counting the number of attached cells demonstrated that plasma polymerized films varied in their ability to support cell attachment. As is shown in figure 3 the greatest number of attached MG-63 cells were found on DND2, in both groups, plain and FN-coated. Surprisingly, pre-adsorption with FN did not improve cell adhesion on materials. Only on controls (CG) and DND1 FN pre-adsorption increased the number of attached cells while on PPHMDS and DND2 even reducing in cell attachment was observed. Cell attachment characterized only partially cell-material interactions. More information can be received from cell spreading, which is expression of an active physiological process. The extent of cell spreading was quantified by measuring the average cell area following digital image analysis of phase-contrast pictures. MG-63 cells demonstrated reduced spreading on DND1 compared to PPHMDS and DND2 ($p < 0.05$) in both, plain and FN-coated materials (figure 4).

On plain surfaces the highest cell spreading area was estimated on DND2 suggesting that the second method of modification of the polymer with DND stimulated better spontaneous spreading of MG-63 cells.

After coating with FN the spreading area on all materials significantly increased, assuming that the materials adsorbed and organized well FN, which in turn promotes cell attachment and spreading. However, the highest average spreading area of MG-63 cells was found to be on PPHMDS films despite their hydrophobicity.

### 3.3. Cell proliferation

Another parameter characterizing cell behaviour in contact with biomaterials is cell growth. In general, MG-63 cells grew well on all tested materials, because an increase in their LDH activity from the 1st to the 7th day was observed (figure 5). The highest cell proliferation rates were found on both DND composites compared to unmodified PPHMDS and control CG. There was no apparent difference in cell growth on the two composite layers although the cell adhesion and spreading differed significantly.

### 3.4. Alkaline phosphatase activity

Cell function was investigated by measuring the alkaline phosphatase activity of MG-63 cells cultured for 7 days on different plasma polymer films. Alkaline phosphatase (ALP) is a membrane–anchored enzyme, widely used as a marker for early osteogenic differentiation. It enhances the local concentration of inorganic phosphate and thus participates in the initiation of the mineralization of the bones. As can be seen from figure 6 MG-63 cells expressed low levels of alkaline phosphatase activity on all studied materials which did not increase significantly after one week incubation. On the first day, the enzyme activity was highest in the control cells (CG) and the lowest in DND2 however the differences were not statistically significant. On day 7, the cells plated on DND-modified materials
showed higher ALP-activity compared to the unmodified polymer and control. The highest enzyme activity was measured in MG-63 cell incubated on DND2 materials suggesting that the second method of modification induced spontaneous osteogenic differentiation of cells.

**Figure 5.** Cell proliferation of MG-63 cells cultured for 7 days on different surfaces.

**Figure 6.** ALP activity of MG-63 cells cultured for 7 days on different surfaces.

### 4. Conclusion

Generalizing our results we can conclude that the modification of biomaterials with nanodiamond particles can improve the adhesion, growth and function of osteoblast-like cells and therefore these materials can be used to induce osteogenic differentiation even without the addition of soluble osteogenic factors. The method of the modification however results in different surface properties that alter the biological response of cells.

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