Study of detonation nanodiamond – plasma polymerized hexamethildisiloxan composites for medical application

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Abstract. The present study reports on how detonation nanodiamond (DND) - plasma poly(hexamethyldisiloxane) composites (PPHMDS) affect osteoblast cell behavior. It has been established that various modified DND nanoparticles (Ag-DND and Si-DND) can be readily integrated into virtually all polymer matrices. In particular, PPHDMS composites have been developed over the past few years because of the variety of their application as medical devices and implants. By incubation of MG-63 osteoblast-like cells on the surface of DND (Ag-DND and Si-DND) - PPHMDS composite, we tested the hypothesis that DND-based polymer composites can influence the adhesion behavior of MG-63 osteoblast-like cells. Morphological and structural characterization of DND, Ag-DND and Si-DND powders was carried out by XRD, HRTEM and EDS. For the study of the composite layers, deposited on cover glass (CG), FTIR spectroscopy has been performed in order to determine if the DND nanofiller can potentially modify the structural and chemical dynamics of the polymer matrix. The kinetic of static water contact angle of composite surfaces as a function of the as-used nanofiller DND’s in polymer matrix was measured The results with MG-63 osteoblast-like cells suggest the potential of using DND-based polymer composites for application in engineering implantable scaffolds and devices.

1. Introduction
An important goal of materials science is the development of interfaces that integrate the functions of living cells and materials. The development of materials that serve as substrates for adherent cells is important in a range of basic and applied programs. Recently there has been a growing interest in the synthesis of composite functional materials exhibiting novel physico-chemical properties, involving integration of inorganic nanomaterials into a polymer matrix [1]. Numerous siloxan-based materials including polymerized hexamethyldisiloxane (PPHMDS) chosen for this study have been developed.
Plasma poly (hexamethyldisiloxane) (PPHMDS) has a long history of exploitation in a variety of applications, because it is easy to prepare with the well established technology of plasma polymerization [2, 3], it is non-toxic, with a very low surface tension, and it neither dissolves nor swells in a cell culture medium [4]. On the other hand, nanodiamond carbon nanostructures are of interest due to the combination of unique properties inherent to diamond and the specific surface structure of particles facilitating its functionalization [1]. One class of nanodiamond particles with 4 nm primary particle size is the so-called detonation nanodiamond (DND), which is synthesized by detonation of carbon-containing explosives [1, 5]. Recently, DND nanoparticles have been modified, by means of appropriate chemical reactions, and silver (Ag) and silicon (Si)-based nanoparticles being produced. The antimicrobial effects of Ag ions are well recognized, as well as Ag nanoparticles being highly efficient against bacterial colonization and at the same time allowing adhesion and spreading of mammalian cells [2]. Production of (Si)-based nanoparticles is proposed to prevent DNDs from forming aggregates [6].

The aim of this work is the design of novel thin polymer films filled with modified DND nanoparticles. The importance of studying the interaction of osteoblast cells with polymers and polymer-based nanocomposites is their increasing use in many biological and medical applications [2, 4 and 8].

2. Materials and methods

2.1. Substrate

Prior to deposition of the films, the cover glass substrates (of diameter 12 and 16 mm) were cleaned by standard wet chemical procedure and for 5 min in a reactor chamber by plasma at 133 Pa air pressure and 0.08 mA/cm² current density.

2.2. Surface modification of DND nanoparticles

DND particles were synthesized by detonation of trinitrotoluene and hexogen at high pressure and high temperature [5]. A method to produce DNDs with a uniform metal particle distribution over the DND aggregate surface has recently been proposed [9]. The method is based on the interaction of metal salts (in our case AgNO₃) with the functional carboxyl groups (-COOH) present on the surface of the aggregates in DND suspensions [6]. For the production of the second group of DND (Si-DND), the method of silanization was applied.

2.3. Deposition of DND (Ag-DND and Si-DND) - PPHMDS composite

For plasma polymerization, the plasma polymerization equipment was used as describe in [8]. The monomer used in this experiment was a guaranteed grade of hexamethyldisiloxan (HMDS) [(CH₃)₃-Si-O-Si-(CH₃)₃] → 99% Merck (Germany). The CG substrates were placed on a Teflon plate between the electrodes. The plasma polymerization conditions were controlled by two parameters: the monomer flow rate and the current density of the glow discharge [3]. The deposition rates of plasma-polymerized (PP) films were determined by the quartz crystal microbalance technique. Using the several polymerization conditions for HMDS, it was found that the deposition rates were proportional to the polymerization time [10]. For deposition of the composites on the CG substrate, plasma polymerization equipment was used [3, 10] and the following technological regime was applied: DND particles were added to the HMDS monomer in the concentration of 0.01 g/100 ml and prior to deposition by ultrasonic shaking for 15 min. After that the container with the suspension was placed on the magnet stirrer and continuously stirred at 25°C and 275 r.p.m. Plasma polymerization was performed for 20 minutes at 0.08 mA/cm² current density at about 133 Pa gas phase pressure. The thickness of the composite layers was approximately 200 nm as determined by a Dektak Stylus Profile System (Veeco Instruments Inc., Woodbury, NY, USA). After the deposition, the samples were washed under a flow of Milli-Q pure water and dried in air. Three groups of samples were thus
prepared named in the text as 6-DND_PPHMDS, Ag-DND_PPHMDS and Si-DND_PPHMDS. As controls PPHMDS deposed on CG substrate were also prepared.

2.4. FTIR spectroscopy measurement
The surface functional groups of the composite layers were studied by FTIR spectra obtained on a Bruker-Vector 22 FTIR-spectrometer by averaging 128 scans. All spectra were recorded at room temperature using standard instrument settings. The assignment of the absorption bands is based on experience with molecular organic compounds and literature data. The frequencies quoted are believed to be within 2 or 3 cm⁻¹ of the true values.

2.5. Cell culture and cell adhesion assay
Human osteoblast-like MG-63 cell line (purchased from ATCC, USA) was used as a model system to examine the effects of surface coatings on osteoblast adhesion, spreading and overall morphology, as well as on FN matrix organization. Cells were maintained in Dulbecco’s modified Eagle medium (Gibco, 11960-044) supplemented with 10% FBS, 1% penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate (Gibco, 11360-039), in a humidified atmosphere of 5% CO₂ in air. The culture medium was exchanged every second day. Upon reaching confluence the cells were detached with 0.05% trypsin-EDTA (Gibco, 25200-072), inactivated with FBS after detachment (approx. 5 min), and the cells being re-cultured or used for the experiments. To investigate the overall morphology and initial cell adhesion and spreading, 2 x 10⁴ cells/well were seeded to each surface in 2.0 ml serum free medium. One set of samples has been pre-coated with proteins as described above while another one was studied as plain controls. After 2 h of incubation, the cells were stained for actin and vinculin, using specific antibodies (phalloidin and mouse anti-human vinculin, respectively). Focal adhesions formed after 2 h of incubation of MG-63 cells on the samples were visualized using specific mouse monoclonal antibody (Sigma) against vinculin (1:800 dilution) in 1% bovine serum albumin (BSA). This step was followed by goat anti-mouse Cy3 conjugated secondary antibody (Jackson ImmunoResearch). After incubation with the antibodies, the samples were mounted and viewed on an inverted fluorescent microscope and at least three representative images were obtained.

3. Results and discussion

3.1. Surface contact angle measurements
Figure 1 shows the alteration of the statistic contact angle of the PPHMDS and composite films DNDs(6-DND, Ag-DND, Si-DND) studied as a function of time. The contact angle of the polymer (PPHMDS on CG) films decreased when modified DND (Si-DND, Ag-DND) were used. Decreasing
of the contact angle means that the surface of the grown polymer film becomes more hydrophilic. The increases in the case of the 6-DND nanofiller are explained with the increased amount of oxygenated residues on the surface of the nano-particles [11]. When Ag-cations are introduced on the DND surface, the total polymer film surface becomes even more hydrophilic due to the covalent bonds formed between the Ag cations and Si-O residues.

### 3.2. FTIR spectroscopy measurements of composites

On figure 2 and 3, the FTIR spectra of the DND powders and composite layers are presented respectively. One can note the significant differences in the surface functionality between the used initial DND powders and the prepared polymer layers on the CG substrate. The spectrum of plasma grown composites on the CG substrate possessed the characteristic adsorption bands of polymer methyl groups as stretching and deformation modes from the C–H bond in various different tetrahedral arrangements of the CH₃ groups connected to the silicon atoms as (Si-(CH₃)₃, Si-(CH₃)₂, Si-(CH₂)n-Si) with a maxima between 400 - 600 and 950–900 cm⁻¹; the Si–O rocking mode of the Si–O–Si bond at 460 cm⁻¹ and an asymmetric stretching vibration of the Si–O–Si group with a maximum between 1130–1040 cm⁻¹; a stretching vibration of the Si–C bond with a maximum at 780 cm⁻¹ from the Si–CH₃ group and carbonyl and hydroxyl groups (–OH, >C=O) in the region 1500-1700 cm⁻¹. In the spectrum, the characteristic peaks for the sp bonded carbon of the DND 1550 cm⁻¹, a peak due to nanocrystalline diamond at approximately 1200 cm⁻¹ and sp² bonded carbon at 1160 cm⁻¹ can be clearly observed. So, the surface of the CG DND_PPHDMS is characterized with many functional groups of the type –OH, >C=O, Si–O–Si, Si–O–C and different tetrahedral CH₃ which provide the surface with both hydrophobic and hydrophilic centers. The appearance of the pronounced peak at 550 cm⁻¹ in the spectrum of the CG_Ag-DND_PPHMDS indicates the existence of Ag cations. The splitting of the peak at 1550 cm⁻¹ into two peaks and the appearance of a broad band at 1800 cm⁻¹ confirmed the location of Ag cations at the grain boundaries of the diamond nanoparticles due to interaction of the silver ions either with the polymer Si-OH groups, or with the diamond functional groups (–OH, >C=O, Si–O–C). In the case of CG_Si-DND_PPHMDS, definite peaks at around 500, 1200-1300, 1470 and 1600 cm⁻¹ point towards an increased content of amorphous carbon in the grown layer due to the silanization of the DND surface.

**Figure 2.** FTIR spectra of the DND powders: 6-DND (1); Si-DND (2) and Ag-DND (3).

**Figure 3.** FTIR spectra of the composites: CG_6-DPPHMDS (1); CG_Ag-DND_PPHMDS (2); CG_Si-DND_PPHMDS (3).
3.3. Cell experiments

3.3.1. FTIR spectra of coated with FN composites

In the present study we employed the FTIR measurements to evaluate the structural changes of composite surfaces after their covering with FN. The FTIR absorption spectra, shown in figure 5, demonstrated that both composite surfaces Si-DND_PPHMDS and Ag-DND_PPHMDS interact with the protein (FN). In both cases, a significant increase of the 1800 cm\(^{-1}\) peaks and rearrangement of the peak’s intensity in the region 1400 - 1650 cm\(^{-1}\) occur in comparison with the spectra without FN shown in figure 3. This observation is due to an enhanced reaction between the FN molecule and the existing functional groups on the composite surfaces and its irreversible adsorption on the surface of the material. In figure 4 the protein easy interaction of the initial CG substrate covered with FN for 30 minutes is demonstrated. After 24 hours of intensive washing in DI water the same samples were measured again. Both the FTIR spectra slightly differ, which confirm that an irreversible adsorption occurs even at the beginning of the CG treatment with the FN solution.

![Figure 4. FTIR spectra of initial CG substrate (1); CG_FN after 30 min. treatment with FN (2); the same CG_FN sample measured again after 24 hours.](image)

![Figure 5. FTIR spectra of CG_Si-DND_PPHMDS (1) and CG_Ag-DND_PPHMDS (2). composite surfaces, coated with FN.](image)

3.3.2. Cytotoxicity test

To estimate the cytotoxicity of DND materials we incubated a suspension of DND (6-DND) and DND-modified particles (Si-DND and Ag-DND) with osteoblast-like cells (MG63) for 24 hours in a serum-containing medium.

![Figure 6. Overall morphology of MG63 osteoblasts cells incubated for 24 hours on plain cover glass slips with suspension of 6-DND and modified Si- and Ag-DND nanoparticles respectively; magnification 20x.](image)
At the end of incubation the overall cell morphology was visualized by fluorescent diacetate (FDA) - a dye agent that stains only vital cells (figure 6). Our results showed some decrease in the number of viable cells in all the tested samples, more pronounced in cells cultured with pure 6-DND particles while in cell cultures incubated with Si- and Ag-DND particles the reduction in cell numbers was lower. Nevertheless, the attached cells were well spread, even better than the control samples, suggesting unaffected cell functions. Therefore, DND powders seem to be promising materials for some applications with cells. However, to learn more about how they affect the specific cell adhesion and functions, more detailed investigations have to be performed.

3.3.3. Cells experiments with MG-63 osteoblast cells cultured on the composite surfaces

The purpose of the presented study was to evaluate the protein adsorption and cell adhesion on the PPHMDS-DNDs composites.

![Figure 7](image)

Figure 7. Actin staining of MG63 osteoblast-like cells, cultured for 2 hours on plain surfaces (a) CG-control; (b) Si-DND_PPHMDS (c) Ag-DND_PPHMDS; and coated with FN surfaces (d), (e) and (f) respectively; magnification 20 x.

The results from actin staining, shown in Fig. 7 revealed that the FN coating improved significantly cell adhesion and spreading (d,e,f) in contrast to the uncoated (plain) composite surfaces (a,b,c), suggesting that on the composite surfaces the FN most probably adsorbs in conformation that is more suitable for the cells. Vinculin staining of MG63 osteoblast-like cells, cultured for 2h on the composite surface cover glass controls revealed that vinculin was also better expressed on composite surfaces. It was reported [12, 13] that PPHMDS is an hydrophobic material and incubated MG63 cells are rounder, while on the PPHMDS, filled with DNDs, the cell spreading improved (figure 6 and 7- d, e, f). On the other hand, hidrophilization of the composite surfaces results in a slight decrease in spontaneous cell adhesion (Fig. 7 and 8-a, b, c). Previously we have reported that plasma polymerization of Hexamethildisiloxan (HMDS) under certain conditions [10, 12]; followed by NH$_3$ plasma treatment was effective for decreasing the PPHMDS surface contact angle and effective for protein adsorption as well as cell proliferation. In the present study we demonstrated that DND-based polymers are most effective for protein and cells attachment. The plasma polymerization of
DNDs PPHMDS composites possess a potential for surface modification of various implant materials. The adsorption behavior of cell adhesive proteins, particularly fibronectin (FN), on the surface of novel composites (DND-based polymer) subsequently treated with NH₃ plasma are in progress.

![Image](image1)

**Figure 8.** Vinculin staining of MG63 osteoblast-like cells cultured for 2 hours on plain surfaces (a) CG-control; (b) Si-DND_PPHMDS; (c) Ag-DND_PPHMDS; and coated with FN surfaces (d), (e) and (f) respectively; magnification 63 x

### 4. Conclusion

Novel composites have been produced using modified DND (Ag-DND, and Si-DND) nanoparticles as fillers in PPHMDS layers and their biological response using the MG63 osteoblast cells model has been tested. It was shown that by varying the DND filler, the chemical property of hydrophobic PPHMDS films can be modified. This study demonstrates that via appropriate material design it is possible to generate surfaces allowing considerably better attachment of the osteoblast cells.

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