TÜBİTAK

Turkish Journal of Biology

http://journals.tubitak.gov.tr/biology/

Research Article

Turk J Biol
(2018) 42: 195-203
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doi:10.3906/biy-1711-26

Increased elastic modulus of plasma polymer coatings reinforced with detonation nanodiamond particles improves osteogenic differentiation of mesenchymal stem cells

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Received: 08.11.2017 • **Accepted/Published Online:** 29.03.2018 • **Final Version:** 27.04.2018

Abstract: In the present study we demonstrated that composite PPHMDS/DND coatings with elastic moduli close to those of mature bone tissue (0.2–2.8 GPa) stimulated growth and osteogenic differentiation of human adipose-derived mesenchymal stem cells (hAD-MSCs). Composite coatings were prepared by a method of plasma polymerization (PP) where detonation nanodiamond (DND) particles in different amounts (0.1, 0.5, and 1 mg/mL) were added to hexamethyldisiloxane (HMDS) before plasma deposition. This method allows variation only in the reduced elastic modulus (Er´) with increase in the particle concentration, while the other surface properties, including surface wettability and topography, did not change. The response of hAD-MSCs to the increasing stiffness showed an effect on adhesion and osteogenic differentiation but not on cell proliferation. Matrix mineralization and cell spreading were maximized on PPHMDS/DND coatings with the highest elastic modulus (2.826 GPa), while the differences in proliferation rates among the samples were negligible. In general, PPHMDS/DND coatings provide better conditions for growth and osteogenic differentiation of hAD-MSCs in comparison to glass coverslips, confirming their suitability for osteo-integration applications. Additionally, our findings support the hypothesis that biomaterials with elasticity similar to that of the native tissue can improve the differentiation potential of mesenchymal stem cells.

Key words: Detonation nanodiamonds, organosilicone, bone implants, stiffness, cell adhesion and growth

1. Introduction

The demand for orthopedic implants shows strong growth, as a result of the aging population and the widespread prevalence of physically active lifestyles (Woolf and Pfleger, 2003; Iorio et al., 2008; Lu et al., 2009; Bhandari and Schemitsch, 2010; Fayaz et al., 2011; Amini et al., 2012). Despite the large number of materials produced for orthopedics (metallic, plastic, ceramics, composites, etc.) their extensive clinical application is limited by poor tissue integration (Viceconti et al., 2000; Reyes et al., 2007; Nuss and von Rechenberg, 2008; Chen and Thouas, 2015). To address this issue many efforts have focused on the modulation of implant properties and stimulation of bone–implant interface mineralization (Navarro et al., 2008; Agarwal and García, 2015; Civantos et al., 2017). One approach to improve implant surface is deposition of plasma polymerized coatings with favorable properties. Plasma polymerization is a process of monomer fragmentation and recombination in plasma with easy control of the process parameters and uniform and high-dense surface deposition. The high branched and crosslinked structure of plasma polymers leads to excellent adhesion ability to solid substrates. Thus the need for additional "glue" material between the coatings and the implants is avoided (Pihan et al., 2009; Szili et al., 2011; Sardella et al., 2016). Furthermore, by adding fillers to the polymer network new plasma polymers with stable mechanical properties can be developed (Picraux and Pope, 1984; Ratner, 1992; Sioshansi and Tobin, 1996).

Recently mechanical properties (e.g., elasticity, stiffness) have been recognized as a factor guiding mesenchymal stem cell (MSC) differentiation (Engler et al., 2006; Clause et al., 2010; Li et al., 2011; Kshitiz et al., 2012). Some papers have reported enhanced osteogenic

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differentiation of MSCs on stiffer materials (Chen and Jacobs, 2013; Vertelov et al., 2016). It seems most likely materials with elastic modulus close to those of the natural bone tissue will enhance osteogenic differentiation. Therefore, implant coatings should have a high elastic modulus (in the range scale of GPa) similar to that of the natural bone tissue (Yu et al., 2015).

Organosilicone polymers are frequently considered for biomedical application because of their low cost, easy fabrication, and favorable biological properties such as low inflammatory and immunogenic response (McInnes and Voelcker, 2009). However, drawbacks for their use in bone tissue engineering are their high hydrophobicity, which hampers cell adhesion, and poor mechanical properties. Reinforcement with nanoparticles (NPs) can improve the mechanical properties of polymers (Domun et al., 2015). When NPs interact with the polymer matrix, this results in a larger contact area compared to macrosized fillers. If this area is strong, the load will be transferred from the polymer matrix to the NPs (Sun et al., 2016). Appropriate selection of the NPs along with certain treatment can significantly increase the quality of the contact surface and thus leads to a great improvement in mechanical properties. Among different types of NPs (carbon nanotubes, grapheme, nanoclay, nanosilica, etc.) detonation generated nanodiamond (DND) has gained worldwide attention as a reinforcing material because of its inexpensive large-scale synthesis, extreme hardness combined with nanosize, and excellent biocompatibility (Holt, 2007; Burleson et al., 2009; Lim et al., 2009; El-Say, 2011). A dramatic increase in tensile modulus, fracture energy, and elastic modulus has been achieved by addition of small amounts of DND to different polymers (PVA, PC, PMMA, epoxy, and PLA) (Maitra et al., 2009; Kurkin et al., 2010; Zhao et al., 2010; Neitzel et al., 2011, Protopapa et al., 2011; Neitzel et al., 2012; Mochalinand Gogotsi, 2015). However, PP composites based on hexamethyldisiloxane (HMDS) and DND particles with different stiffness have not been reported to date.

The aim of the present work was to reinforce HMDS with DND particles to develop PPHMDS/DND composite coatings with elastic modulus in GPa scale and improved osteogenic potential. HMDS is an organosilicone widely used for plasma polymerization. Previously, we have shown that it can be easily modified with different NPs and the composite layers had better cell-contacting properties than the pure polymer (Pramatarova et al., 2011). We studied here the adhesive behavior, growth, and differentiation potential of hAD-MSCs because of a lack of information in the literature about the effect of increased stiffness of plasma deposited composite coatings reinforced with nanodiamond particles on the osteogenic potential of hAD-MSCs. Thus, the present work would enlighten the field of biomaterials knowledge and be valuable for future development of bone implants with improved cell-contact properties.

2. Materials and methods

2.1. Composite coatings preparation

Composite PPHMDS/DND coatings with three different concentrations of DND particles (0.1, 0.5, and 1 mg/mL) were prepared. DND particles were first mixed with the monomer (HMDS, purity >99.99%, Merck, Germany), followed by plasma polymerization and deposition for 10 min at a current density 0.32 mA/cm^2 on commercially available glass coverslips (CG, Menzel-Glaeser). The process is described in detail in Pramatarova et al. (2011). After the deposition, the samples were washed with deionized water and air-dried. For cell proliferation and mineralization studies, the materials were pre-coated with an adhesive protein, fibronectin, in order to ensure attachment of sufficient cells for the cell experiments.

DND powder was obtained from detonation soot, delivered from YTM ARGE A.S. (İstanbul, Turkey) and purified at the University of Tasmania, Australia, from non-diamond carbon and metal impurities with a mixture of HNO_3, H_2SO_4, and HCl with ratio 70%/20%/10% as described in detail in Mitev et al. (2013, 2014).

2.2. Elastic modulus, water contact angle, and surface roughness measurements

Nanomechanical analysis was performed by the Hysitron TI950 TriboIndenter (Hysitron, Inc., Minneapolis, MN, USA) using a diamond Berkovich tip as an indenter probe. Continuous measurement mode (CMX) was used to obtain depth profiles of the mechanical properties of the PPHMDS/DND coatings with three different concentrations of DND particles. CMX was carried out by applying a small dynamic force oscillation continuously superimposed and scaled to a quasistatic force during loading. The harmonic force and the corresponding displacement signal were analyzed according to nanometric dynamic mechanical analysis, which gave a variety of mechanical parameters such as storage modulus and hardness (Rokosz et al., 2015; Sepitka et al., 2016). The CMX indentation function was prescribed by quasistatic force (Pqstat) in the range from 0.25 to 250 μN. The dynamic actuation force (Pdyn) was prescribed in the range from 0.35 to 10.68 μN at a frequency of 200 Hz. For each sample, average values were obtained from 16 indents in a 4 × 4 matrix with a separation step of 10 μm.

Water contact angle (WCA) measurements were done by the sessile drop shape method under ambient conditions. The values of the static water contact angles were measured on a Drop Shape Analyzer DSA10 (Krus GmbH, Germany).

Topographic features of the pure PPHMDS used as control and composite (PPHMDS/DND) samples with different nanodiamond concentrations were examined by atomic force microscopy (AFM) (Solver PRO, NT-MDT, Russia) in tapping mode in air. Samples were scanned with the standard Si cantilever with a force constant of 22 N/m and at a resonance frequency of 325 kHz (tip radius was 10 nm and the tip length was 95 μm). Average surface roughness (Ra) was measured from representative images on 3 × 3 μm^2 area and the scan rate was set at 1.3 Hz.

2.3. Cell culture

Human adipose-derived mesenchymal stem cells (Lonza, Spain), passage 2, were used in this study. The cells were maintained in Dulbecco's modified Eagle medium (DMEM, Invitrogen, USA), supplemented with 10% fetal bovine serum (FBS, Invitrogen) at 37 °C in a humidified atmosphere containing 5% carbon dioxide. The medium was exchanged every second day. After reaching 70% confluence the cells were detached with 0.1% trypsin/EDTA solution and seeded on FN precoated materials at a density of 2 × 10^4 cells/mL for proliferation and differentiation experiments. Osteogenic differentiation was induced on day 7 by replacing DMEM with osteogenic medium (DMEM, supplemented with 10% fetal bovine serum, 150 μM ascorbic acid, 10 mM β-glycerophosphate, and 10 nM dexamethasone) for up to 14 days.

2.4. Initial cell adhesion assays

The initial adhesion of hAD-MSCs to the composites was evaluated at 2 h of incubation on plain and FN-coated PPHMDS/DND composites and control glass coverslips with respect to overall cell morphology, average cell spreading area, and expression and organization of actin cytoskeleton and focal adhesion contacts. For this, cells were triple stained with phalloidin, vinculin, and Hoechst to visualize the actin cytoskeleton, focal contacts, and cell nuclei. At the end of incubation the samples were washed with phosphate buffer saline (PBS), and the attached cells were fixed and permeabilized with 4% paraformaldehyde solution and 0.5% Triton X-100. Nonspecific binding was blocked with 2% bovine serum albumin (Sigma, Spain). The cells were then incubated with primary antibody (mouse anti-human vinculin, Sigma, Spain), followed by a mixture of secondary antibody (Alexa 555-conjugated goat anti-mouse antibody) for vinculin, FITC-labeled phalloidin for actin, and Hoechst (10 μg/mL, Invitrogen) for nuclei detection. The cells were finally mounted in Fluoroshield mounting media (Sigma) and imaged on an Axio Observer Z1 (Zeiss, Germany) microscope, supplied with a digital camera. Micrographs at magnification 10× were captured and further used for quantification of the cell spreading area by Image J software.

2.5. Cell proliferation assay

The growth of hAD-MSCs was examined by using a Cell Counting Kit (CCK-8, Sigma-Aldrich) following the manufacturers' instructions. Briefly, on day 4 of incubation onto the composites the cells and materials were transferred to new plates, fresh medium containing CCK-8 reagent in ratio 1:10 was added, and the cells were incubated for 4 h at 37 °C in the dark. Newly synthesized yellow formazan dye was determined spectrophotometrically at 450 nm wavelength using a standard microplate reader (BioRad).

2.6. Matrix mineralization assay

Osteogenic differentiation of hAD-MSCs was evaluated on day 14 of osteogenic stimulation by their capacity to mineralize the extracellular matrix (ECM). The extent of mineralization was determined by Alizarin Red S staining (Sigma). At the end of incubation, the cells were fixed with 4% paraformaldehyde solution for 10 min, washed with PBS, and incubated with Alizarin Red S dye (2% in distilled water, pH 4.1–4.3) for 15 min. The unbound dye was removed by an extensive wash with distilled water and the samples were observed microscopically. For quantification of the calcium deposits the incorporated Alizarin Red S dye was dissolved with cetylpyridinium chloride solution and measured at λ = 570 nm in a multiplate spectrophotometer (BioRad).

2.7. Statistical analysis

All experiments were carried out in triplicate. The error bars indicate standard deviations. Statistical significance between the groups was calculated using ANOVA. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Surface characteristics

Surface properties of PPHMDS/DND substrates were characterized with respect to their surface elasticity, surface hydrophobicity, and topography. The resulting reduced storage modulus (Er′) values for each of the substrates used in this study in the contact depth hc = 100 nm are shown in Figure 1. A significant increase in storage modulus (more than eightfold) was evident after adding the highest concentration of DND particles of 1 mg/mL.

To determine if different amounts of DND particles in HMDS would alter the surface wettability and topography of the PPHMDS/DND composites we measured the materials' water contact angles and roughness. There was no difference in WCA between the substrates with different storage modulus. All composites were hydrophobic, with WCA higher than 90°, as was expected for the chemical nature of the polymer (Figure 2). This indicates that the addition of DND particles in different concentrations to HMDS did not affect the wettability of the resulting composites.

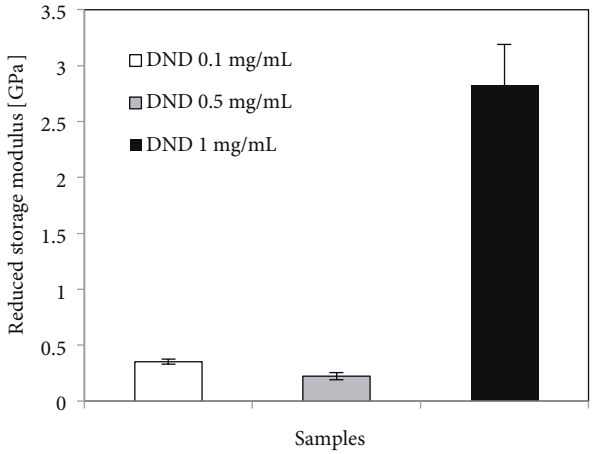

Figure 1. Reduced storage modulus of PPHMDS/DND composite materials with different concentrations of DND particles.

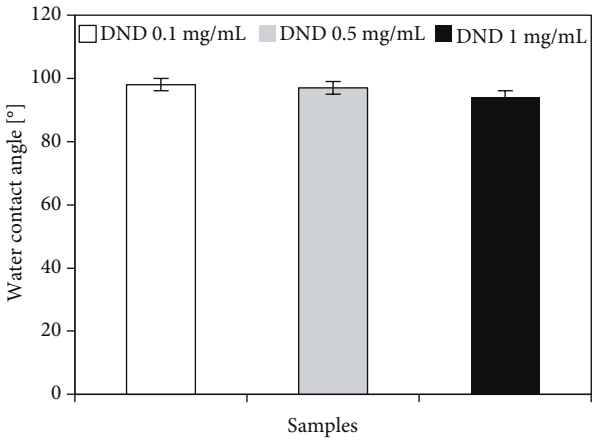

Figure 2. Water contact angle of PPHMDS/DND composite films with different concentrations of DND particles.

Representative AFM images of the sample surfaces, shown in Figure 3 and quantitative roughness analysis, revealed that the surface morphology of the pure polymer (PPHMDS) and composites is very similar. The measured average surface roughness (Ra) of all samples is about 0.6 nm, meaning that the materials' surface is flat and did not change with addition of the NPs. The similarity in the surface morphology among the pure polymer and the composites indicates that the nanodiamonds are embedded in the polymer matrix.

3.2. Initial cell adhesion

Using substrates of three different values of Er′, we assessed first the influence of substratum elasticity on the initial adhesion of the hAD-MSCs. The cells were seeded onto the plain and FN-coated PPHMDS/DND films and control cover glasses at a subconfluent density to minimize intercellular interaction. On the plain materials hAD-MSCs were found to attach and spread randomly and their morphology was rounded or stellate-like with many cytoplasmic extensions (Figure 4, upper panel). Slightly larger cells were observed on the composites with the highest Er′. The absence of vinculin staining, which reflects cell-to-substrate adhesion sites, and actin stress fibers suggests poor cell adhesion on the plain composites. After pre-coating of the PPHMDS/DND composites with FN (Figure 4, lower panel) an increase in cell spreading and assembly of actin stress fibers with increasing of the concentration of DND particles was observed. On the composites with DND concentration 0.1 mg/mL (Er′ = 0.352 GPa) the cells were smallest in size, exhibited

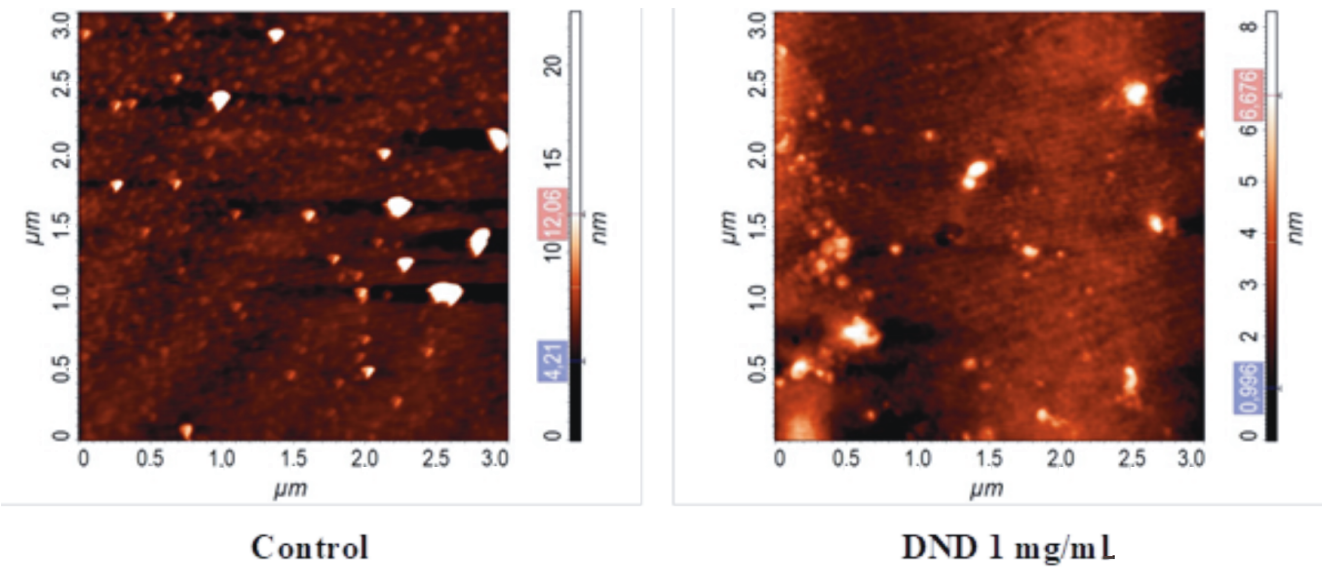

Figure 3. AFM micrographs of control and PPHMDS/DND composite films with 1 mg/mL concentrations of DND particles.

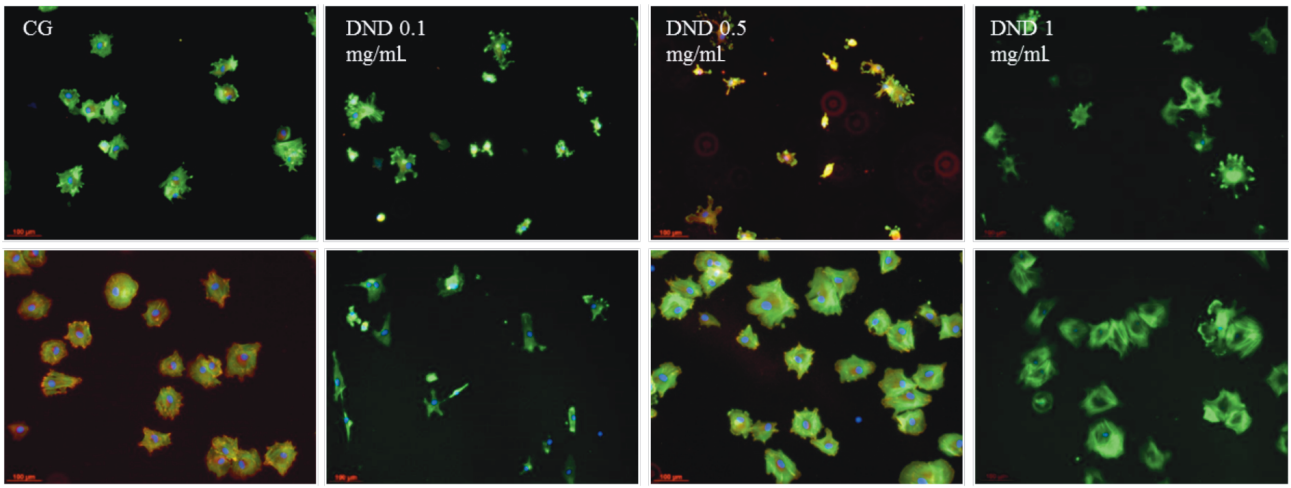

Figure 4. Initial adhesion of hAD-MSCs after 2 h incubation in serum-free medium on plain (upper panel) and FN-coated (lower panel) PPHMDS/DND composite films with different concentrations of DND particles; triple staining for DNA (blue), filamentous actin (green), and vinculin (red).

mainly elongated and irregular morphology, and spread randomly similar to the cells on the plain composites. On the composites with DND concentration 0.5 mg/mL the cells became larger and rounded similar to the cells on the control CG but also many cells with polygonal shape were observed. On the composites with DND concentration 1 mg/mL the cells acquired polygonal, well-spread, and flattened morphology. F-actin was poorly expressed and not well organized on the composites with concentration of DND 0.1 mg/mL, while on the substrates with concentration of DND of 0.5 mg/mL actin was well expressed but only a small amount of it was organized in stress fibers. On the substrates that were eight times stiffer (Er′ = 2.826 GPa) the cells assembled the actin cytoskeleton into a robust stress fiber network. Focal contacts were visible mostly on DND with 0.5 mg/mL and 1 mg/mL, while on the composites with DND with 0.1 mg/mL vinculin was not expressed.

The results from the quantitative morphometric analysis of cell area clearly showed an increase in cell spreading area with increase in concentration of DND particles in both groups: plain and FN-coated composites (Figure 5), although the differences among some samples are not statistically significant (between 0.1 and 0.5 mg/mL for the plain and between 0.5 and 1 mg/mL for FN-coated composites). Expectedly, the cells spread better on FN precoated cover glass and composites (the right group of columns) when compared to the plain (uncoated) cover glass and composites (the left group of columns).

3.3. Cell proliferation

The effect of matrix stiffness on the proliferative capacity of the hAD-MSCs was investigated next (Figure 6). The analysis of the data revealed that on day 4 after cell seeding on FN-coated substrates the proliferation rate did not increase as the materials' stiffness increased. The cells exhibited comparable proliferation rates on all FN-coated composites with statistically insignificant ($P > 0.05$) lower values for the composites with lowest Er′ (0.223 GPa). This suggested that the proliferation of hAD-MSCs was not dependent on the materials' stiffness. Compared to the control cover glass, however, the cell growth on DND/PPHMDS composites was enhanced approximately twice ($P < 0.05$), meaning that PPHMDS/DND composites can promote hAD-MSCs proliferation.

3.4. Osteogenic differentiation

The final step in this study was to determine whether substratum elasticity influenced osteoblastic differentiation of hAD-MSCs. The cells were seeded on FN-coated PPHMDS/DND substrates with three different stiffness values and on a cover glass and after reaching the confluence on day 7 the culture medium was supplemented with osteogenic factors for the next 14 days of culture. Using the Alizarin red staining method to determine the extent of ECM mineralization, which is an indicator of a mature osteoblast phenotype, we found that hAD-MSCs incubated on all tested PPHMDS/DND composites deposited mineral to a greater extent compared to the control CG (Figure 7) ($P < 0.05$ for the control and PPHMDS/DND with Er′ = 2.826 GPa). This suggests that PPHMDS/DND composites can favor osteogenic differentiation of hAD-MSCs. The cells on the surface with the lowest and highest storage modulus (Er′ = 0.223 GPa and Er′ = 2.826 GPa) mineralized the ECM to the smallest and greatest degree, respectively.

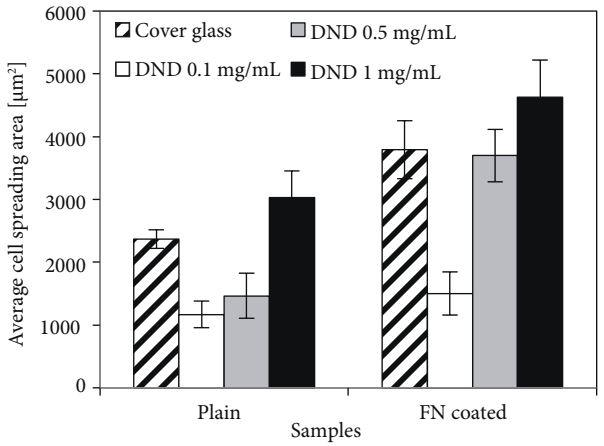

Figure 5. Spreading of hAD-MSCs after 2 h incubation in serum-free medium on plain and FN-coated PPHMDS/DND composite films with different concentrations of DND particles.

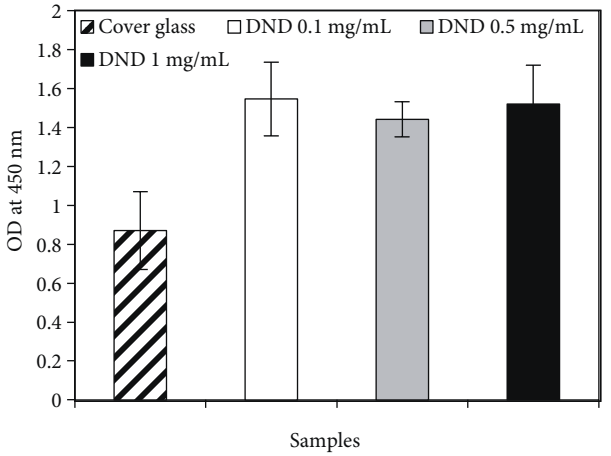

Figure 6. Growth of hAD-MSCs after 4 days incubation on FN-coated PPHMDS/DND composite films with different concentrations of DND particles.

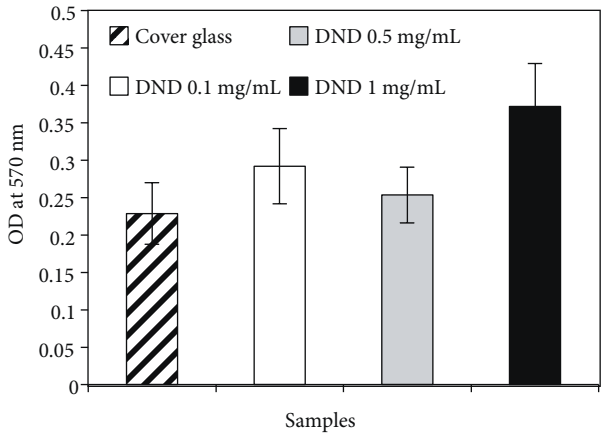

Figure 7. Mineralization of ECM by hAD-MSCs after 21 days incubation on PPHMDS/DND composite films with different concentrations of DND particles.

4. Discussion

Because of the great interest in the development of biomaterials capable to control stem cell differentiation for bone tissue engineering (O'Keefe and Mao, 2011; Wang et al., 2014) the present study was designed to understand whether manipulating matrix stiffness by the addition of DND particles in a polymer matrix influences the behavior and osteogenic differentiation of hAD-MSCs. This objective comes from the hypothesis that extracellular matrix elasticity regulates stem cell differentiation and is supported by a number of recent reports (Engler et al., 2006; Lv et al., 2015). However, the results in the literature are controversial mostly because of the different materials and cells used for the different in vitro studies. To address the response of the hAD-MSCs to matrix stiffness we used an organosilicone composite, PPHMDS/DND, based on the fact that its mechanical properties can be manipulated easily by varying the relative amounts of nanoparticles. We added DND particles in three different concentrations: 0.1, 0.5, and 1 mg/mL, and obtained composite films with values of the elastic modulus close to those of natural bone tissue. To investigate, however, the role only of substratum elasticity in stem cell differentiation it would be ideal to tailor the substrate elastic modulus without altering other material properties such as surface wettability, chemistry, and roughness. To achieve this we used here a method of plasma polymerization where DND particles were first mixed with HMDS before plasma deposition and thus PPHMDS/DND composite films with different elasticity but with similar other surface properties were prepared. The choice of the method was based on our recent work comparing two incorporation approaches of the nanodiamond particles in the PPHMDS matrix during plasma polymerization. We found that when the NPs were embedded inside the polymer matrix (first approach) the resultant composite films possessed surface properties similar to those of the pure polymer, while the deposition of DND particles as an outer layer onto plasma polymerized HMDS resulted in alteration in the material's surface properties (Keremidarska et al., 2015). Therefore, when varying the concentration of DND particles in HMDS by the first approach as expected only the elastic modulus of the different PPHMDS/DND composites changed. The surface hydrophilicity and roughness were not influenced as was confirmed by water contact angle and AFM measurements. It is possible the increase in the elastic modulus with DND content was not linear because the processes running during the formation of composite coating are very complicated and are influenced by many

factors: the degree of interaction between DND surface groups and polymer matrix, DND content, dispersion, and surface functional groups. The nonlinear increase in elastic modulus of our composites with the increased DND concentration might be attributed to the possible aggregation and uneven distribution of DND particles in the polymer matrix. It decreases the effective area of DND particles to interact with the polymer and leads to weak bonding between DND particles and PPHMDS and inability to form a stable network at low particle concentration.

Many authors have shown that materials with different mechanical properties support cell spreading to different degrees (Discher et al., 2005; Chowdhury et al., 2010). Since cell functions have been reported to be directly linked to cell spreading (Spiegelman and Ginty, 1983; Janmey, 1998) it could be argued that the effects of substratum elasticity are actually due to a change in cell spreading (McBeath et al., 2004; Mammoto and Ingber, 2010). Here we investigated the ability of hAD-MSCs to spread on plain and FN-coated PPHMDS/DND composites with elastic modulus in the range of 0.223–2.826 GPa. An improved cell spreading with an increase in the concentration of DND particles was found on both plain and FN-coated composites. However, the average cell spreading area on plain composites was smaller in comparison to those on the FN-coated film, suggesting that the adhesive ligand (FN) increased the sensitivity of hAD-MSCs to substratum elasticity. This was not surprising because cells actually interact through their integrin receptors with the protein layer adsorbed on the materials surface and formation of the adhesion complexes depends on the quantity and conformation of the coated protein (Geiger et al., 2001). Thus the cells "feel" the mechanical characteristics of the substrate and respond with different morphology and spreading area (Wozniak and Chen, 2009). Cells cultured on stiff substrates are characterized with increased assembly of actin stress fibers and by the recruitment of vinculin from cytoplasmic pools to the sites of focal adhesion. Probably ECM rigidity regulates cytoskeletal tension by controlling the spreading of the cells, which in turn is a critical determinant of cell fate.

Despite the different cell spreading and morphology cellular proliferation demonstrated lack of sensitivity to substratum elasticity within the studied variation in reduced storage modulus. Our results coincide with the observations reported by Rowlands et al. (2008), who found that over a certain Young's modulus value materials' elasticity does not affect MSC proliferation. Hosseini et al. (2012) also obtained similar results for endothelial cells when cultured on polydimethylsiloxane/nanocilica composites with different NP concentrations. However, it must be highlighted that the authors used materials with much lower range of elasticity, in kPa and MPa scale, as well as different cell types.

In contrast to cell proliferation osteogenic differentiation demonstrated sensitivity to differences in substratum elastic modulus. The strongest matrix mineralization was detected for stem cells cultured on the composite with the highest DND concentration. This suggests that the mechanical queues of the 1 mg/mL DND sample, together with the soluble osteogenic factors and FN as adhesive ligand, exert the best synergistic effect on the MSCs' fate, triggering osteogenic differentiation processes. Our data are in agreement with the results obtained by other authors showing that the stiffer are the substrates, the higher is the osteogenic differentiation degree of the MSCs (Gandavarapu, 2014).

Finally, of particular interest is the finding that both cell proliferation and osteogenic differentiation of hAD-MSCs demonstrated increased rates compared to the control CG. It means that these materials are able to provide conditions that support the cell expansion of adipose-derived mesenchymal stem cells and their mineralization. This is a critical step in biomaterial development because in tissue engineering a large amount of precursor cells is required to restore the damaged bone tissue.

In summary, the results presented herein demonstrate that variation in elastic modulus of PPHMDS/DND composites in the range of 0.2 to 2.8 GPa influences the adhesion and differentiation but not proliferation of human adipose-derived mesenchymal stem cells and that FN precoating enhanced the sensitivity of stem cell to material's stiffness. PPHMDS/DND composites with the highest elastic modulus (2.826 GPa) stimulated to the greatest degree bone matrix mineralization in hAD-MSCs. The obtained composite PPHMDS/DND coatings induced sufficient proliferation and osteogenic differentiation in the hAD-MSCs especially after FN-precoating. These data suggest our composites could be considered suitable materials for modification of already practically applied bone tissue implants. Moreover, they can be used as model substrata to study the effect of a material's elasticity on cell behavior, because the deposition by plasma polymerization of the mixture containing DND particles in HMDS was established as a perspective approach for preparation, tailoring, and alterations of material properties, allowing variation only in the elastic modulus but not the other surface properties.

Acknowledgments

This work was supported by the National Scientific Council of Bulgaria [project No. DFNI BO-18/28.11.2012] and by the Czech Science Foundation [project No. 16-14758S]. Production and characterization of the nanodiamond samples used in this work was supported by the Australian

Research Council [grant DP110102046 to UTAS]. The authors would like to dedicate this work in honor of Assoc Prof Lilyana Pramatarova. She collaborated with us from the beginning of this study but sadly passed away before the manuscript preparation. We are grateful for her cooperation, enthusiasm, and support. Prof Dr George Altankov (IBEC, Barcelona) is acknowledged for his kind gift of human adipose-derived mesenchymal stem cells and for allowing access to the microscopy apparatus.

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