

Effect of nanodiamond modification of siloxane surfaces on stem cell behaviour

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Abstract. Mesenchymal stem cells (MSCs) hold a great promise for use in many cell therapies and tissue engineering due to their remarkable potential to replicate indefinitely and differentiate into various cell types. Many efforts have been put to study the factors controlling stem cell differentiation. However, still little knowledge has been gained to what extent biomaterials properties influence stem cell adhesion, growth and differentiation. Research utilizing bone marrow-derived MSCs has concentrated on development of specific materials which can enhance specific differentiation of stem cells e.g. osteogenic and chondrogenic. In the present work we have modified an organosilane, hexamethyldisiloxane (HMDS) with detonation nanodiamond (DND) particles aiming to improve adhesion, growth and osteodifferentiation of rat mesenchymal stem cells. HMDS/DND films were deposited on cover glass using two approaches: premixing of both compounds, followed by plasma polymerization (PP) and PP of HMDS followed by plasma deposition of DND particles. We did not observe however an increase in rMSCs adhesion and growth on DND-modified PPHMDS surfaces compared to unmodified PPHMDS. When we studied alkaline phosphatase (ALP) activity, which is a major sign for early osteodifferentiation, we found the highest ALP activity on the PPHMDS/DND material, prepared by consequent deposition while on the other composite material ALP activity was the lowest. These results suggested that DND-modified materials were able to control osteodifferentiation in MSCs depending on the deposition approach. Modification of HMDS with DND particles by consequent plasma deposition seems to be a promising approach to produce biomaterials capable to guide stem cell differentiation toward osteoblasts and thus to be used in bone tissue engineering.

1. Introduction

About 40 years ago Friedenstein described for the first time spindle shaped stromal cells in bone marrow that proliferated and form colonies [1]. Cells were named colony-forming unit-fibroblasts (CFU-F) but later different authors used different synonyms such as mesenchymal stem cells (MSCs), skeletal stem cells [2], stromal stem cells, mesenchymal progenitor cells, etc. to define almost the same cell population [3]. Under defined *in vitro* conditions MSCs are able to differentiate into multiple cell types, e.g. osteoblasts, chondroblasts, adipocytes. Easy isolation and multipotent properties of MSCs make them an attractive choice for different clinical applications [4].



Trauma-related skeletal deformities and disorders are among the most widely spread diseases in the world - over 40 % of the population suffers from them. Many of the patients require bone graft substitutes to repair the injury or defect [5]. An ideal bone-graft substitute must provide biomaterials and growth factors for osteoinduction and progenitor cells for osteogenesis. Until recently the growth factors and cytokines were considered as the most potent factors directing *in vitro* differentiation of MSCs. However differentiation driven by the substrate would be a better proposition in terms of implanting tissue-engineered constructs to elicit a specific cellular response [6]. Synthetic materials offer a greater degree of control of material's properties, reproducibility and scalability over naturally occurring materials. Organosilanes, a class of synthetic polymers, meet with a number of indicators the requirements of medical practice, including excellent inertness, smoothness, stability and biocompatibility. They have a rare combination of properties that makes them unique: high hydrophobicity, solubility in non-polar solvents, high chain flexibility, low cohesive energy, very low surface tension, good thermal stability and resistance to UV. In particular, PPHMDS has the advantages to be easily processed and fabricated by the method of plasma polymerization and its properties can be readily controlled by varying process parameters. However, the low mechanical stability and high hydrophobicity are serious disadvantages for bone mimicry [7]. Modification with nanodiamonds (NDs) seems to be promising in improving mechanical and cell-contacting properties of siloxanes due to the superior mechanical features and good biocompatibility of nanodiamonds [8]. ND particles can be deposited onto polymer surface or can be implicated into monomer structure. Here, we have compared both methods of modification of PPHMDS with detonation generated ND particles and how both methods affect surface properties and consequently, the stem cell behaviour. Although there are some papers concerning effect of nanodiamond coatings on osteoblast adhesion [9] there is currently a lack of knowledge about how nanodiamond-modified materials can control stem cell adhesion and the subsequent related osteodifferentiation.

2. Materials and methods

2.1. Synthesis and modification of PPHMDS surfaces

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. Preparation and characterization of the materials are described in [10]. As control surfaces cover glasses (CG) were used.

2.2. Cell culture

Rat mesenchymal stem cells were isolated from bone marrow of femurs and tibias of 4-week-old male rats according the centrifugation method of Dobson et al. [11]. Cells were cultured under conditions previously described in [12].

2.3. Cell adhesion assay

To assess initial cell adhesion rat mesenchymal stem cells were seeded in a concentration of $2 \cdot 10^4$ cells/ml onto tested materials, precoated for 30 minutes with 20 $\mu\text{g/ml}$ fibronectin (Sigma, Germany) and were incubated for 2 hours in serum-free media. Phase-contrast micrographs of the attached cells were taken by Zeiss Axiovert 25 inverted microscope (Germany). The pictures were further used for measuring the cell spreading area by image analysis software (Image J).

2.4. Cell growth assay

Evaluation of cell proliferation ability of rMSCs was performed by measuring the activity of the cytoplasmic enzyme lactate dehydrogenase (LDH) with LDH assay (Roche Diagnostic, Germany). Cells were seeded in initial concentration of $2 \cdot 10^4$ cells/ml and cultured for one week on the films in

media supplemented with 10% foetal bovine serum (Sigma, Germany). At the first and the seventh day cells were treated according to the manufacturer's instructions. LDH activity was assessed by measuring the optical density with a plate reader at wavelength $\lambda=492$ nm.

2.5. Alkaline phosphatase activity assay

At the 1st and 7th day of incubation of the cells on the materials the levels of expression of the enzyme alkaline phosphatase (ALP) were evaluated. For this aim cells were rinsed with Tris buffered saline (TBS) and lysed with Triton X-100. 50 μ l of cell lysates were mixed with 100 μ l enzyme substrate-p-nitrophenylphosphate (Sigma, Germany) and incubated at 37° C for 2 hours. Optical density was measured at wavelength $\lambda=405$ nm.

2.6. Statistical analysis

All experiments were conducted in triplicate. The results are presented as mean values $\pm$ standard deviation (SD). For statistical evaluation the Student's test has been applied. The significance was determined at $p \leq 0.05$.

3. Results and discussion

3.1. Characterization of PPHMDS surfaces

Engineering a successful biomaterial to establish optimal interactions with cells for good osteointegration requires knowledge about materials and how their properties influence cell behaviour [13]. Surface topography and wettability are the major materials' factors affecting cell-biomaterial interactions [14]. Therefore, we have characterized first PPHMDS surfaces in respect to their surface roughness and hydrophilicity by AFM and goniometry, respectively and then have studied stem cell behaviour. AFM measurements (data not shown here) revealed that nanodiamond modification increased the surface roughness of the materials, especially in the case of DND2 samples. Water contact angle data showed that the samples, obtained by consequent deposition of PPHMDS and DND were hydrophilic while the other PPHMDS/DND films were hydrophobic with WCA similar to pure PPHMDS film (figure1). In summary, both composite films have different roughness and wettability depending on the method of preparation.

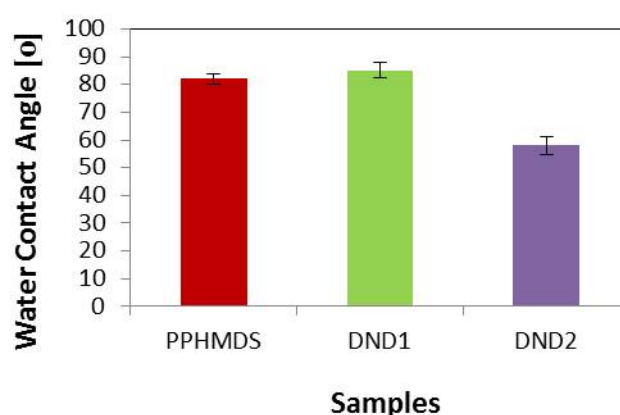


Figure 1. Water contact angle of the polymeric films.

3.2. Initial cell adhesion

Since cell processes such as adhesion, proliferation and differentiation are directly influenced by material's properties they can be used to assess the effect of material modification on cell behaviour. The initial cellular events taking place on biomaterials surface mimic to a certain extent the natural

adhesive interactions of cells with the extracellular matrix [15]. In fact, living cells do not interact directly with foreign materials. Upon contact of a biomaterial with physiological fluids *in vivo* or culture medium *in vitro* the first event that happened is adsorption of proteins such as fibronectin (FN), vitronectin (VN), fibrinogen (FG) on followed by attachment of the cells to the adsorbed protein layer. In order to mimic physiological conditions we have pre-adsorbed our materials with FN which is one of the most important proteins mediating cell adhesion [16]. Representative phase contrast images of living rMSCs incubated for 2 hours on various substrates are shown in figure 2. Surprisingly, well spread cells with polygonal morphology, suggesting better interactions with material, were observed mainly on unmodified PPHMDS films. Although the largest cells were observed on DND2 surfaces, in general the cells on DND-modified materials were mainly round with many protrusions.

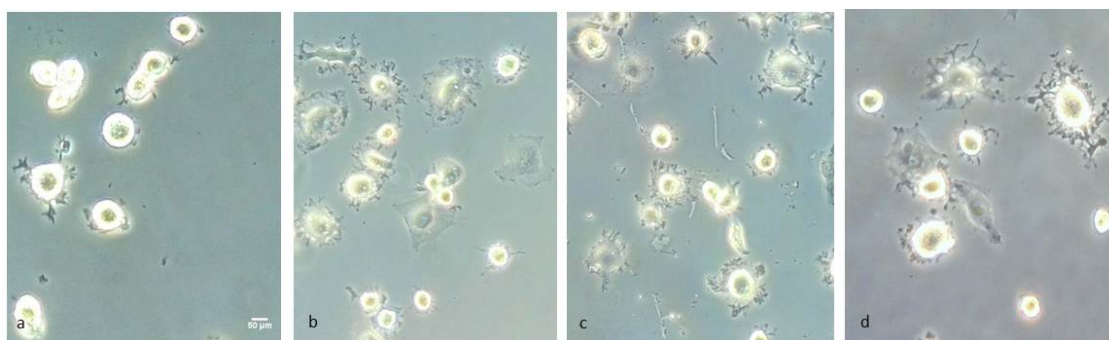


Figure 2. Phase contrast micrographs of rMSCs after 2 hours incubation on FN coated a)CG, b)PPHMDS, c)DND1 and d) DND2. Mag. 20x.

These results can be explained with adsorption and organization of FN. It is well known that fibronectin molecules adsorb preferentially on hydrophobic surfaces but usually they are tightly connected to the material and their cell binding domains stay hidden for the cells [17]. Therefore it is not uncommon that cells on the most hydrophobic DND1 showed the smallest average spreading area as it can be seen from figure 3.

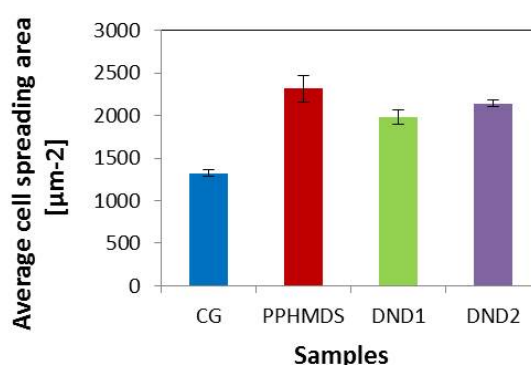


Figure 3. Image J measurement of cell spreading area of rMSCs after 2 hours incubation on FN coated polymeric surfaces.

Opposite, on hydrophilic surfaces FN undergoes greater extension of its dimer providing more binding sites for the mesenchymal stem cells and thus strength the cell-material interactions. In this context moderately hydrophilic DND2 should provide better substrate for cell adhesion. Moreover, DND2 surface is rougher than the other materials and there is data in literature about improved stem cell attachment on rough surfaces [18]. However our results from overall cell morphology and analysis

of cell spreading area surprisingly demonstrated that the hydrophobic and the smoothest PPHMDS favours better the cell binding and spreading of rMSCs compared to both DND-modified surfaces. Therefore not only hydrophobicity and surface roughness but also more surface properties must be taken into account to explain differences in stem cell behaviour on DND-modified and unmodified PHMDS surfaces.

3.3. Cell growth

Further, we have studied cell growth and differentiation in order to make connection between cell adhesion and growth and function of stem cells. We observed an increase in cell growth over the entire culture period (7 days) on all surfaces tested meaning that DND modification did not adversely affect rat MSCs. Among siloxane surfaces the highest proliferative ability in rMSCs at the 1st day was found to induce DND1 while at the 7th day - PPHMDS films (figure 4). However, compared to the control surfaces (cover glasses) the cell growth on siloxane and DND-modified siloxane surfaces was suppressed.

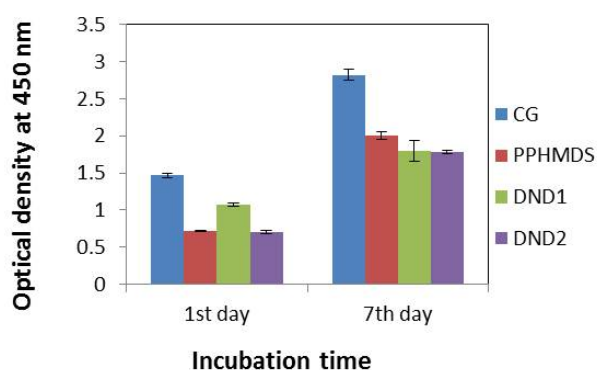


Figure 4. LDH assay of rMSCs on polymeric films.

3.4. Alkaline phosphatase activity

To fully characterize rMSCs behaviour on PPHMDS surfaces we measured ALP activity on days 1 and 7. Although rMSCs demonstrated comparatively low expression of the enzyme at the first day with insignificant differences between materials at day 7 dramatic increase in ALP activity was detected. As it can be seen in figure 5 the cells, except these, cultured on DND1, showed almost three times higher levels of the alkaline phosphatase. The strongest ALP activity in rMSCs was provoked by DND2 materials. Keeping in mind that activity of this enzyme is among the major markers for early osteodifferentiation [19], these results suggest that DND2 managed guide the stem cells toward osteogenesis.

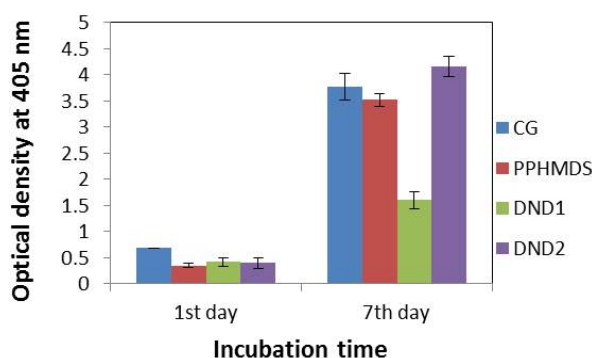


Figure 5. ALP assay of rMSCs on polymeric films.

4. Conclusions

Among the materials studied in this work unmodified PPHMDS films supported the greatest adhesion and proliferation of rMSCs. However, the most significant indication for osteodifferentiation stem cells exhibited after cultivation on the composite films obtained by consequent PP of HMDS and NDs. Therefore, we can conclude that for bone tissue engineering applications modification of polymeric films with nanodiamond particles by consequent deposition of the polymer and nanoparticles should be the method of choice because it induced in greatest degree differentiation of mesenchymal stem cells toward osteoblast cells.

Acknowledgements

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