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Adhesion, Viability and Differentiation of Adipose Tissue Derived Mesenchymal Stem Cells onto Micro/Nanostructured Polystyrene Substrates

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Abstract - Mesenchymal stem cells exhibit unique properties such as self-renewal and differentiation into different types of cells. To study *in vitro* these properties, micro/nanostructured surfaces that mimic the morphology of *in vivo* extracellular environment have been employed as substrates for stem cells culture and differentiation. In this work, micro/nanostrutured polystyrene surfaces fabricated through oxygen plasma etching were used to study the topography effect on adhesion, viability and differentiation of mesenchymal stem cells derived from rat adipose tissue (rMSCs). For this purpose, rMSCs were cultured on micro/nanostructured surfaces with average peak-to-valley height ranging from 50 to 500 nm, for 14 and 21 days, with either standard culture or osteogenic differentiation medium. Regarding cells cultured with standard medium, it was found that both their viability and morphology was negatively affected when cultured to micro/nanostructured surfaces with average peak-to-valley height ≥270 nm, while mineralization, as determined by Alizarin Red S staining, was slightly increased on the micro/nanostrutured surfaces compared to flat ones, indicating that micro/nanotopography did not affect osteogenic differentiation. On the other hand, when rMSCs were cultured with osteogenic differentiation medium on micro/nanostrutured surfaces, their viability and morphology were strongly affected, and a 30% reduction in mineralization compared to flat polystyrene was determined on surfaces with average peak-to-valley structures ranging between 90 and 480 nm. On the contrary, when cells were cultured with osteogenic differentiation medium on surfaces with peak-to-valley structures of 50 nm, mineralization was 1.7 times higher compared to flat surfaces. These results indicate that surface roughness ≥90 nm has a strong negative effect on viability, morphology and mineralization of rMSCs cultured in both media, while surface roughness of 50 nm exhibits a ~70% increase in mineralization of rMSCs compared to flat substrates when cultured in osteogenic medium. Thus, micro/nanotopography could be a useful tool to promote MSCs differentiation *in vitro*.

Keywords**:** mesenchymal stem cells, micro/nanostructured surfaces, polystyrene, oxygen plasma, morphology, mineralization, differentiation

1. Introduction

Mesenchymal stem cells (MSCs) are a powerful tool in the field of tissue engineering as well as of regenerative and personalised medicine, due to their unique characteristics and especially self-renewal and differentiation into specific types of cells (osteoblasts, myoblasts, adipocytes and chondrocytes) [1]. *In vivo*, the maintenance of stem cell phenotype or their differentiation to certain lineages is determined by their microenvironment, which is known as stem cell niche [2]. When cultured *in vitro* with traditional culture methods, however, stem cell differentiation is uncontrolled and inefficient leading to a heterogeneous cell population [3]. In order to overcome this issue several techniques have been exploited, such as

addition in the culture medium of appropriate growth factors and cytokines and more recently the use of micro/nanostructured surfaces as substrates for stem cell culture [4].

It is well documented that micro/nanostructured surfaces which mimic the morphology of *in vivo* extracellular environment could regulate many cell functions like adhesion, proliferation, morphology and differentiation [5]. To this end, micro/nanostructured substrates have been employed to investigate the role of topography in the differentiation pathway of MSCs [6-12]. It has been shown that micro/nanotopography could promote differentiation of MSCs, compared to flat substrates, even without the use of growth factors and biochemical cues [8, 13, 14]. It has also been reported that circular structures with diameter <50 μm promote MSC differentiation to adipocytes, whereas structures of different shapes with dimensions ranging between 50-100 μm favour differentiation to osteoblasts and chondrocytes [15-18]. Successful differentiation of MSCs has been also achieved onto micro/nano- pillars, pores, ridges grooves as well as on random structures [19].

E-beam lithography, laser interference lithography, laser holography, photolithography, soft lithography followed by embossing, polymer demixing, electrospining and plasma etching are some of the techniques so far employed to create micro/nanostructured surfaces to be implemented as cell culture substrates [20, 21]. Amongst these, plasma etching is gaining ground as a method for constructing micro/nanostructured surfaces for MSCs differentiation. For example, poly ether etherketone (PEEK) surfaces micro/nanostructured in oxygen plasma have been successfully used as substrates for the differentiation of MSCs to osteoblasts [22].

Our team has applied gaseous plasma to create hierarchical random micro/nanostructures on different polymeric surfaces, leading to substrates with controlled chemistry and topography, which have been exploited as substrates for protein microarrays and cell culture [23-26]. Regarding application of these substrates for cell culture, it was found that a roughness threshold above which the number of attached cells decreased dramatically existed for both primary human skin fibroblasts and 3T3 cells when cultured onto oxygen plasma nanostructured poly(methyl methacrylate) surfaces (PMMA) with different roughness height and fractal dimension [25]. Such micro/nanostructured surfaces have been also used as substrates for the enrichment of different cancer cells (A431 skin cancer, HT1080 fibrosarcoma, PC3 prostate cancer and A549 lung cancer cells) from mixtures with normal skin and lung fibroblasts, increasing enrichment ratios of cancer to normal cells in co-cultures ranging from by approximately 40 to 80 times [26].

Taking into account the literature reports regarding differentiation of MSCs to osteoblasts, as well as the wide variety and unique properties of the polymeric micro/nanostructured surfaces fabricated by gaseous plasma treatment from our team, in this work we investigated the differentiation potential of MSCs derived from rat adipose tissue (rMSCs) to osteoblasts onto oxygen-plasma micro/nanostructured polystyrene slides. In particular, rMSCs were cultured on micro/nanostructured polystyrene slides as well as on flat slides in presence of standard culture or osteogenic differentiation medium and several indicators of cell adhesion, viability and differentiation were determined at different time intervals.

2. Experimental

2.1. Materials

Bovine serum albumin (BSA), 4',6'-diamidino-2-phenylindol solution (DAPI), phalloidin Atto 488, alizarin Red-S and cetylpyridinium chloride were purchased by Sigma-Aldrich Co. (Taufkirchen, Germany). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin and 0.05%/0.02% (w/v) trypsin-EDTA solution in PBS were from Biochrom GmbH (Berlin, Germany). Polystyrene slides and StemPro Osteogenesis differentiation kit were from Thermo Fisher Scientific (Whaltham, MA). p-Phenylenediamine antifade mounting gel (Vectashield) was purchased from Vector Laboratories Inc. (Burlingame, CA). Paraformaldehyde was from AppliChem Inc. (Maryland Heights, MO).

2.2. Preparation and characterization of micro/nanostructured surfaces

Polystyrene slides, 1-mm thick, were etched with O_2 plasma in a homemade plasma reactor based on an Alcatel ICP MET instrument [27], under the following conditions: ICP power 2000 W, bias power 300 W, operating pressure 6 mTorr, mTorr, oxygen gas flow rate 100 sccm, electrode temperature 15 ^oC, and etching time 1, 2, 3 and 4 min. Surface roughness roughness was evaluated through scanning electron microscopy imaging with a JSM-7401F SEM instrument (JEOL, Europe) Europe) working at 30 kV. Samples were cleaved and observed at an angle θ of 45^o allowing the determination of the peak-peak-to-valley height from SEM images by dividing the observed height with sinθ to correct for the tilt.

2.3. Cell culture and seeding

Mesenchymal stem cells derived from rat adipose tissue (rMSCs) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine and 1% (v/v) penicillin/streptomycin at 37 ^oC in a saturated humid atmosphere containing 5% CO_2, until 70-80% confluence. Then, the cells were detached from the culture flasks using trypsin-EDTA, counted with a Neubauer hematocytometer and diluted with DMEM culture medium at a concentration of 3000 cells/cm^2 for seeding onto the polystyrene surfaces which were previously sterilized in a laminar flow hood through exposure to ultraviolet light for 20 minutes. The medium was renewed or replaced with osteogenic differentiation medium (OM) after 1-day culture, and from then on DMEM and OM was renewed every 3 days, until 14 or 21 days of culture were reached. All experiments were repeated at least three times in quadruplicates and the results were expressed as mean value of cells per cm^2 ± standard deviation (SD). The results were analysed statistically by the paired Student's t-test and considered significantly different at p values lower than 0.05.

2.4. Staining of cells cytoskeleton and nucleus

At the end of each culture period, the slides were washed with phosphate buffer saline, pH 7.4 (PBS), and the adherent cells were fixed with a 4% (w/v) paraformaldehyde solution, permeabilized with 0.1% (v/v) Triton X-100 and blocked with a 5% (w/v) BSA solution in PBS. After washing with PBS, cells were incubated with a 100 nM Phalloidin Atto 488 solution in PBS for 1 h, rinsed with PBS, and incubated with a 100 ng/ml DAPI solution in PBS for 5 min, for cytoskeleton and nucleus staining respectively. After washing with PBS, p-phenylenediamine antifade mounting gel was added onto the slides and coverslips were placed on top for observation under an epifluorescence microscope (Axioskop 2 Plus; Carl Zeiss, Hamburg, Germany) facilitated with appropriate filter pairs and a MicroPublisher 3.3 RTV CCD camera (QImaging, Surrey, BC, Canada) for image acquisition and processing with the Image Pro Plus v6.0 software.

2.5. Determination of mineralization

After each incubation period, cells were fixed with a 4% (w/v) paraformaldehyde solution for 20 min and incubated with a 2% (w/v) aqueous Alizarin Red-S solution, pH 4.2, for 30 min in the dark, under gentle rotation. Then, the surfaces were rinsed 4 times with water, incubated for 15 min with phosphate buffered saline in order to reduce non-specific Alizarin Red-S stain and observed under an optical microscope (Axioskop 2 Plus; Carl Zeiss, Hamburg, Germany). For the extraction and quantification of calcium mineral content, the surfaces were incubated with a 10 % (w/v) cetylpyridinium chloride solution in 10 mM sodium phosphate buffer, pH 7.0, for 15 min, and the alizarin Red-S concentration in the extracts was calculated by absorbance measurement at 560 nm using a Fluoroscan Ascent microtiter plate reader (Labsystems Diagnostics, Vantaa, Finland).

3. Results and Discussion

3.1 Characterization of micro/nanostructured polystyrene surfaces

The surface roughness, expressed as average peak-to-valley height of nanostructures, of polystyrene slides etched with oxygen plasma at bias power of 300 W for 1, 2, 3 and 4 min was determined by the respective SEM images presented in figure 1. As shown, the height of the micro/nanostructures increased with etching time. More specifically, after image analysis the average peak-to-valley height of nanostructures created was found to be 50, 90, 270 and 580 nm for etching times of 1, 2, 3 or 4 min, respectively.

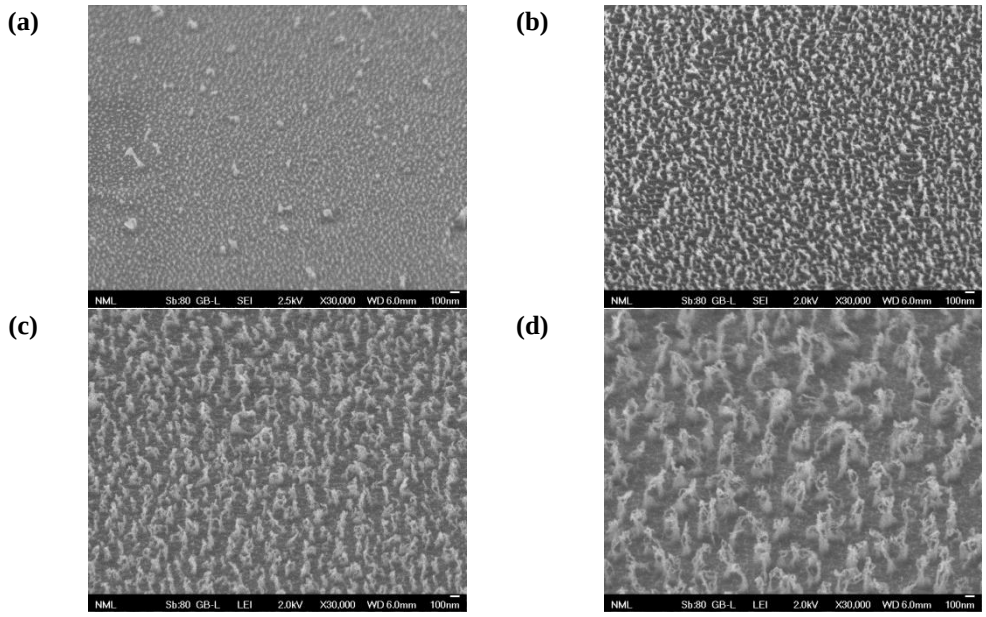

Fig. 1. SEM images of polystyrene surfaces treated with bias power 300 W for (a) 1 min, (b) 2 min, (c) 3 min and (d) 4 min. Images are titled at 45°. The scale bar corresponds to 100 nm.

3.2. Adhesion and proliferation of rMSCs on polystyrene micro/nanostructured surfaces

For the evaluation of nanotopography effect on rMSCs, cell adhesion and proliferation was initially determined. For this purpose, rMSCs suspensions were seeded on untreated and oxygen plasma treated polystyrene surfaces at a concentration of ~3000 cells/cm^2 and cultured for 14 and 21 days. In figure 2, the number of rMSCs adherent per surface area after 14 and 21 days of culture on the surfaces with different roughness either with standard culture medium (Fig. 2a) or osteogenic differentiation medium (Fig. 2b) is presented.

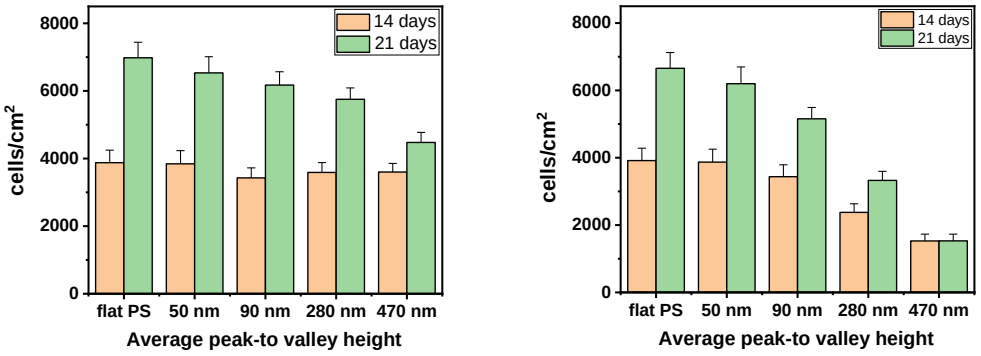

Fig. 2. Number of rMSCs adherent per surface area after 21 days of culture with (a) DMEM or (b) OM on untreated and O_2 plasma treated polystyrene surfaces with different average peak-to-valley height values.

As shown in figure 2a, when rMSCs were cultured in DMEM, the number of adherent cells after 14 days of culture were similar in all surfaces tested. However, when the culture was prolonged to 21 days the number of adherent cells

on the surfaces with roughness ≥ 90 nm was decreased compared to flat and 50 nm rough surfaces indicating decreased proliferation rate.

Regarding rMSCs proliferation and viability when cultured with osteogenic differentiation medium, it was found that that nanotopography exhibited a strong effect on the number of adherent cells after 14 days of culture and furthermore the the proliferation rate when the cells were cultured for 21 days was considerably inhibited, especially for the surfaces with with roughnes ≥90 nm. More specifically, as shown in figure 2b, on surfaces with roughness higher than 90 nm, the number number of adherent differentiated rMSCs after 21 days of culture was reduced by 50% compared to the untreated surfaces, while on the surfaces with the highest average peak-to-valley height (480 nm) the adherent cell population was reduced by 80% compared to flat surfaces indicating considerable inhibition of proliferation.

These findings indicate that the non-differentiated rMSCs were less affected from the surface nanotopography compared to osteogenic differentiated ones. Nonetheless, surfaces with roughness up to 50 nm seems not to affect considerably differentiated rMSCs viability and proliferation.

3.3. Effect of micro/nanotopography on rMSCs morphology

The effect of nanotopography on adherent rMSCs morphology was also investigated. Therefore, after culture for 14 and 21 days both with standard culture and osteogenic differentiation medium, the adherent cells were stained with fluorescent dyes for the visualization of their cytoskeleton and nucleus. In figure 3, fluorescence microscope images of rMSCs cultured for 21 days on flat and oxygen plasma treated polystyrene surfaces with average peak-to-valley structures height of 50 nm and 270 nm are provided. Regarding rMSCs cultured with standard culture medium, slight shrinkage of the cells was observed when cultured on 50 nm rough surfaces whereas on 270 nm rough surfaces considerable shrinkage and change of the cells morphology to an asteroid-like shape was observed. The morphology of rMSCs cultured with osteogenic differentiation was also influenced by nanotopography. More specifically, on surfaces with average peak-to-valley height structures of 50 nm, the adherent cell morphology was the same with that of cells cultured on flat surfaces, whereas at surfaces of higher roughness (e.g., 270 nm), the adherent rMSCs cytoskeleton was shrunk compared to those grown on flat surfaces. Thus, a 20% cytoskeleton shrinkage was determined for rMSCs cultured on surfaces with average peak-to-valley height 50 nm compared to cells adherent on flat surfaces. Taking into account, cell viability and proliferation as well as their morphology, oxygen plasma treated polystyrene surfaces with average peak-to-valley height structures of 50 nm were selected for further experimentation in order to define the effect of surface micro/nanotopography on rMSCs differentiation.

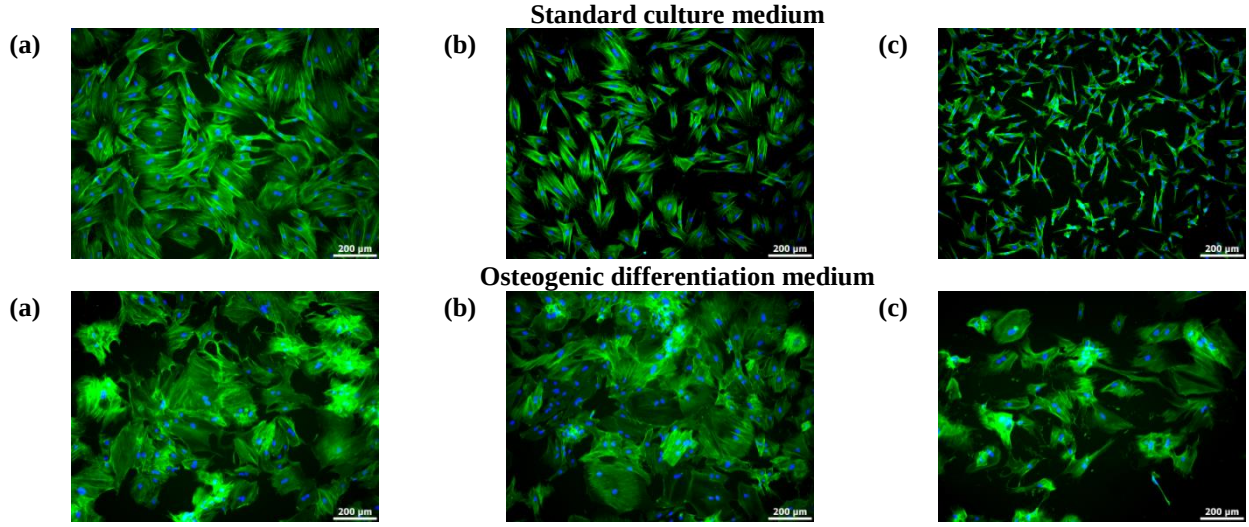

Fig. 3. Fluorescence microscope images of adherent rMSCS after a 21-day culture with standard culture (top row) or osteogenic differentiation medium (bottom row) on (a) flat and rough surfaces with average peak-to-valley height of (b) 50 nm or (c) 270 nm.

3.4 Effect of surface micro/nanotopography on rMSCs differentiation

The differentiation of rMSCs cultured on plasma treated polystyrene surfaces with average peak-to-valley height structures of 50 nm with respect to flat surfaces was evaluated by determining mineralization through alizarin staining In figure 4, representative optical microscope images of alizarin stained rMSCs cultured for 21 days with standard medium and osteogenic differentiation medium on flat and nanostructured (average peak-to-valley height 50 nm) surfaces are illustrated. From these images, it can be concluded that rMSCs cultured, with either standard culture or osteogenic differentiation medium, on nanostructured surfaces presented higher mineralization compared to cells cultured onto flat surfaces.

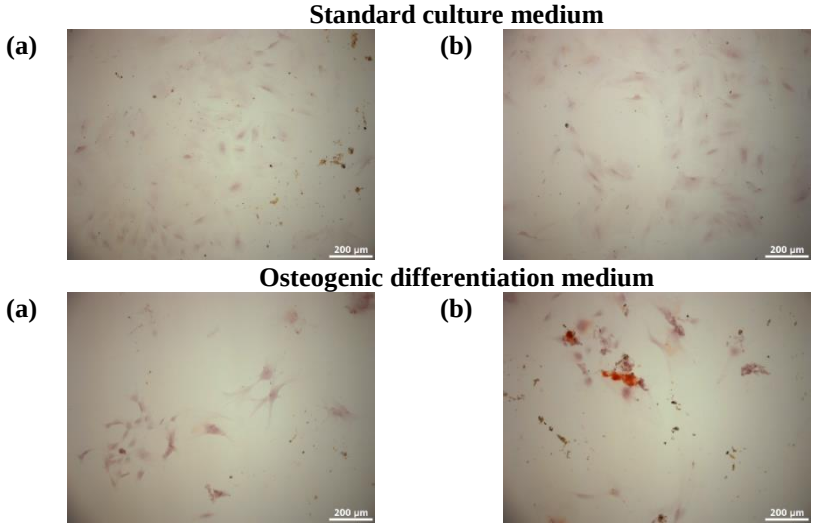

Fig. 4. Optical microscope images of Alizarin Red S-stained rMSCS cultured for 21 days with standard culture medium or with osteogenic differentiation medium on (a) flat or (b) plasma-treated surfaces with average peak-to-valley height 50 nm.

This finding was confirmed by quantification of alizarin after extraction from the adherent cells through incubation with cetylpyridinium chloride and measurement of extract absorption at 560 nm. As shown in figure 5, mineralization was 1.7 and 1.4 times higher for cells cultured with osteogenic medium and standard culture medioumn, respectively, on nanostructured surfaces compared to those grown onto the flat ones. Thus, it can be concluded that rMSCs differentiation was enhanced by surface nanotopography.

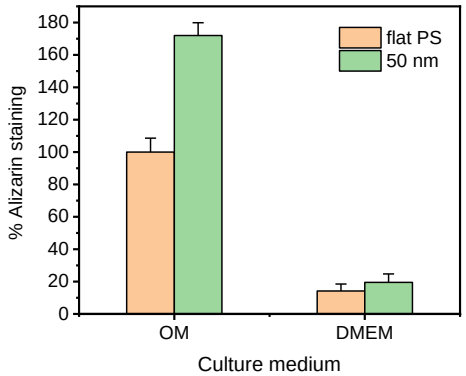

Fig. 5. Percent Alizarin Red S values obtained for cells cultured with OM or DMEM on both flat and plasma treated polystyrene surfaces (average peak-to-valley height structures 50 nm) normalized to Alizarin Red S value received for cells cultured with OM on flat polystyrene. Bars represent mean value ± SD of 4 experiments.

4. Conclusion

Nanostructured polystyrene surfaces fabricated through oxygen plasma etching have been evaluated as substrates for stem cell culture and differentiation. It was found that nanostructured surfaces with average peak-to-valley height of 50 nm enhanced differentiation of cells cultured in osteogenic medium compared to flat surfaces. Although further experimentation is required to verify the effect of nanotopography to stem cells differentiation, through determination of additional differentiation markers expression, the results obtained so far are promising and indicate that nanostructured surfaces promote differentiation of MSCs and could find application in the field of tissue engineering and regenerative medicine.

Acknowledgements

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