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Regionally-selective adhesion and growth of human osteoblast-like MG 63 cells on micropatterned fullerene C_{60} layers

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Fullerenes C_{60} were deposited on to microscopic glass coverslips using the Leybold Univex-300 vacuum system through metallic masks with rectangle openings. Below the openings, the C_{60} formed prominences 128 ± 8 nm (A), 238 ± 3 nm (B), 326 ± 5 nm (C) or 1043 ± 57 nm (D) in height. Human osteoblast-like MG 63 cells in the cultures on samples A, B and C were distributed almost homogeneously, while on samples D, they were localized almost exclusively in the grooves among the prominences. Thus, fullerene C_{60} films can act as substrates for guided cell adhesion and growth.

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1. Introduction

Fullerenes have been considered as promising materials for industrial as well as biomedical applications, such as quenching oxygen radicals, photodynamic anticancer and antimicrobial therapy and controlled drug or gene delivery [1-3]. However, relatively little is yet known about the influence of these nanoparticles on cell-substrate adhesion. Continuous layers of fullerenes C_{60} deposited on carbon-carbon composites, i.e. materials considered as promising for construction of bone implants [4], stimulated the spreading of human osteoblast-like MG 63 cells in cultures on these materials, though the total number of attached cells was lower than on uncoated composites and on tissue culture polystyrene dishes. The latter was probably due to the relatively high hydrophobicity of non-functionalized fullerenes [5]. An interesting idea is whether fullerenes could be used for constructing patterned surfaces for regionally-selective adhesion and directed growth of cells. Adhesive domains for cells can be created using moderately hydrophilic compounds (e.g., acrylic acid), attachment of various chemical functional groups (particularly those containing oxygen) or functionalization of the surface of the material by oligopeptidic ligands for cell adhesion receptors. These oligopeptidic ligands can contain amino acid sequences such as RGD, bound by many cell types, or KRSR, recognized preferentially by osteoblasts [5-7]. In addition, the affinity of fullerenes to the bone tissue can be achieved by deriving them with amide bisphosphonate in conjunction with multiple hydroxyl groups [3].

Higher wettability and attractiveness for cells can also be achieved by irradiation of certain regions on the material surface by plasma, ions or ultraviolet light [8]. Repulsive regions for cells can be prepared using hydrophobic or extremely hydrophilic compounds, such as octadiene, polysiloxanes or polyethylene oxide [6, 7, 9]. Patterned surfaces can also be produced by changing the surface roughness and topography, i.e., by creating prominences and hollows of different size, shape and depth [10, 11]. In this study, micropatterned layers were prepared by deposition of fullerenes C_{60} on microscopic glass coverslips through contact metallic masks, and the adhesion and growth of human osteoblast-like MG 63 cells were then studied in the cultures on these surfaces.

2. Experimental

Preparation of the fullerene C_{60} layers. Fullerenes C_{60} (purity 99.5 %, SES Research, U.S.A.) were deposited on to microscopic glass coverslips (Menzel Glaser, Germany; diameter 12 mm) by evaporation of C_{60} in the Univex-300 vacuum system (Leybold, Germany) in the following conditions: room temperature of the substrates, C_{60} deposition rate $\leqslant$ 5 Å/s, temperature of C_{60} evaporation in Knudsen cells about 450^{o} C, time of deposition up to 50 min [5]. The thickness of the layers increased proportionally to the temperature in the Knudsen cell and

the time of deposition. Four types of micropatterned layers of increasing thickness were created using metallic masks with rectangular holes with an average size of 128 ± 3 μm per 98 ± 8 μm (about 12,500 μm^2) and 50 μm in distance (samples A, C, D) or 470 x 440 μm (about 207,000 μm^2), distance 200 μm (sample B). The thickness of the C_{60} layers was measured by atomic force microscopy (AFM, Digital Instruments CP II Veeco, U.S.A.). A scratch was made in the layer and its profile was measured in contact mode. A Veeco CONT20A-CP scanning probe with spring constant 0.9 N/m was used. Repeated scanning of the same area of the scratch (5 times) was used for thickness measurement with error max. ± 5%. An example of scratches, measured on sample B, was chosen as an example and is shown in Fig. 1.

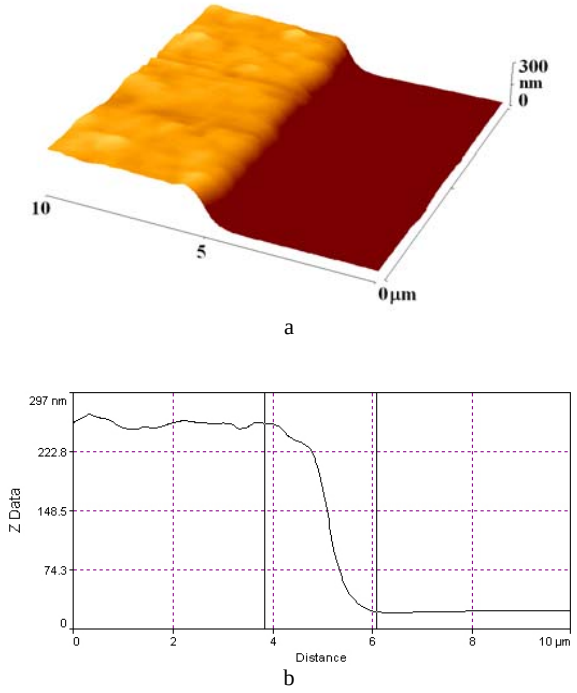

Fig. 1. Surface morphology of a scratch into the fullerene C_{60} sample B (a) and the line analysis (thickness 238.7 nm) (b)

The surface wettability of the films was estimated from the contact angle measured by a static method in a material-water droplet system using a reflection goniometer (SEE System, Masaryk University, Brno, Czech Republic). For cell culture, the samples were sterilized by 70% ethanol for 1 h, and after this procedure the quality of the C_{60} films was checked by Raman spectra measured in the back-scattering geometry at room temperature using a Raman microscope Ramascope 1000 (Renishaw, UK) with a 514.5 nm excitation wavelength Ar-ion laser.

Cell culture on the fullerene C_{60} layers. The sterilized samples were inserted into 24-well polystyrene multidishes (TPP, Switzerland; diameter 15 mm) and seeded with human osteoblast-like MG 63 cells (European Collection of Cell Cultures, Salisbury, UK). Each dish contained 5,000 cells (i.e., about 2,830 cells/cm^2) and 1.5 ml of Dulbecco's modified Eagle's Minimum Essential Medium (DMEM; Sigma, U.S.A., Cat. N^o D5648) supplemented with 10% of foetal bovine serum. The cells were cultured at 37^o C in a humidified air atmosphere containing 5% of CO_2. On days 1, 3 and 7 after seeding, the cells were rinsed with phosphate-buffered saline (PBS; Sigma, U.S.A.), fixed with 70% ethanol (-20°C, 5 min) and stained with hematoxylin and eosin (Fluka). The number, shape and distribution of the cells on the material surface (namely their relation to the grooves and bulges on the micropatterned surfaces) were evaluated on days 1, 3 and 7 after seeding on microphotographs taken under an IX-50 microscope, equipped with a DP-70 digital camera (both from Olympus, Japan). The obtained values of cell number were expressed as cells/cm^2 and used for the construction of growth curves. In addition, the total cell number as well as the viability of the cells, i.e. the percentage of viable cells in the cell population, was determined by the LIVE/DEAD viability/cytotoxicity kit for mammalian cells (Invitrogen, Molecular Probes, U.S.A.). Briefly, the cells were incubated for 5 to 10 min at room temperature in a mixture of two of the following probes: calcein AM, a marker of esterase activity in living cells, emitting green fluorescence, and ethidium homodimer-1, which penetrated into dead cells through their damaged membrane and produced red fluorescence.

Statistical analysis. The quantitative data on the physicochemical properties of the material was presented as mean ± S.D. (Standard Deviation) from 5 to 6 measurements for each experimental group. The quantitative data obtained in the cells was presented as mean ± S.E.M. (Standard Error of the Mean) from 9 to 21 measurements. If two experimental groups were compared, Student's t-test for unpaired data was used, whereas multiple comparison procedures were performed by the One Way Analysis of Variance (ANOVA), Student-Newman-Keuls method, using SigmaStat software (Jandel Corp. U.S.A.). *P* values equal to or less than 0.05 were considered significant.

3. Results

Physicochemical properties of the fullerene C_{60} layers. As revealed by AFM (Fig. 1) , the thickness of the layers on sites underlying the openings of the grid was 128 ± 8 nm, 238 ± 3 nm, 484 ± 5 nm and 1043 ± 57 nm in the samples A, B, C and D, respectively (data presented as mean ± standard deviation). Fullerene layers were also formed below the metallic part of the grid, where their thickness was either within the size of the standard deviations (layers A, B and D) or it amounted to 158 ± 5 nm (layer C). Therefore, in layer C, the effective height of the fullerene prominences was 326 ± 5 nm.

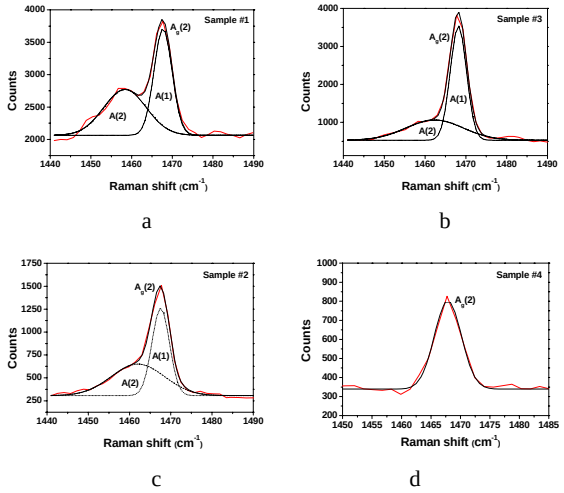

Fig. 2. Gaussian peak fit analysis of the Raman spectra obtained on micropatterned C_{60} layers A (prominences of 128 ± 8 nm), B (238 ± 3 nm), C (484 ± 5 nm) and D (1043 ± 57 nm) after 1-hour sterilization with 70% ethanol. The satellite peak A(2) on A, B and D is a sign of oxidation and polymerization of some fullerene C_{60} molecules.

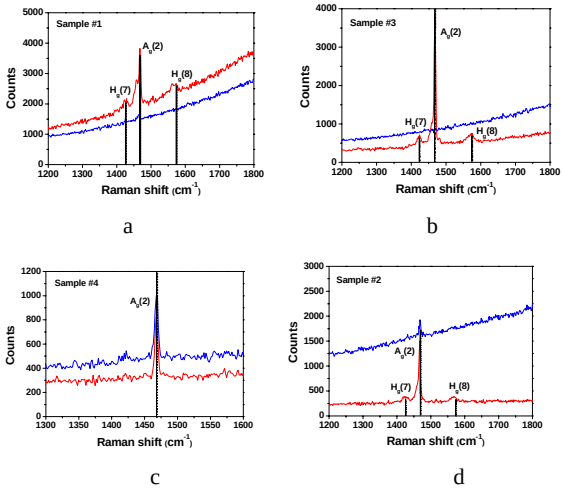

Fig. 3. Raman spectra of micropatterned C_{60} layers below the openings (red line) or bars (blue line) of the metallic lattice, obtained after 1-hour sterilization of the layers with 70% ethanol. (As revealed by AFM, the layer thickness below the openings and bars was 128 ± 8 and ≤ 8 nm, respectively, in the sample A, 238 ± 3 nm and ≤ 3 nm in B, 484 ± 5 nm and 158 ± 5 nm in C, and 1043 ± 57 nm and ≤ 57 nm in D).

Reflection goniometry showed that all fullerene C_{60} layers were relatively highly hydrophobic. All micropatterned layers had similar water drop contact angles, i.e., 93.6 ± 3.6° (A), 94.5 ± 5.5° (B), 95.3 ± 3.1° (C) and 95.6 ± 3.8° (D).

Raman analysis showed that after sterilization with ethanol, the fullerene layer C was almost chemically intact, as confirmed by a high peak $A_g(2)$ at wavenumber 1468 cm^{-1}, low peaks $H_g(7)$ and $H_g(8)$ and absence of D (disorder, ~ 1350 cm^{-1}) and G (graphitic, ~ 1600 cm^{-1}) bands, which are signs of fragmentation and graphitization of C_{60}, respectively (Fig. 2). However, in the other micropatterned layers, an analysis of the vibration mode $A_g(2)$ showed that some of the C_{60} molecules reacted with oxygen or polymerized, as indicated by the satellite peak A(2). The proportion of C_{60} molecules involved in these chemical changes was determined by the ratio A(2)/A(1), and reached about 30% in sample B and 50% in samples A and D (Fig. 2 A, B, D). Similarly as in AFM, fullerenes were also found below the metallic bars of the grid, although in the thinner layers A and B, the amount was very low and barely detectable (Fig. 3).

Optical microscopy revealed that below the openings of the grids, the fullerene prominences were brownish in color, and color intensity increased with layer thickness. In samples A, B and C, the layers were relatively well transparent in a conventional light microscope. Thus, the cells growing on these layers were well observable in native or hematoxylin- and eosin-stained cultures, and the fullerene bulges and grooves were also well distinguishable (Fig. 4 A, C, E). On the other hand, the thickest fullerene prominences on sample D were less transparent (Fig. 4 G), thus the presence of absence of cells on the prominences were better observable after fluorescence staining (Fig. 4 H).

Adhesion, viability and growth of cells on fullerene C_{60} layers. From day 1 to 7 after seeding, the cells on the layers with the fullerene prominences of 128 ± 8 nm, 238 ± 3 nm as well as 326 ± 5 nm were distributed almost homogeneously over the entire material surface (Fig. 4 A-F), thus the cell population densities on the prominences and in the grooves were similar (Fig. 5 A-C). However, the cells on the layers with prominences of 1043 ± 57 nm were found preferentially in the grooves (Figs. 4 G, H and 5 D). These grooves contained from 75.0 ± 13.4 % to 99.6 ± 0.3 % of cells, although they occupied only 44 ± 3 % of the material surface. The cell population density in the grooves was about 5 to 192 times higher than on the bulges, and these differences increased with time of cultivation (Fig. 5 D).

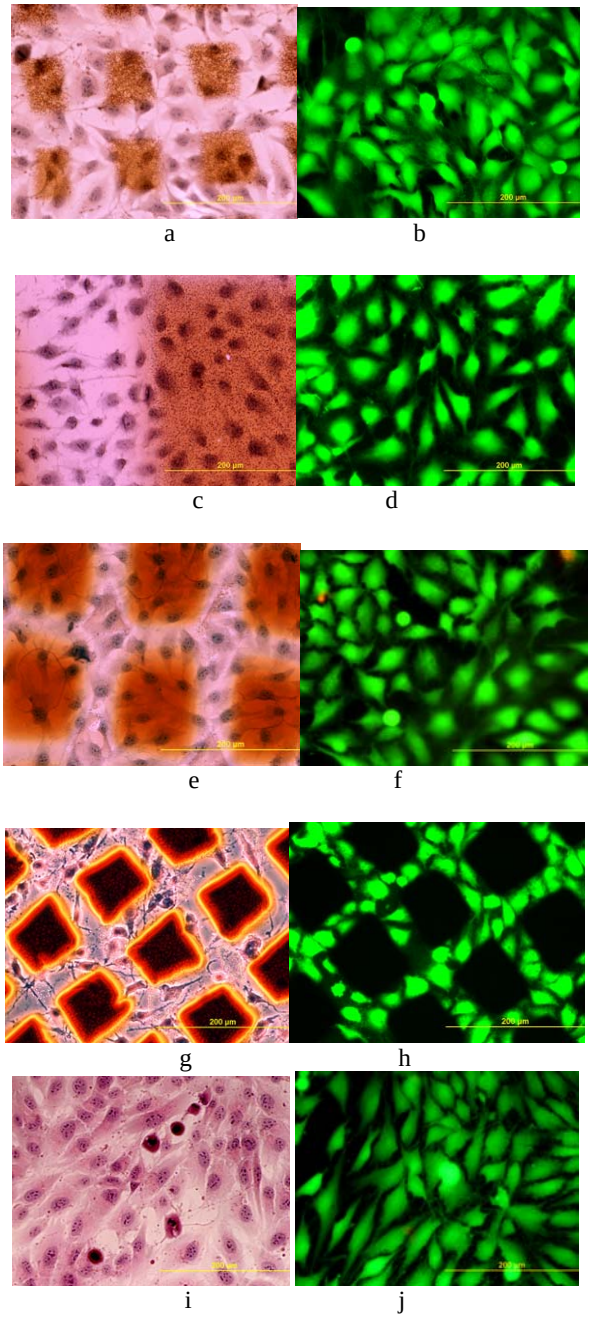

Fig. 4. *Human osteoblast-like MG 63 cells on day 7 after seeding on fullerene layers micropatterned with prominences 128 ± 8 nm in height (**A, B**), 238 ± 3 nm in height (**C, D**), 326 ± 5 nm in height (**E, F**), 1043 ± 57 nm in height (**G, H**), microscopic glass coverslip (**I, J**) or polystyrene culture dish (**K, L**). Stained with hematoxylin and eosin (**A, C, E, G, I, K**) or LIVE/DEAD viability/cytotoxicity kit (**B, D, F, H, J, L**). Microscope Olympus IX 50, digital camera DP 70, obj. 20x, bar = 200 µm except J, where bar = 100 µm.*

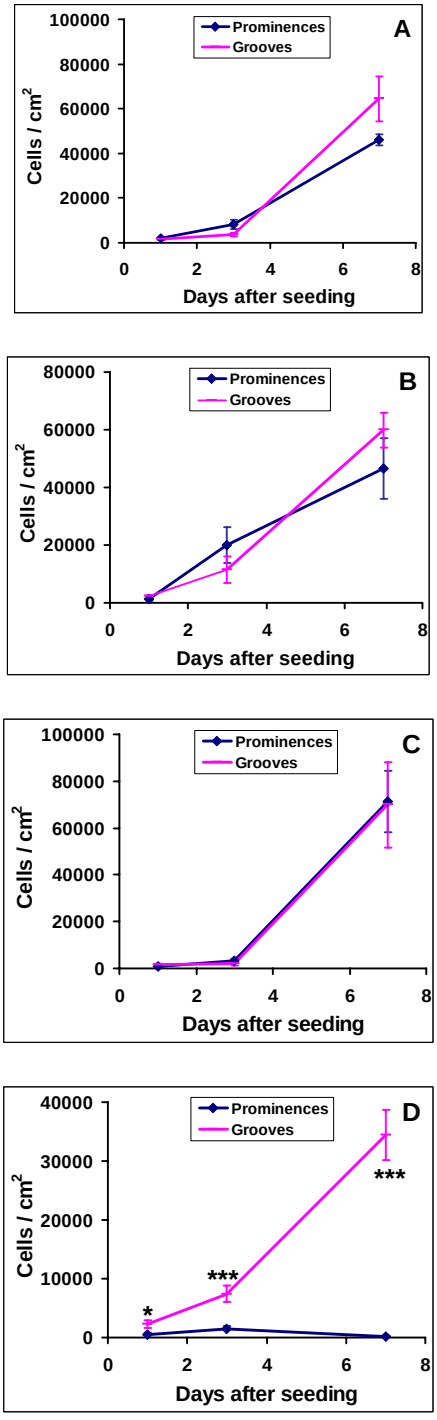

*Fig. 5. Population densities of MG 63 cells on prominences and grooves on fullerene layers micropatterned with prominences 128 ± 8 nm in height (A), 238 ± 3 nm in height (B), 326 ± 5 nm in height (C) or 1043 ± 57 nm in height (D). Mean ± Standard Error of Means (S.E.M.) from 9 to 21 microphotographs, Student t-test, statistical significance: *p≤0.05, ***p≤0.001.*

Fluorescence staining of cell membranes and nuclei using the LIVE/DEAD kit revealed that, on day 1 after seeding, the percentage of viable cells on the fullerene layers ranged between 80 ± 10 % and 87 ± 20 %, and was similar to the values obtained on the control tissue culture polystyrene dishes (96 ± 21%) and microscopic glass coverslips (67 ± 16 %). It can be assumed that the presence of a certain number of dead cells was due to the stress to which the cells were submitted during the cell seeding procedure, particularly during trypsinization and keeping the anchorage-dependent cells in suspension, rather than due to a cytotoxic action of the fullerenes. Accordingly, the percentage of viable cells increased with time of cultivation, reaching 97 ± 25 % to 99 ± 15 % on day 7 after seeding on all tested surfaces. Moreover, the growth dynamics of the cells on micropatterned layers A, B and C were similar to those on the control tissue culture polystyrene dishes and microscopic glass coverslips (Fig. 6). Only on sample D, where the cells grew preferably in the grooves, the total cell number obtained on day 7 after seeding was significantly lower than on the other samples (Fig. 6).

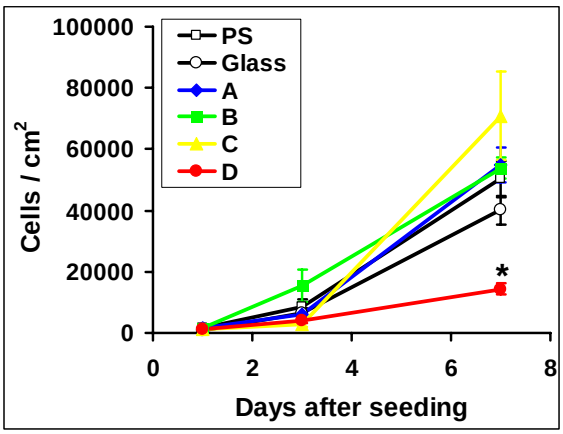

*Fig. 6. Growth curves of MG 63 cells on polystyrene culture dish (PS) microscopic glass coverslip (Glass), and micropatterned fullerene C_{60} films with prominences 128 ± 8 nm in height (A), 238 ± 3 nm in height (B), 326 ± 5 nm in height (C) or 1043 ± 57 nm in height (D). Mean ± S.E.M. from 9 to 21 microphotographs, ANOVA, Student-Newman-Keuls Method, statistical significance: *$p \leq 0.05$ in comparison with all other samples.*

4. Discussion

All micropatterned fullerene C_{60} layers created in this study proved to be good substrates for the adhesion and growth of human osteoblast-like MG 63 cells. On layers containing prominences between 128 ± 8 nm and 326 ± 5 nm in height, the cells adhered and grew to an extent comparable to that on tissue culture polystyrene dishes and microscopic glass coverslips, and were distributed almost homogeneously on the entire material surface (Fig. . In addition, the viability of the cells on the fullerene films was high and similar to the values found on the standard cell culture substrates. These results are, at least in part, consistent with our earlier findings obtained in MG 63 cells cultured on C_{60}-coated composites with carbon matrix reinforced with carbon fibres, i.e. materials considered as promising for construction of bone implants and bone tissue engineering [4]. On the C_{60}-coated composites, the cells adhered in a lower number than on the uncoated material, polystyrene dishes and glass coverslips, but were better spread, i.e., they were attached by a larger cell-material contact area [5]. Also in studies by other authors, fullerene C_{60} layers deposited on to polystyrene culture dishes supported adhesion, spreading, growth and viability of breast epithelial cells [1], and C_{60} molecules grafted onto polyurethane promoted adhesion and activation of platelets [2].

The beneficial effects of fullerene C_{60} layers on cell colonization could be explained by their surface nanostructure, mimicking the nanoarchitecture of the natural extracellular matrix [5, 12]. Moreover, due to the micropatterning, the fullerene layers in our study were constructed with a hierarchically-organized micro- and nanostructure, i.e., with a similar architectonic principle as natural tissues. In these tissues, nanoscale structures, such as protein molecules or inorganic crystals in the bone, are integrated into microscopis and then macroscopic forms. Similarly, in our studies, nanoscale fullerene molecules were organized into micrometer scale structures. It is believed that materials organized on two or more length scales bear a closer resemblance to biological matrices than those with single scale features, and that these materials should be more advantageous in biomedical applications [10]. The presence of oxygen, suggested in some of our fullerene layers by Raman spectroscopy (Fig. 2 A, B, D), might also contribute to the supportive effects of these films on cell adhesion and growth. Oxygen-containing chemical functional groups have been repeatedly shown to enhance the colonization of various materials with cells [4, 6, 8, 9].

Only on the surface with the highest fullerene bulges of 1043 ± 57 nm was the final cell population density significantly decreased. This was probably due to the fact that the cells grew preferentially in the grooves among the prominences, and thus they used less space for proliferation. Preferential growth of bone cells in grooves, pits and other types of hollows has been observed on various artificially microfabricated or primary rough polymeric, metallic or carbon-based materials [4, 10, 11]. However, the cells were also able to colonize the prominences on these surfaces, although these prominences were usually much higher (i.e., several micrometers or even tens of μm) than those on the thick micropatterned fullerene layers in our present study (only about 1 μm). Surprisingly, on our fullerene C_{60} layers, the MG 63 cells were not able to “climb up” relatively low prominences only about 1 μm in height, even at a relatively late culture interval of 7 day after seeding. This may be due to a synergistic action of certain physical and chemical properties of the fullerene bulges less appropriate for cell adhesion, such as their hydrophobicity, a steep rise as well as the tendency of spherical ball-like fullerene C_{60} molecules to diffuse out of the prominences [13].

5. Conclusions

Fullerenes C_{60} deposited as layers micropatterned with grooves and prominences from 128 ± 8 nm to 326 ± 5 nm supported the adhesion, growth and viability of human osteoblast-like MG 63 cells to a similar extent as standard tissue culture polystyrene dishes and microscopic glass coverslips. The cells on these layers were distributed homogeneously. However, the layers with fullerene prominences 1043 ± 57 nm in height promoted regionally-selective adhesion and growth of MG 63 cells in the grooves located among the prominences. Thus, these films can be used as templates for directed cell adhesion and growth.

Acknowledgements

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