Fullerene C_{60} films of continuous and micropatterned morphology as substrates for adhesion and growth of bone cells

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1. Introduction

Fullerenes, i.e. spheroidal molecules made exclusively of carbon atoms (e.g., C_{60}, C_{70}), display a diverse range of biological activity (for a review, see [1–3]). Their unique hollow cage-like shape and structural analogy with clathrin-coated vesicles in cells support the idea of the potential use of fullerenes as drug or gene delivery agents [4–6]. Fullerenes are able to accept and release electrons. When irradiated with ultraviolet or visible light, fullerenes can convert molecular oxygen into highly reactive singlet oxygen [7]. Thus, they have the potential to inflict photodynamic damage on biological systems, including damage to cellular membranes, inhibition of various enzymes or DNA cleavage [8–11]. This harmful effect can be exploited for photodynamic therapy against tumors, viruses and bacteria resistant to multiple drugs [12,13]. On the other hand, C_{60} is considered to be the world’s most efficient radical scavenger. This is due to the relatively large number of conjugated double bonds in the fullerene molecule, which can be attacked by radical species. Thus, fullerenes would be suitable for applications in quenching oxygen radicals, and thus preventing the damage of various tissues and organs, including the cardiovascular and central nervous systems [1,7,14–19]. In addition, fullerenes emit photoluminescence which could be utilized in advanced imaging technologies [3].

In their pristine unmodified state, fullerenes are highly hydrophobic and water-insoluble. On the other hand, they are relatively highly reactive, which enables them to be structurally modified. Fullerenes can form complexes with other atoms and molecules, e.g., metals, nucleic acids, proteins, synthetic polymers as well as other carbon nanoparticles, e.g., nanotubes. In addition, fullerenes can be functionalized with various chemical groups, e.g. hydroxyl, aldehydic, carbonyl, carboxyl, ester or amine group, as well as amino acids and peptides. This usually renders them soluble in water and intensifies their interaction with biological systems [4,5,8–11,17–21].

Despite all these exciting findings, relatively little is still known about the influence of fullerenes, particularly when arranged into layers and used for biomaterial coating, on cell-substrate adhesion,
subsequent growth, differentiation and viability of cells, especially bone-forming cells. In our earlier study, a layer of fullerene C_{60} molecules, i.e. fullerite, deposited on carbon fibre-reinforced carbon composites (CFRC), enlarged the cell spreading area in human osteoblast-like MG 63 cells in cultures on these materials [2], which was most probably due to the nanostructure of the material surface. Moreover, it is believed that nanostructured surfaces can promote preferential adhesion and growth of osteoblasts over other “competitive” cell types, including fibroblasts, and thus they can prevent fibrous encapsulation and loosening of bone implants. It is considered that the underlying mechanism is higher adsorption to the nanostructured surfaces of vitronectin, an extracellular matrix (ECM) protein preferred by osteoblasts [22,23]. Therefore, it can be expected that carbon nanoparticles, including fullerences, may serve as novel building blocks for creating artificial bioinspired nanosstructured surfaces for bone tissue engineering [24-26]. Proliferation and differentiation of chondrocytes, another cell type important in bone reconstruction, were also promoted by fullerences. C_{60} enhanced the production of specific large proteoglycan ECM molecules, typical for cartilage, in rat embryonic limb bud cells in culture [27]. Interestingly, in a line of human epidermoid carcinoma cells, fullerences protected cells from anoikis, i.e., apoptosis due to adhesion deprivation, by a mechanism supporting the formation of focal adhesion plaques, assembly of the actin cytoskeleton and cell spreading [28]. Enhanced attachment and spreading of platelets was also found on a polyurethane surface grafted with fullerene C_{60} molecules [4].

In the present study, the adhesion, growth, viability and maturation of human osteoblast-like MG 63 cells were investigated in cultures on fullerene C_{60} films of various thickness and morphology, deposited on microscopic glass coverslips. In addition to continuous films, also micropatterned films were prepared by deposition of fullerences through a metallic mask with rectangular thick micropatterned. The micropatterned layers were created by evaporation in the Knudsen cells about 450 °C, time of deposition up to 50 min [2]. The thickness of the layers increased proportionally to the temperature in the Knudsen cell and the time of deposition. Four types of layers of different thickness and morphology were prepared: thin continuous, thick continuous, thin micropatterned and thick micropatterned. The micropatterned layers were created by deposition of fullerences through a metallic mask with rectangular holes with an average size of 128±3 µm/98±8 µm (about 12 430 µm²) and 50 µm in distance (Fig. 1).

Raman spectra of the deposited C_{60} films were 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 [2]. The polarization of the scattered radiation was not analyzed. The thickness of the C_{60} layer 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%. In addition, the surface morphology and root mean square roughness (RMS) was evaluated by AFM.

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 (Sloanview, Masaryk University, Brno, Czech Republic) [2].

2.2. Cells and culture conditions

To cultivate the cells, the fullerene-coated glass coverslips were sterilized by 70% ethanol for 1 h, 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 5000 cells (i.e., about 2,830 cells/cm²) and 1.5 ml of Dulbecco’s modified Eagle’s Minimum Essential Medium (DMEM; Sigma, U.S.A., Cat. N° D5648) supplemented with 10% fetal bovine serum (PBS; Sebak GmbH, Aidenbach, Germany) and gentamicin (40 µg/ml, LEK, Ljubljana, Slovenia). The cells were cultured for 1, 3 and 5 or 7 days at 37 °C in a humidified air atmosphere containing 5% of CO₂.

2.3. Evaluation of the cell number, morphology and spatial distribution on the material surface

On days 1, 3, 5 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) according to the manufacturer’s protocol. The number, shape and distribution of the cells on the material surface (namely their relation to the grooves and bulges on the two micropatterned surfaces) were evaluated on microphotographs taken under an IX-51 microscope, equipped with a DP-70 digital camera (both from Olympus, Japan).

In addition, on days 5 and 7 after seeding, when the cells were becoming confluent and overlapping, the cell number was evaluated by automated counting in a ViCell analyzer (Beckman Coulter, U.S.A.) after they had been detached by a trypsin-EDTA solution (Sigma, U.S.A., Cat. N° T4174) in PBS for 10 min at 37 °C.

The cells on the continuous and micropatterned C_{60} layers were evaluated in two separate experiments performed in triplicate. Since the adhesion and growth activity of MG 63 cells varied slightly in each experiment, internal controls represented by polystyrene culture dishes (TPP, Switzerland) and microscopic glass coverslips (Menzel Glaser, Germany), cleansed with ethanol, were included in each experiment [4].

2.4. Growth curves and cell population doubling time

The cell numbers were expressed as cell population densities/cm² and were used for constructing the growth curves. The cell population doubling time (DT) was calculated as DT=(t−tₚ)log2/log Nₜ−Nₜ₀, where tₚ and t represent earlier and later time intervals after seeding, respectively, and Nₜ₀ and Nₜ represent the number of cells at these intervals.

2.5. Evaluation of cell viability

On days 1, 3 and 7 after seeding, the cells were rinsed with PBS, and their number and viability was determined by the LIVE/DEAD viability/cytotoxicity kit for mammalian cells (Invitrogen, Molecular Probes, U.S.A.) according to the manufacturer’s protocol. 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. Live and dead cells were then counted on microphotographs taken under an epifluorescence microscope (Olympus IX-51, DP-70 digital camera,
In addition, on days 5 and 7 after seeding, the dead cells were detected by the trypan-blue exclusion test performed during cell counting in the ViCell XR analyzer mentioned above.

2.6. Immunofluorescence staining of β₁ integrins, talin, β-actin and osteopontin

On day 3 after seeding, the presence and spatial arrangement of the following molecules in MG 63 cells was evaluated: β₁ integrins, i.e., an important group of cell-matrix adhesion receptors on osteoblasts, supporting their differentiation and their sensitivity to the material surface properties [29]; talin, an integrin-associated protein present in focal adhesion plaques, β-actin, an important component of the cytoplasmatic cytoskeleton; and osteopontin, a non-collagenous calcium-binding extracellular matrix glycoprotein and marker of osteogenic cell differentiation. The cells were rinsed twice in PBS and fixed with cold 70% ethanol (−20 °C, 5 min), pre-treated with 1% bovine serum albumin in PBS containing 0.05% Triton X-100 (Sigma, St. Louis, MO, U.S.A.) for 20 min at room temperature, and then incubated with the following primary antibodies: mouse monoclonal anti-human integrin β₁, mouse monoclonal anti-human talin (both from Chemicon International Inc., Temecula, CA, U.S.A.; Cat No. MAB1981 and MAB3264, respectively), monoclonal anti-β-actin (clone AC-15, Sigma, St. Louis, MO, U.S.A., Cat. No. A-5441) or rabbit polyclonal anti-human osteopontin (Alexis Biochemicals, Cat No. ALX-210-309). The antibodies were diluted in PBS to concentrations of 1:200 to 1:400 and applied overnight at 4 °C. After rinsing with PBS, the secondary antibodies, represented by goat anti-mouse F(ab′)2 fragment of IgG (for samples stained with mouse monoclonal antibodies; dilution 1:1000) or goat anti-rabbit F(ab′)2 fragment of IgG (for samples stained with the rabbit polyclonal antibody; dilution 1:5000) were added for 1 h at room temperature. Both secondary antibodies were conjugated with Alexa Fluor® 488 and purchased from Molecular Probes, Eugene, OR, U.S.A. (Cat. No. A11017 and A11070, respectively). After incubation with secondary antibodies, the cells were rinsed twice in PBS, mounted under microscopic glass coverslips in a Gel/Mount permanent fluorescence-preserving aqueous mounting medium (Biomedica Corporation, Foster City, CA, U.S.A.) and evaluated under an epifluorescence microscope (IX-51, Olympus, Japan) equipped with a digital camera (DP-70, Olympus, Japan).
2.7. Measurement of cell adhesion area

Cells immunostained against β-actin were used for an evaluation of the size of the cell spreading area, i.e., the cell-material contact area. The cells were photographed using a IX 51 microscope (obj. 20) equipped with a DP 70 digital camera (Olympus, Japan) in 20 randomly selected microscopic fields (size approx. $1.38 \times 10^{-3}$ cm$^2$) for each experimental group. The size of the area projected on the material was measured using Atlas software (Tescan Ltd., Brno, Czech Republic). The cells that developed intercellular contacts were excluded from the evaluation. For each experimental group, 3 independent samples (containing 35 to 113 cells in total) were evaluated.

2.8. Statistical analysis

The quantitative data on the physical properties of the material was presented as mean±S.D. (Standard Deviation). The quantitative data obtained in the cells was presented as mean±S.E.M. (Standard Error of the Mean). 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

3.1. Physical and chemical properties of the fullerene C$_{60}$ layers

Raman analysis was performed on micropatterned samples immediately after deposition and then after sterilization with ethanol. Immediately after deposition, the Raman spectra showed that the fullerene films were prepared with high quality, 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, \(-1350 \text{ cm}^{-1}\)) and \( G \) (graphitic, \(-1600 \text{ cm}^{-1}\)) bands, which are signs of fragmentation and graphitization of \( C_{60} \), respectively (Fig. 2A). After sterilization with ethanol, the thin micropatterned fullerene layers were almost intact (Fig. 2B), and a considerable amount of fullerenes was found not only on sites underlying the openings of the grid, but also below its metallic part (Fig. 2C). However, in thick 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 \( A_{g}(2) \) on Fig. 2D. The proportion of \( C_{60} \) molecules involved in these chemical changes was determined by the ratio \( A_{g}(2)/A_{g}(1) \), and reached about 50%. Moreover, the amount of fullerenes below the metallic bars of the grid was very low, though still detectable (Fig. 2E).

As revealed by AFM, the thickness of the thin and thick continuous layers was 505 ± 43 nm and 1090 ± 8 nm, respectively. In both micropatterned layers, the fullerenes formed bulge-like prominences below the holes of the metallic grid. The thickness of these bulges was 484 ± 5 nm and 1043 ± 57 nm in the thin and thick film, respectively. A fullerene \( C_{60} \) film was also found below the metallic part of the grid, especially in the thin layer, where its thickness amounted to 158 ± 5 nm. Therefore, the effective height of the fullerene domains in the thin micropatterned layer was only 326 ± 5 nm. In the thick micropatterned layers, the thickness of the fullerene film formed below the metallic portion of the grid was less than 57 nm. The surface of both continuous and micropatterned layers which contained nanosize clusters of the RMS value from 4.97 to 6.32 nm (Fig. 1B and F).

Optical microscopy revealed that the fullerene layers were brownish in color (Fig. 1A, D and E). The color intensity increased with layer thickness, while the transparency of the layers in a conventional light microscope decreased (Fig. 1D and E). Despite of this, the cells on both continuous layers were well observable, even those native and non-stained (Fig. 1D and E). On thick micropatterned layers, the bulge-like prominences were relatively dark, and the contrast between the bulges and grooves was relatively high (Fig. 1E). In addition, it was not possible to focus the cells on bulges and in grooves simultaneously, whereas the fluorescence signal from both groups of cells was observable. Thus, the presence and morphology of cells on bulges and in grooves was evaluated using fluorescence microscopy (Fig. 3F).
Reflection goniometry showed that all fullerene C\textsubscript{60} layers were relatively highly hydrophobic. The continuous and micro-patterned layers had similar water drop contact angles ranging from 95.3±3.1° to 100.6±6.8°.

3.2. Adhesion and proliferation of cells on the fullerene C\textsubscript{60} layers

On day 1 after seeding, the cells on both continuous thin and thick fullerene layers adhered at similar numbers (3420±420 cells/cm\textsuperscript{2} and 2880±440 cells/cm\textsuperscript{2}, respectively), which was comparable to the values found on standard cell culture substrates, represented by the tissue culture polystyrene dish (3080±290 cells/cm\textsuperscript{2}) and the microscopic glass coverslip (2560±310 cells/cm\textsuperscript{2}). On both micropatterned thin and especially thick fullerene layers, the average cell population densities tended to be lower (by 11 to 43%) than both polystyrene and glass, but these differences were not statistically significant (Fig. 4). However, the cells on the thick micropatterned layers were less spread and adhered over a significantly smaller cell adhesion area (983±34 µm\textsuperscript{2} compared to 1815±131 µm\textsuperscript{2}, 1757±16 µm\textsuperscript{2}, 1878±193 µm\textsuperscript{2} and 2286±148 µm\textsuperscript{2} in thin continuous, thick continuous and thin micropatterned layers, respectively, and 2286±148 µm\textsuperscript{2} on microscopic glass coverslips). These cells often had a roundish or elongated spindle-like shape, whereas the cells on both the continuous and thin micropatterned fullerene layers were mostly polygonal (Fig. 3).

From days 1 to 5 after seeding, the cells on both continuous C\textsubscript{60} films, polystyrene and glass, proliferated with a similar cell population doubling time (Table 1), and on day 5 they reached similar cell population densities (from 91 600±9500 cells/cm\textsuperscript{2} to 112 700±9800 cells/cm\textsuperscript{2}). However, on the thick micropatterned fullerene layers, the doubling time was significantly longer and the final cell population density (measured on day 7 after seeding) was significantly lower than the values obtained on the thin micropatterned films (Fig. 4). The attenuated growth of the cells on thick micropatterned layers was probably due to the presence of relatively high prominences and deep grooves.

![Fig. 4. Growth curves of human osteoblast-like MG 63 cells in cultures on polystyrene dishes, microscopic glass coverslips, continuous thin and thick fullerene C\textsubscript{60} layers (A) and micropatterned thin and thick fullerene C\textsubscript{60} layers (B). Mean±S.E.M. from 9 to 30 measurements. Statistical analysis: ANOVA, Student–Newman–Keuls Method. Statistical significance: ▲■: p≤0.05 in comparison with the sample of the same symbol.](image)

![Fig. 5. Population density of human osteoblast-like MG 63 cells on day 1 (A), 3 (B) and 7 (C) after seeding on thin and thick fullerene (Full) C\textsubscript{60} layers micropatterned with grooves and bulges. Mean±S.E.M. from 9 measurements. Statistical analysis: Student t-test for the unpaired data. Statistical significance: ⁎⁎: p≤0.01 and ⁎⁎⁎: p≤0.001 in comparison with the values on the bulges.](image)

Table 1

<table>
<thead>
<tr>
<th>Sample/Doubling time</th>
<th>I. Polystyrene</th>
<th>II. Glass</th>
<th>Continuous C\textsubscript{60} films</th>
<th>Micropatterned C\textsubscript{60} films</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Day 1 to 5</td>
<td>19.6±0.5</td>
<td>18.6±0.3</td>
<td>19.1±0.4</td>
<td>19.0±0.6</td>
</tr>
<tr>
<td>B. Day 1 to 7</td>
<td>21.7±1.1</td>
<td>19.8±0.4</td>
<td>18.5±0.9, I</td>
<td>22.2±0.9, II</td>
</tr>
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</table>

Means±S.E.M. Statistical analysis: ANOVA, Student–Newman–Keuls Method. Statistical significance: I, II, III, IV, p≤0.05 in comparison with the sample of the same number.
The cells colonized practically exclusively the grooves (Fig. 3F), thus they used less space for their proliferation. Although the grooves occupied only 41±1% of the material surface, they contained from 80±4% to 98±1% of the total cells on the material surface. The cell population density in the grooves was about 5 to 57 times higher than on the bulges, and these differences increased with time of cultivation (Fig. 5). On the other hand, on the thin micropatterned films, the cells colonized homogeneously the entire surface of the sample (Fig. 3E), and the percentage as well as the population density of cells in the grooves and on the bulges were similar (Fig. 5).

3.3. Cell viability on the fullerene C$_{60}$ layers

As revealed by staining the adhered cells with calcein and ethidium homodimer using the LIVE/DEAD kit, the percentage of viable cells on days 1 to 7 after seeding on all fullerene layers ranged from 80±10% to 100±15%, and was similar to the values obtained on the polystyrene culture dishes (99±24% to 100±15%), and also the microscopic glass coverslips (88±21% to 99±26%). The trypan-blue exclusion test, performed on the trypsinized cells while they were being counted in the ViCell Analyser, also showed similar proportions of viable cells on the fullerene layers (82±11% to 89±13%), as well as standard cell culture surfaces (80±13% to 95±13%). The average viability of the cells tended to increase with time of cultivation.

3.4. Presence and spatial arrangement of $\beta_1$ integrins, talin, $\beta$-actin and osteopontin in cells on fullerene C$_{60}$ layers

As revealed by immunofluorescence, MG 63 cells on both continuous and micropatterned fullerene layers were intensively stained for $\beta_1$ integrins and talin, i.e. molecules participating in cell-substrate adhesion, $\beta$-actin, an important component of the cytoplasmic cytoskeleton, as well as osteopontin, a marker of...
osteogenic cell differentiation. This staining intensity was similar as in cells on the control polystyrene culture dish and microscopic glass coverslips (Fig. 6). All these molecules (particularly extra-
cellular matrix protein osteopontin) were found in fine granular
distribution throughout the cells, often preferentially located in
the perinuclear region. In addition, both β1 integrins and talin
also formed dot- or streak-like focal adhesion plaques, visible
mainly on the cell periphery. Beta2 integrin-containing focal
adhesion plaques were particularly well developed and were
often located on fine long protrusions formed by cells, which was
accompanied by the formation of a fine mesh-like β-actin
cytoskeleton. No apparent differences in the staining intensity
and distribution of all molecules mentioned here were found
between cells growing on thin and thick micropatterned fullerene
layers or in cells in grooves and on bulges.

4. Discussion

This study has shown that fullerene C60 layers acted as good
substrates for adhesion, growth and maturation of human osteoblast-
like MG 63 cells, comparable to standard cell culture materials, such as
tissue culture polystyrene and microscopic glass coverslips. These
results are consistent with the earlier findings of the supportive effects
of fullerene C60 layers formed on polystyrene culture dishes by the
evaporation of methanol from colloidal C60-methanol suspensions, on
the adhesion, proliferation and assembly of an actin cytoskeleton in
several lines of normal and malignant breast epithelial cells [3]. Grafting
C60 molecules onto polyurethane surfaces also increased the number of
adhered platelets and enhanced their activation [4].

The supportive effects of fullerene C60 layers on cell colonization
could be explained by their surface nanostructure, mimicking the
nanoarchitecture of the natural extracellular matrix (ECM), i.e. a
physiological substrate for cell adhesion [22–26]. In the case of thick
micropatterned layers, the cell colonization could be supported also
by the presence of oxygen, revealed by Raman spectroscopy. Oxygen-
containing groups have repeatedly been found to enhance the
colonization of various materials with cells [30,31]. Both the
nanostructure and the oxygen content in our C60 layers may act
synergistically by enhancing the adsorption of cell adhesion-mediating
ECM molecules, such as vitronectin, fibronectin and collagen, provided by the serum of the culture medium or synthesized by the
cells [22–24,30]. The spatial conformation of these molecules on our
films may also be more appropriate for the accessibility of specific
sites on these molecules by cell adhesion receptors, e.g. integrins [22–
24,30]. As revealed by immunofluorescence staining of the MG 63 cells
on our samples, β1 integrins (i.e., receptors for collagen, fibronectin
and vitronectin), and talin, an integrin-associated protein, were
assembled in focal adhesion plaques, and these processes were
accompanied by the formation of β-actin cytoskeleton and the presence of a considerable amount of osteopontin, a marker of
osteogenic cell differentiation. All these events can be considered as
signs of active cell-substrate interaction followed by signal transduc-
tion inside the cells. Moreover, β1 integrins have been reported to
enhance osteogenic cell differentiation, which is manifested by higher
levels of osteocalcin, higher activity of alkaline phosphatase and a
more pronounced response to 1,25-dihydroxyvitamin D3 [29].

Similarly, a water-soluble fullerene derivative, C7-fulleropyrroli-
dicarboxylic acid, supported the formation of focal adhesion plaques, an assembly of an actin cytoskeleton and cell
spreading in human epidermoid carcinoma cells exposed to UV
light, probably by its antioxidative action [28]. The free radical
scavenger activity of fullerenes and their derivatives also
protected keratinocytes from the apoptosis induced by ultraviolet
light B [5], minimized the oxidative damage of small bowel
transplants [14], decreased neuronal degeneration and death,
prevented focal cerebral ischemia [15,16] and attenuated ische-
mia-reperfusion-induced lung injury [17]. Adducts of the C60
fullerenes with poly(N-vinyl-pyrrolidone) prevented the distur-
ance of long-term memory consolidation in rats induced by
cytochrome c. Inhibitor of protein synthesis [18]. However,
photopolymerized fullerene derivatives protected cells against
oxidative stress only at low concentrations (10–50 µM in cell
culture media), while at high concentrations (1 and 1.5 mM), they
toed cell death [17]. Hydroxylated fullerenes, or water-soluble
nano-C60, i.e. a fullerene aggregate that readily forms when
pristine C60 is added to water, induced oxidative damage to the
human vascular endothelial cells, water-soluble hydroxylated fullerenes caused cell death probably by the activation of ubiquitin-autophagy pathways [11].
Suspensions of colloidal C60 fullerenes were genotoxic for human
lymphocytes [9]. These harmful effects of fullerenes C60, usually
potentiated by the light, especially ultraviolet light, could be
utilized for antimicrobial therapy or photodynamic therapy against
tumor cells [12,13].

Nevertheless, as shown by Raman spectroscopy, the fullerene
layers in this study and also in our earlier study [2], were relatively
stable and resistant to sterilization and cell cultivation procedures.
Similarly, fullerene molecules grafted onto polyurethane remained
intact after tests of their interaction with platelets [4]. Therefore,
it can be supposed that if the fullerenes were released from our films
into the cell culture media (which remains to be further investiga-
ted), only low and harmless concentrations of these molecules
were achieved. On the other hand, MG 63 cells on thick
micropatterned fullerene layers adhered over a significantly smaller
area than those on the control glass coverslips. However, this lower
spreading of cells, coupled with their elongated morphology, can be
explained by the preferential growth of cells in relatively narrow
grooves rather than by cytotoxic effects of the fullerene layer. On
thin micropatterned fullerene layers, i.e. surfaces with relatively
low irregularities, the MG 63 cells were normally spread in a similar
extent as on the control cell culture substrates. Another factor
supporting lower cell spreading on fullerene films may be the
relatively high hydrophobicity of these materials. Highly hydro-
phobic surfaces have been often shown to be less adhesive for cells
[30,31].

Preferred growth of primary osteoblasts as well as osteo-
genic cell lines in grooves, pits and other types of hollows has
been observed on various polymeric and metallic materials
applicable for construction of bone and dental implants [32–35].
However, the cells were also able to colonize the prominences on
these surfaces, represented e.g. by ridges or pillars, although
these prominences were usually much higher (i.e., several µm or
even tens of µm) than those on the thick micropatterned
fullerene layers in our present study (only about 1 µm). In
addition, bone cells, including MG 63 cells, were also able to
spread over pore entrances from 40 to even 100 µm in diameter
on the surface of scaffolds constructed for bone tissue engineering
[36,37]. Surprisingly, on our fullerene C60 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 days after seeding. This might be due to a synergistic action of
hydrophobia and other physicochemical properties of the full-
ere bulges less appropriate for cell adhesion, such as their steep
rise and the tendency of spherical ball-like fullerene C60
molecules to diffuse out of the prominences towards the grooves
[38].

Thus, micropatterned fullerene films could be used as templates for
regionally-selective cell adhesion and growth. Moreover, the osteogenic
cells growing in hollows have been reported to be more active in
phosphorylation of various kinases and transcription factors, signal
transduction and differentiation [32–35]. Microstructured fullerene layers (if they adhere strongly to the underlying materials and are resistant to the release of fullerene molecules) could be used as a bioactive coating for bone implants in order to improve their integration with the surrounding bone tissue.

5. Conclusion

Fullerenes C₆₀ deposited as continuous films or layers micro-patterned with grooves and bulges clearly supported the adhesion, growth, viability and maturation of human osteoblast-like MG 63 cells to a similar extent as standard tissue culture polystyrene dishes and microscopic glass coverslips. In addition, thick micro-patterned layers (i.e., 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.

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