no burst release in both cases. On the other hand, BSA release appeared faster from the hydrogel obtained at 37°C than from the one obtained at 50°C, even though the gelation time was longer for the former than for the latter. Nearly

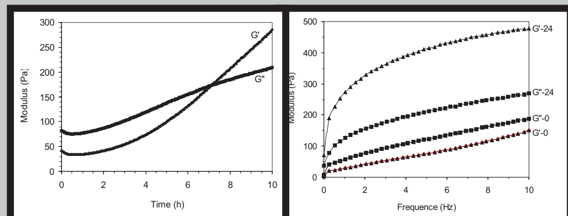

FIG. 1. A) Time-dependent changes of storage modulus (G') and viscous modulus (G") of a 14% 1L/1D sample at 25°C and at 1Hz; B) Changes of storage modulus (G') and loss modulus (G") of a 14% 1L/1D solution as a function of frequency at t=0 and t=24h at 25°C.

38% and 24% of BSA were released after 360 h, respectively. The difference can be assigned to the fact gelation at 50°C led to much more consistent hydrogel structure than at 37°C, which disfavored drug diffusion.

Circular dichroism (CD) was used to determine whether BSA molecules were denatured after the gelation procedure. Figure 2B shows the CD spectra of original BSA and of BSA released from the hydrogel obtained at 50°C. The two spectra appeared almost identical, indicating that the gelation procedure at 50°C did not denature BSA proteins.

Conclusion

Bioresorbable hydrogels were prepared from aqueous solutions containing both PLLA/PEG and PDLA/PEG block

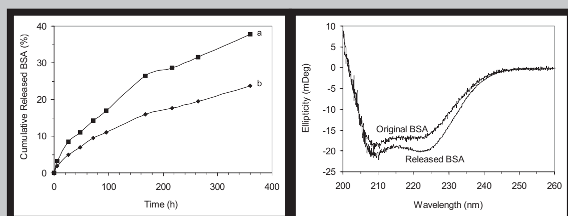

FIG. 2. A) drug release profiles of 20% 2L/2D hydrogels containing c.a. 40mg of BSA: a) gelation for 90h at 37°C, b) gelation for 24h at 50°C; B) Circular dichroism (CD) spectra of original BSA and of BSA released from the hydrogel obtained at 50°C.

copolymers due to interactions and stereocomplexation between PLLA and PDLA blocks. Rheological studies showed that both storage and loss moduli depend not only on the polymer properties such as the molar mass and EO/LA ratio, but also on the factors such as the concentration, temperature, time and frequency. The gelation process is time- and temperature-dependent and the hydrogel is a dynamic and evolutive system because of continuous formation/destruction of crosslinks and degradation. Drug release studies show that the release rate can be adjusted by changing the gelation conditions and factors such as drug load, polymer concentration and molar masses.

References

[1] S. Li, M. Vert, in Encyclopedia of Controlled Drug Delivery, E. Mathiowitz ed., John Wiley & Sons : New York, 1999, p 71.
[2] R.L. Dunn, in Biomedical Applications of Synthetic Biodegradable Polymers, J.O. Hollinger ed. CRC Press: Boca Raton, 1995, pp 17-31.
[3] S. Li, J. Biomed. Mater. Res., Appl. Biomater, 48, (1999), 142-153.
[4] S. Li, M. Vert, Macromolecules, 36,(2003), 8008-8014.
[5] S. Li, Macromol. Biosci., 2003, 3, 657-661.
[6] S. Li, A. El Ghzaoui, E. Dewinck, Macromol. Symp., 222, (2005) 23-35.

ADHESION OF BONE AND VASCULAR CELLS ON CARBON FIBRE-REINFORCED CARBON COMPOSITES COATED WITH A FULLERENE LAYER

Lucie Bacakova*, Lubica Grausova*, Jiri Vacik, Ivana Jungova*****

*Institute of Physiology, Acad.Sci.CR, Prague, Czech Republic, lucy@biomed.cas.cz
**Nuclear Physics Institute, cad.Sci.CR, Prague, Czech Republic; vacik@ujf.cas.cz
***2ND Medical Faculty, Charles University, Prague, Czech Republic; jungovai@seznam.cz

Abstract

Carbon fibre-reinforced carbon composites (CFRC), i.e. materials promising for hard tissue surgery, were coated by a fullerene layer in order to strengthen the material surface and create its nanostructure pattern which is known to be attractive for colonization with bone cells. The fullerene layer was relatively resistant to wear, at least swabbing with cotton, rinsing with liquids and exposure to cells and proteolytic enzymes. Both human osteoblast-like MG 63 cells and rat vascular smooth muscle cells (VSMC) in 1- and 2-day-old cultures adhered to these surfaces in lower numbers in comparison with the control uncoated material and tissue culture polystyrene. In addition, the VSMC on the fullerene-coated surfaces were less spread. The lower cell adhesion was probably due to a relatively high hydrophobicity of fullerenes. On the other hand, the spreading of MG 63 cells was comparable to that observed on the control surfaces, and these cells also assembled dot-like vinculin-containing focal adhesion plaques and relatively rich fine filamentous beta-actin cytoskeleton. We suppose that the cell adhesion may be enhanced by derivatization of fullerenes with specific chemical functional groups or peptidic ligands for cell adhesion receptors.

[Engineering of Biomaterials, 47-53,(2005),3-6]

Introduction

Fullerenes are spheroidal molecules made exclusively of carbon atoms (e.g., C_{60}, C_{70}). Similarly as carbon nanotubes, and nanodiamonds, fullerenes are considered

as promising building blocks for the construction of novel nanomaterials, especially for molecular electronics. In addition, these molecules display a diverse range of biological activity. Their unique hollow cage-like shape and structural analogy with clathrin-coated vesicles in cells as well as their reactivity allowing for immobilization of various molecules on their surface support the idea of the potential use of fullerenes as drug or gene delivery agents [1, 2]. In their pristine unmodified state, fullerenes are highly hydrophobic, water-insoluble, able to accept and release electrons, and relatively highly reactive, which enables them to be structurally modified. When irradiated with UV or visible light, fullerenes can convert molecular oxygen into highly reactive singlet oxygen. Thus, they have the potential for inflicting photodynamic damage on biological systems, including damage to cellular membranes or DNA cleavage. This harmful effect can be exploited for photodynamic therapy against tumors, viruses and bacteria resistant to multiple drugs. 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 [3,4].

However, relatively little is known about the influence of fullerenes on cell-substrate adhesion. A thin layer of fullerene molecules may enhance the cell adhesion on various biomaterials by its nanostructure arrangements (i.e., the presence of fullerene clusters or crystallites less than 100 nm), which resembles the architecture of physiological extracellular matrix (ECM), i.e. a structure composed from nanoscale proteins, and in the case of bone, also hydroxyapatite and other inorganic nanocrystals [5-7]. Therefore, we investigated adhesion of human osteoblast-like cells of the line MG-63 in cultures on carbon fibre-reinforced carbon composites, i.e. materials considered as promising for bone tissue engineering, coated with a thin fullerene layer. In addition, we studied adhesion of vascular smooth muscle cells, i.e. another important cell type present in the bone, moreover capable of osteogenic differentiation [8].

Material and methods

Two-dimensionally reinforced CFRC were prepared in the Institute of Rock Structure and Mechanics, Acad. Sci. CR, Prague [3]. Commercially available woven fabric made of carbon fibres Toray T 800 was arranged in layers, infiltrated with a carbon matrix precursor (phenolic resin UMAFORM LE, Synpo Ltd., Pardubice, CR), pressed, cured, carbonised at 1000°C, graphitised at 2200°C and finally graphitised at 2200°C. In order to decrease the material surface micro-scale roughness, non-appropriate for cell spreading [8], the CFRC were ground using metallographic paper of 4000 grade.

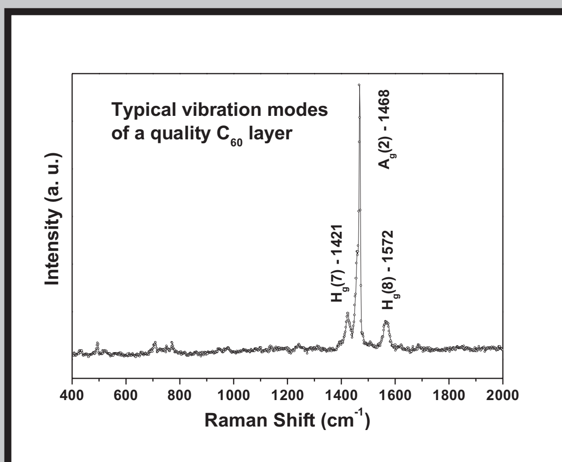

FIG. 1. Raman analysis of a thin C60 layer deposited on a CFRC substrate.

Thin fullerene (C60) layers, deposited on the CFRC, were prepared by evaporation of C60 in the Leybold Univex-300 vacuum system. In order to synthesize high quality C_{60} coverings, suitable deposition kinetics is necessary. Thus, the C_{60}/CFRC samples were prepared at room temperature (of the substrates) with a deposition rate $\leq$1 A/s (the temperature of C_{60} evaporation in the Knudsen cells was 400°C), and time of deposition of about 15 min. The thickness of the fullerene layers was estimated to be <100 nm. FIG.1 (showing a typical result of the Raman analysis) confirms that the fullerene films were prepared with a high quality with no fragmentation or graphitization of C_{60}. One third of the CFRC samples (3x3 cm) was protected of fullerene coating using a mask. Other completely uncoated CFRC samples as well as tissue culture polystyrene dish served as controls.

The samples were repeatedly rinsed in phosphate-buffered saline and inserted on the bottom of polystyrene Petri dishes (diameter 5 cm; GAMA, Ceske Budejovice, CR). The sterilization was avoided in order to prevent possible damage of fullerenes by heat, irradiation or chemicals, and the samples were used for short-term cell culture only. They were seeded with human osteoblast-like MG 63 cells (European Collection of Cell Cultures, Salisbury, UK) or vascular smooth muscle cells (VSMC) obtained from the thoracic aorta of adult male Wistar rats [3]. Each dish contained 300,000-500,000 cells (i.e., 15,000-25,000 cells/cm^2 and 7 ml of Dulbecco Minimum Essential Medium supplemented with 10% of fetal bovine serum and 40 μg/ml of gentamicin. Cells were cultured for 1-2 days 37°C in humidified air atmosphere containing 5% of CO_2, fixed with 70% ethanol and stained with propidium iodide. In addition, two molecules participating in the process of cell adhesion and spreading, namely vinculin, an integrin-associated protein, and beta-actin, important component of the cytoplasmatic cytoskeleton, were visualized by immunofluorescence staining [5,8,10].

Results and discussion

Grinding with metallographic paper of 4000 grade lowered the surface roughness of CFRC about twice. As measured by a profilometer (Rank Taylor Hobson Ltd., England), the departures of the roughness profile from the mean line (i.e., R_a parameter) decreased from 6.5±1.8μm to 3.5±0.6μm, and the mean spacing of the adjacent local peaks (parameter S) lengthened from 38±11μm to 96±49μm. Fullerene coating did not change significantly this surface microroughness but created a nanostructured pattern on the pre-existing microarchitecture of the CFRC surfaces. This nanopattern was expected to enhance adhesion of cells, especially those bone-derived, because recently, a very interesting finding has been reported that nanostructured surfaces can enhance the adsorption of vitronectin, i.e. an extracellular matrix protein mediating preferential adhesion of osteoblasts over other cell types [5, 6]. However, on day 2 after seeding, the MG-63 cells adhered to the fullerene-coated CFRC in a significantly lower number than on uncoated CFRC, glass or standard tissue culture polystyrene. Similar reaction was also observed in 1-day-old cultures of VSMC (FIG.2A,B). In addition, the VSMC on

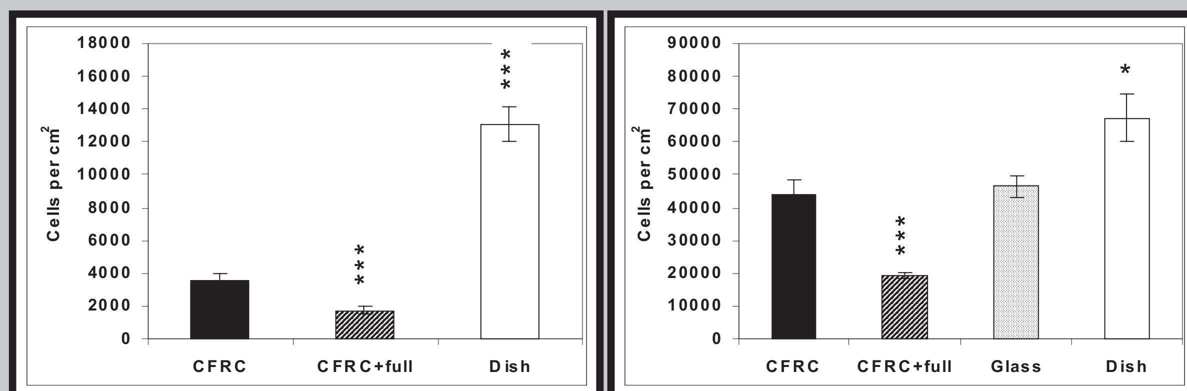

FIG. 2. Number of rat aortic smooth muscle cells (A) and osteoblast-like MG 63 cells (B) on carbon fibre-reinforced carbon composites (CFRC), CFRC coated with fullerenes (CFRC+full), glass coverslips (Glass) and standard polystyrene tissue culture dish (Dish) on day 2 (A) and 1 (B) after seeding. Average ± SEM from 12-21 microscopic fields, Student's t-test for unpaired data. Statistical significance: ***p≤0.001, *p≤0.02 in comparison with CFRC.

an important limitation of the potential biomedical use of CFRC [8].

In comparison with VSMC, the cell spreading area of MG 63 cells on the fullerene-coated CFRC was found to be significantly higher (3182±670 μm^2) than that on the uncoated material (1888±400 μm^2) and even on polystyrene dishes (1300±102 μm^2). This could be explained by the low cell population density on the fullerene layer, which provided the cells with more space for their spreading. On the other hand, the fullerene layer might promote the preferential adhesion of osteoblasts by its nanostructure [5,6]. On the fullerene layer, these cells formed fine dot-like vinculin-containing focal adhesion plaques and fine network of actin microfilaments (FIG.4), which is a sign of effective binding between cell adhesion receptors and ECM proteins adsorbed on the material surface [5,10]. In the future experiments, we plan to reduce the hydrophobia of fullerenes by their derivatization with oxygen-containing and amine groups, or to induce the receptor-mediated cell adhesion by functionalization of fullerenes with oligopeptides containing amino acid sequences RGD or osteoblast-specific KRSR [5,6,10]. A water soluble fullerene derivative, C3-fullero-tris-methanodicarboxylicacid, maintained the integrity of focal adhesion plaques and actin cytoskeleton in a UV-irradiated line of human epidermoid carcinoma cells by quenching oxygen radicals [4]. Enhanced attachment and spreading of platelets was also found on a polyurethane surface grafted with fullerene C_{60} molecules [11]. In addition, fullerene derivatives selectively targeting the bone tissue, delivering diagnostic and therapeutic agents and influencing bone tissue mineralization can also be constructed [1].

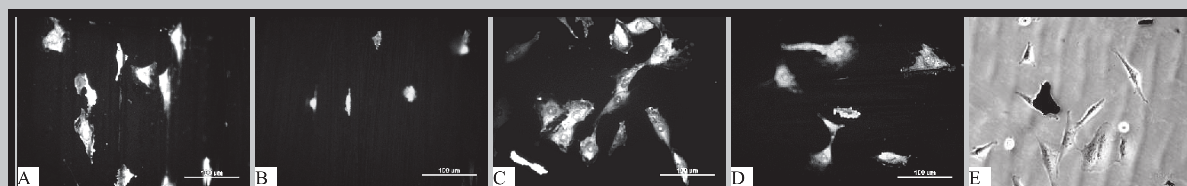

Fig. 3. Rat aortic smooth muscle cells on CFRC (A), CFRC coated with fullerenes (B), standard polystyrene tissue culture dish (C), uncoated region of a fullerene-coated CFRC sample (D), polystyrene dish with a fullerene-coated CFRC sample (E). Day 1 after seeding; fixed and stained with propidium iodide (A-D) or living unfixed cells

the fullerene layer were less spread, i.e. round or spindle-shaped, whereas on the pure CFRC and especially on the tissue culture polystyrene, they were mainly polygonal and of a larger cell-material contact area (FIG.3A-D).

The lower cell adhesion on the fullerene layer was probably due to the hydrophobic character of the non-derivatized fullerene molecules [3]. It is known that hydrophobic materials promote preferential adsorption of cell non-adhesive proteins from the serum of the culture media, such as albumin. In addition, the cell adhesion-mediating ECM proteins are adsorbed in relatively rigid state, thus their specific amino acid sequences are less accessible for cell adhesion receptors [10]. Penetration of fullerenes into cells and their cytotoxic action is less probable, because the cell attachment and spreading on the control uncoated regions of the fullerene-modified CFRC (FIG.3D), as well as on the bottom on polystyrene dishes containing fullerene-coated samples (FIG.3E), were similar as in control polystyrene dishes without fullerene samples. In addition, the fullerene layer was resistant to mild wear, represented by swabbing with cotton, rinsing with liquids (water, phosphate-buffered saline, culture media) and exposure to cells and proteolytic enzymes used for cell harvesting. The fullerene-coated CFRC surfaces seemed to be stronger and less prone to release carbon particles (the latter see on Fig. 3E), which is

Conclusions

Coating of carbon-fibre reinforced carbon composites, i.e. promising materials for bone tissue engineering, with a fullerene layer reduced the number of osteoblast-like MG 63 cells or vascular smooth muscle cells adhering to these materials in vitro. The latter cell type was also less spread, whereas the spreading of MG 63 cells was comparable to that observed on the uncoated composites or tissue culture polystyrene. The cell adhesion may be further enhanced by derivatization of fullerenes with chemical functional groups or oligopeptidic ligands for cell adhesion receptors.

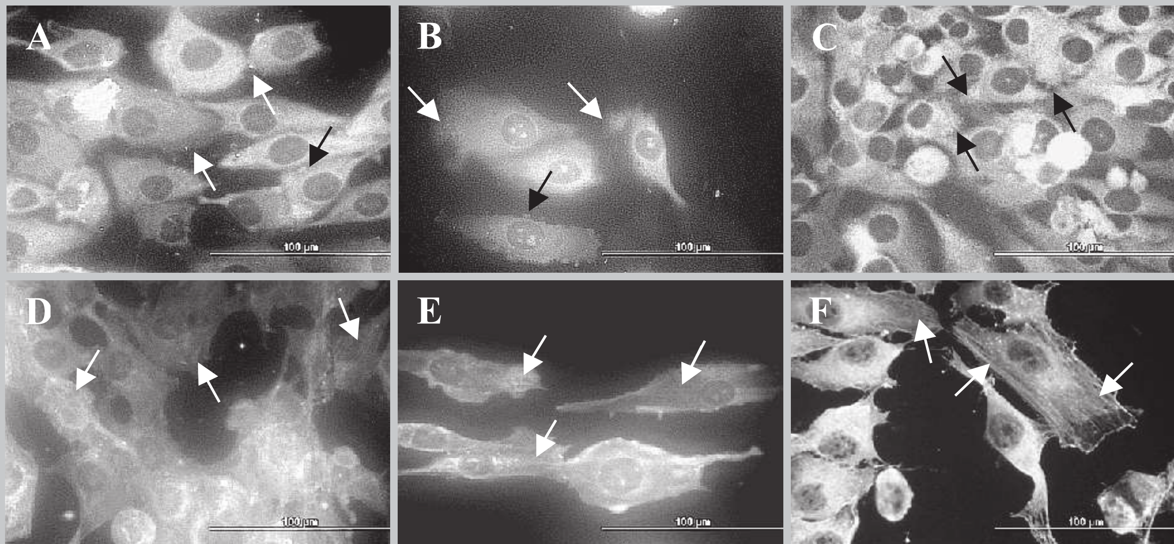

FIG. 4. Immunofluorescence staining of vinculin (A-C) and beta-actin (D-F) in MG 63 cells on day 2 after seeding on CFRC (A,D), fullerene-coated CFRC (B,E) or standard polystyrene tissue culture dish (C, F). Arrows indicate vinculin-containing focal adhesion plaques (A-C) or beta-actin filament bundles (D-F).

Acknowledgements

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References

[1] Gonzalez KA, Wilson LJ, Wub W, Nancolla GH: Bioorg Med Chem 10: 1991-1997, 2002.
[2] Martin CR, Kohli P.: Nat Rev Drug Discov 2: 29-37, 2003.
[3] Jensen AW, Wilson SR, Schuster DI: Bioorg Med Chem. 4:767-779, 1996.
[4] Straface E, Natalini B, Monti D, Franceschi C, Schettini G, Bisaglia M, Fumelli C, Pincelli C, Pellicciari R, Malorni W: FEBS Lett 454: 335-340, 1999.
[5] Webster TJ, Ergun C, Doremus RH, Siegel RW, Bizios R: J Biomed Mater Res 51: 475-483, 2000.
[6] Price RL, Ellison K, Haberstroh KM, Webster TJ: J Biomed Mater Res 70A: 129-138, 2004.
[7] Tan J, Saltzman WM: Biomaterials 25: 3593-3601, 2004.
[8] Bacakova L, Stary V, Kofronova O, Lisa V: J Biomed Mater Res. 2001; 54(4):567-78.
[10] Bacakova L, Filova E, Rypacek F, Svorcik V, Stary V. Cell adhesion on artificial materials for tissue engineering. Physiol Res 53 Suppl 1: S35-45, 2004.
[11] Lin JC, Wu CH: Biomaterials 20: 1613-1620, 1999.

Human osteoblast-like cells MG 63 in cultures on polymer-carbon-alginate composites

Lubica Grausova*, Lucie Bacakova*, Ewa Stodolak**, Stanislaw Blazewicz**, Teresa Mikołajczyk***

*Institute of Physiology, Acad. Sci. CR, Videnska 1083, 142 20 Prague 4-Krc, Czech Republic;
grausova@biomed.cas.cz
**AGH - University of Science and Technology, Faculty of Materials Science and Ceramics, Department of Biomaterials, Krakow, Poland
***Technical University of Lodz, Faculty of Textile Engineering and Marketing, Department of Man-Made Fibres, Lodz , Poland

Abstract

Adhesion and growth of human osteoblast-like MG 63 cells (seeding density of 17964 cells/cm^2) was studied in cultures on the following 7 groups of artificial materials developed for bone tissue engineering: terpolymer of polypropylene, polytetrafluorethylene and polyvinyldifluoride (CP0), terpolymer reinforced with carbon fibres (CP4) or carbon fabric (CP5), terpolymer reinforced with carbon fibres and with pores created by addition and dissolution of alginate fibres (CP6) or powder (CP7), terpolymer reinforced with carbon fibres and containing the alginate powder (CP8) or fibres (CP9). On day 1 and 3 after seeding, the numbers of MG63 cells on all tested materials was similar to the values obtained on the control polystyrene culture dish (PS). However, on day 7, the cell number, ranging from 17766±3180 to 67002±6850 cells/cm^2, increased in the following order: CP0<PS<CP6<CP8<CP7<CP5<CP4<CP9, which suggest a supportive role of carbon and alginate components of the materials on their colonization with cells.
[Engineering of Biomaterials, 47-53,(2005),6-9]

Introduction

In recent years, artificial materials are of growing importance in medicine and biology. A modern scientific interdis-

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