HUMAN BONE-DERIVED CELLS ON C-C COMPOSITES TREATED BY GRINDING, a-C:H COATING, LASER IRRADIATION AND LASER PERFORATION

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Abstract

Carbon fiber-reinforced carbon (CFRC) composites were modified by grinding with abrasive papers, laser irradiation, coating with diamond-like carbon (DLC), creation of pores by laser perforation and various combinations of these treatments, and seeded with human osteoblast-like MG 63 cells. Twenty four hours after seeding, the lowest cell numbers were obtained on the non-ground, non-coated composites with Type 1 laser perforations (diameter 0.1 mm, depth 0.2 mm, spacing 0.3 mm), i.e., on samples with relatively high surface roughness (R_a = 17.7 ± 0.9 μm, S = 93.0 ± 2.0 μm). On the other hand, the cells on these samples were well-spread, adhering with a relatively large cell-material projected area. In comparison with these samples, a significantly higher number of cells were obtained on composites treated with a DLC coating, especially those with laser perforation 2 (diameter 0.4 mm, depth 0.8 mm, spacing 1.2 mm). The cells on both types of laser-perforated samples started to colonize pores created by the laser beam.

[Engineering of Biomaterials, 75, (2008), 2-6]

Introduction

Over a period of more than 30 years, carbon fiber-reinforced carbon (CFRC) composites have proven to be suitable materials for technical applications requiring high mechanical, thermal and chemical resistance, such as rocket motor nozzles, reentry heatshields, aircraft disk brakes, and turbine engines [1]. In addition, the physical properties of these materials, such as specific weight, porosity, modulus of elasticity as well as flexural and shear strength, can be tailored to be similar to the corresponding parameters of bone tissue [2,3]. Therefore, CFRC composites have been considered promising materials for application in orthopedics, dentistry and surgery of the spine, e.g., for the construction of hip joint prostheses [4-7], spinal cages [8] or endosseous dental implants [2]. For hard tissue surgery applications, great attention has also been paid to related materials containing carbon fibres reinforcing various types of polymeric matrices, such as polyetheretherketone (PEEK, [9], liquid crystalline polymer [10], polyamide [11] or poly(methylacrylate) [12]. Some carbon fiber-reinforced polymeric materials have been used clinically, e.g., as cages for lumbar interbody fusion for treatment of spondylolisthesis [13,14], for spine reconstruction after spondylectomy [15] or reconstruction of cranial defects [16]. Carbon fiber modular implants filled with cortical and cancellous bone grafts have been applied for reconstruction of vertebrae in patients suffering from primary or metastatic tumors of the spine [17]. All these materials are radiolucent, which facilitates the examination of the implants with X-rays, computed tomography or magnetic resonance [14,16,18]. However, these materials also have some disadvantages, particularly the tendency to release carbon particles, which is due to the brittle nature of both carbon fibres and matrix. In addition, due to the prominence of carbon fibres over carbon matrix, the surface microroughness of CFRC composites can be relatively high, and this can hamper the adhesion, spreading and growth of bone cells [3,19]. These problems can be minimized by polishing the composites and/or coating these materials with strong and mechanically resistant biocompatible layers, e.g., pyrolytic carbon or graphite [19,20], carbon-titanium [3], zirconium nitride [21] or fullerite [22,23]. Integration of CFRC composites with the surrounding bone tissue could be further enhanced by the presence of pores in these materials, which would enable the ingrowth of bone tissue [24]. Therefore, in the present study, we investigate the adhesion and spreading of human osteoblast-like MG 63 cells in cultures on CFRC composites modified by grinding, coating with amorphous hydrogenated diamond-like carbon (a-C:H, also referred to as DLC), laser irradiation, creation of pores by laser perforation, and various combinations of these treatments.

Material and Methods

Preparation of CFRC composites

Preparation of CFRC composites was described in detail in our earlier papers [3,19,21-23]. Briefly, the two-dimensionally reinforced CFRC composites used in this study were prepared at the Institute of Rock Structure and Mechanics, Acad. Sci. CR, Prague. Commercially available woven fabric (made of Toray T 800 carbon fibres) was arranged in layers, infiltrated with a carbon matrix precursor (UMAFORM LE phenolic resin, Synpo Ltd., Pardubice, Czech Republic), pressed, cured, carbonised at 1000°C in N_2, and finally graphitised at 2200°C in Ar. The composites were then cut with a diamond saw into samples 9 x 9 mm in size and 2 mm in thickness, and subjected to one or more of the surface modifications described below. The surface morphology of a pristine unmodified CFRC sample is shown in FIG. 1A.

Surface modifications of CFRC composites

Grinding

The samples were progressively ground by abrasive papers of grain sizes 350, 600, 800, 1000 and 4000. The resulting surfaces were apparently smooth, with a shiny glass-like appearance (FIG. 1B). The samples were then cleaned in an ultrasonic washer.

Laser irradiation

This treatment was used with the aim to improve some properties of CFRC composites that are important for the cell-material interaction, such as consistency of the carbon matrix, which would prevent the release of carbon particles. The laser beam was applied using a MCVL 1000 LASER machining centre (Nd:YAG laser) under the following parameters: output 50W, speed of the laser beam 80 mm/s, beam trail Ø 0.1mm, pulse frequency 4000 Hz, perpendicular hatching, pitch 0.07 mm, crossover count 2. The surface morphology of a laser-irradiated CFRC sample is shown in FIG. 1C.

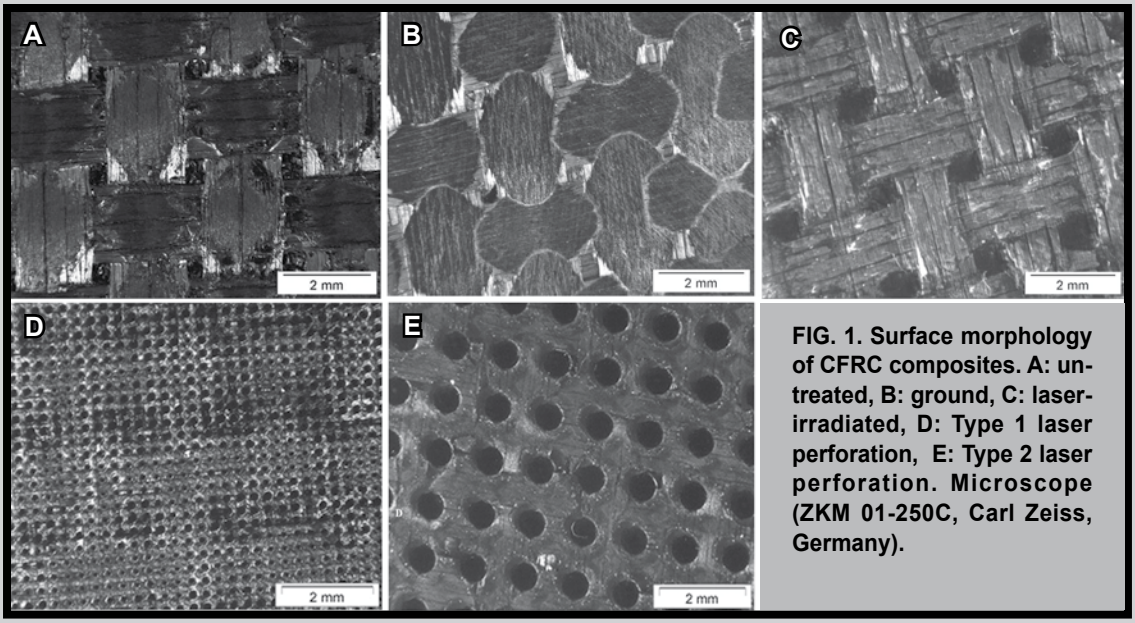

FIG. 1. Surface morphology of CFRC composites. A: untreated, B: ground, C: laser-irradiated, D: Type 1 laser perforation, E: Type 2 laser perforation. Microscope (ZKM 01-250C, Carl Zeiss, Germany).

Deposition of amorphous hydrogenated diamond-like carbon (a-C:H)

DLC films of good adhesion to the CFRC surface and about 200 nm in thickness were created using the plasma-assisted chemical vapor deposition (PACVD) method in a lab-made reaction chamber. First, the samples were cleaned chemically in acetone, then in plasma, using argon. The precursors for creating DLC were CH_4 and H_2. The frequency of the plasma used for both cleaning and deposition was 13.56 MHz. Two types of film were prepared. The differences between them consisted in the bias used for the deposition (-50V or -100V for Type 1 and Type 2, respectively), and for that reason also in the time needed for creating layers of the same thickness (40 and 16 min, for Type 1 and Type 2, respectively). The output was 20 – 25 W for Type 1 and 50 W for Type 2. The other parameters, such as flow of CH_4 (50 sccm), H_2 (30 sccm) and pressure (7.7×10^{-1} Torr) were similar for deposition of both types of DLC layers.

Laser perforation

The samples were modified by a laser beam on an MCVL 1000 LASER machining centre (Nd:YAG laser, output 50 W, speed of the laser beam 80 mm/s, pulse frequency 4500 Hz). Two types of surface texture were created. Perforation 1 represents burning of openings with the following parameters: diameter 0.1 mm, depth 0.2 mm, spacing 0.3 mm. The perforation 2 parameters were: diameter 0.4 mm, depth 0.8 mm, spacing 1.2 mm.

Using these procedures and combinations of them, 27 experimental groups of CFRC samples were prepared in duplicates (TAB. 1). All treatments were performed on one side of the samples, specified in advance.

Surface roughness and morphology of CFRC composites

The surface roughness was characterized by parameter R_a (departures of the roughness profile from the mean line) and parameter S (the mean spacing of the adjacent local peaks). These parameters were determined using a Talysurf profilometer (Talysurf 6, Rank Taylor Hobson Ltd., England). The material surface morphology (FIG. 1) was evaluated using a digitized microscope (ZKM 01-250C, Carl Zeiss, Germany).

TABLE 1. Surface modifications of CFRC composites.

Group No.	Surface processing	Layer deposition	Laser perforation
1	Without processing	Without layer	Without perforation
2	Grinding	Without layer	Without perforation
3	Laser irradiation	Without layer	Without perforation
4	Without processing	a-C:H 1	Without perforation
5	Grinding	a-C:H 1	Without perforation
6	Laser irradiation	a-C:H 1	Without perforation
7	Without processing	a-C:H 2	Without perforation
8	Grinding	a-C:H 2	Without perforation
9	Laser irradiation	a-C:H 2	Without perforation
10	Without processing	Without layer	Perforation 1
11	Grinding	Without layer	Perforation 1
12	Laser irradiation	Without layer	Perforation 1
13	Without processing	aCH 1	Perforation 1
14	Grinding	a-C:H 1	Perforation 1
15	Laser irradiation	a-C:H 1	Perforation 1
16	Without processing	a-C:H 2	Perforation 1
17	Grinding	a-C:H 2	Perforation 1
18	Laser irradiation	a-C:H 2	Perforation 1
19	Without processing	Without layer	Perforation 2
20	Grinding	Without layer	Perforation 2
21	Laser irradiation	Without layer	Perforation 2
22	Without processing	aCH 1	Perforation 2
23	Grinding	aCH 1	Perforation 2
24	Laser irradiation	aCH 1	Perforation 2
25	Without processing	aCH 2	Perforation 2
26	Grinding	aCH 2	Perforation 2
27	Laser irradiation	aCH 2	Perforation 2

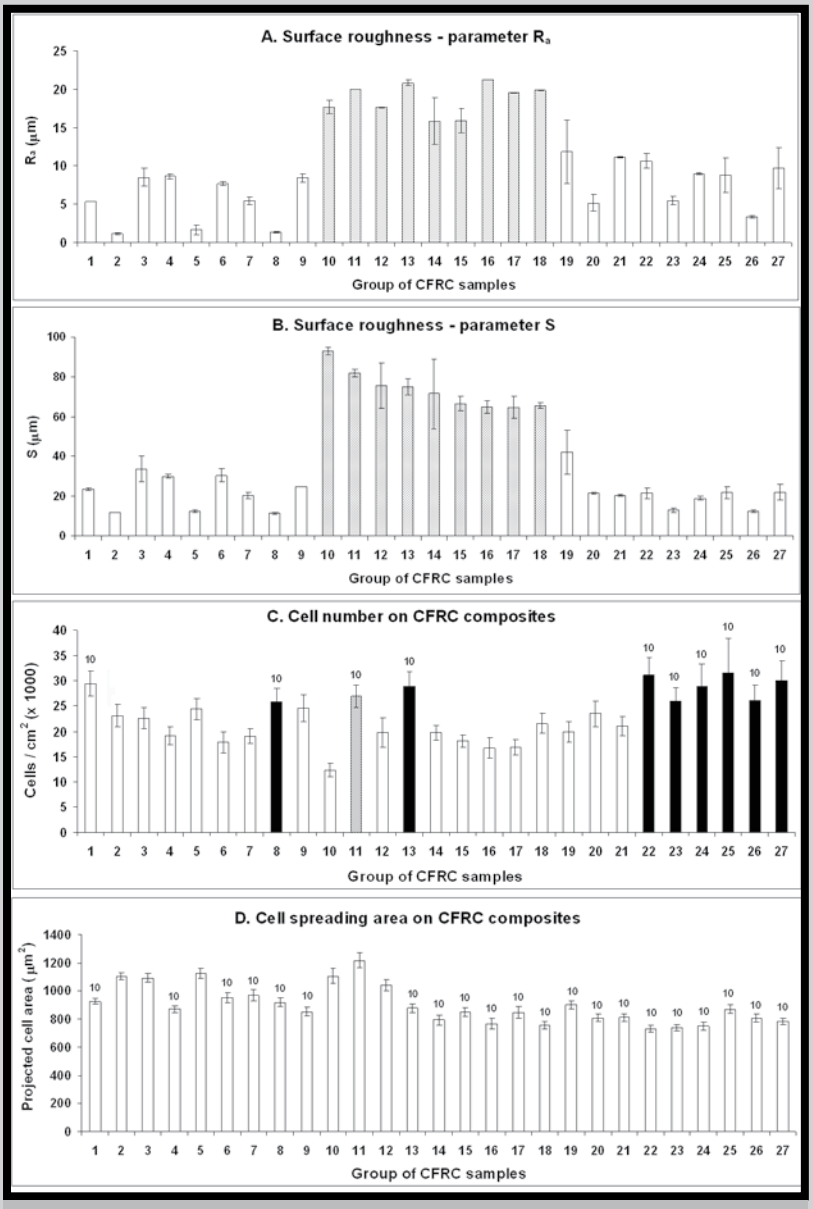

FIG. 2. Parameter R_a (A) and S (B) of the surface roughness correlated with the number (C) and spreading area (D) of human osteoblast-like MG 63 cells. Mean ± S.D. from 2 samples, each measured in 6 areas (A, B) or S.E.M. from 32 measurements (C) or 47-383 cells (D) for each group. ANOVA, Student-Newman-Keuls method. Statistical significance: [10]: p≤0.05 in comparison with the samples of group 10.

Cell culture on the CFRC composites

The CFRC samples were sterilized in 70% ethanol for 2 hours, air dried, inserted into 24-well culture plates (well diameter 15 mm; TPP, Trasadingen, Switzerland) and seeded with human osteoblast-like MG 63 cells (30 000 cells/well, i.e., about 17,000 cells/cm^2) into 1 ml of Dulbecco's modified Eagle's Minimum Essential Medium (DMEM; Sigma, U.S.A., Cat. N° D5648) supplemented with 10% foetal bovine serum (FBS; Sebak GmbH, Aidenbach, Germany) and gentamicin (40 µg/ml, LEK, Ljubljana, Slovenia). The cells were cultured at 37° C in a humidified air atmosphere containing 5% of CO_2.

Twenty four hours after seeding, the cells were rinsed with phosphate-buffered saline (PBS), fixed with 70% cold ethanol (-20°C, 10 min.) and visualized by fluorescence staining. The proteins of the cell membrane and cytoplasm were stained with Texas Red C_2-maleimide (Molecular Probes, Invitrogen, Cat. No. T6008; 20 ng/ml in PBS), and the cell nuclei with Hoechst 33342 (Sigma, U.S.A.; 5 µg/ml in PBS) for 2 hours at room temperature. Digital pictures of the cells were then taken under a conventional fluorescence microscope (Olympus IX 50) and in a confocal microscope (DM 2500, Leica, Germany). The latter enables precise focusing of the cells on highly irregular surfaces or inside porous materials using a series of optical sections. Each sample was photographed in 16 fields (0.136 and 2.25 mm^2 in the conventional microscope and in the confocal microscope, respectively) randomly selected but homogeneously distributed over the material surface. In these fields, the cells were counted and their spreading area (i.e., the cell area projected on the material) was measured using Atlas software (Tescan S.R.O., Brno, Czech Republic).

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). 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.

Results and Discussion

Grinding the CFRC composites with abrasive papers markedly decreased the size of the irregularities (parameter R_a) on the material surface as well as their spacing (S) (FIG. 2A and B, group 1 *vs*. group 2). On the other hand, the surface roughness after laser irradiation increased (group 1 *vs*. group 3), which could be explained by more prominent bundles of carbon fibers over the carbon matrix. In other words, the hollows among the carbon fiber bundles seemed to be deeper, probably due to the increased consistency and dense packing of the carbon matrix after laser irradiation (FIG. 1C compared to 1A). Similarly, coating with diamond-like carbon of Type 1 or Type 2 also increased or did not change the surface roughness (FIG. 2A and B, group 1 *vs*. groups 4 and 7).

The highest surface roughness values were achieved on the composites treated with Type 1 laser perforation (diameter 0.1 mm, depth 0.2 mm, spacing 0.3 mm), i.e., on the samples in experimental groups 10 to 18. The lowest numbers of adhering cells were found on group 10, i.e., on non-processed, non-coated composites with laser perforation 1 (see FIG. 2 C). In our earlier studies, a higher surface roughness of CFRC was also associated with a lower number of MG 63 cells and vascular smooth muscle cells [3,19,21].

However, material surface roughness was not the only factor affecting cell adhesion. In comparison with the samples of group 10, significantly higher cell numbers were usually achieved on samples coated with DLC 1 or 2, i.e. groups 8, 13 and 22 to 27 (except the unmodified group 1 and laser 1-perforated group 11 with surface grinding; FIG. 2C). DLC coating therefore seems to be advantageous for supporting the colonization of CFRC composites by cells.

Similar results were also found when CFRCs were coated with a carbon-titanium layer or pyrolytic graphite [3,19].

On the other hand, the cells on samples with the lowest cell population density (group 10) adhered to the material with a relatively large area, which may be due to the fact that these cells were not limited by cell-to-cell contacts, and thus they used more space for their spreading (FIG. 2D). Similar results were also obtained on CFRCs coated with a fullerite layer [23].

The morphology of MG 63 cells on pristine CFRC composites and materials modified by grinding, laser irradiation, DLC coating and laser perforation is shown in FIG. 3. The cells were polygonal or spindle-shaped, often oriented along the carbon fibers prominent on the material surface, also adhering in hollows in the regions where the carbon fiber bundles crossed in the carbon fabrics (FIG. 3A-E). On both types of laser-perforated samples, the cells started to penetrate into the pores, though they usually did not reach the pore bottom due to the relatively short culture interval of only 24 hours (FIG. 3F,G). Ingrowth of the cells into the pores would be beneficial for integration with the surrounding bone tissue [24], if the CFRC composites are used for constructing bone implants.

Conclusion

Grinding CFRC composites with abrasive papers decreased their surface roughness, measured by parameters Ra and S. On the other hand, laser irradiation and especially Type 1 laser perforation (diameter 0.1 mm, depth 0.2 mm, spacing 0.3 mm) markedly increased the surface roughness, which was negatively correlated with the number of human osteoblast-like MG 63 cells adhering to these materials 24 hours after seeding. The lowest number of cells was achieved on non-ground, DLC non-coated and laser 1-perforated samples. DLC coating was usually associated with relatively high cell numbers, especially on composites treated with laser perforation 2 (diameter 0.4 mm, depth 0.8 mm, spacing 1.2 mm). On both types of laser-perforated samples, the cells showed a tendency to colonize the laser-created pores.

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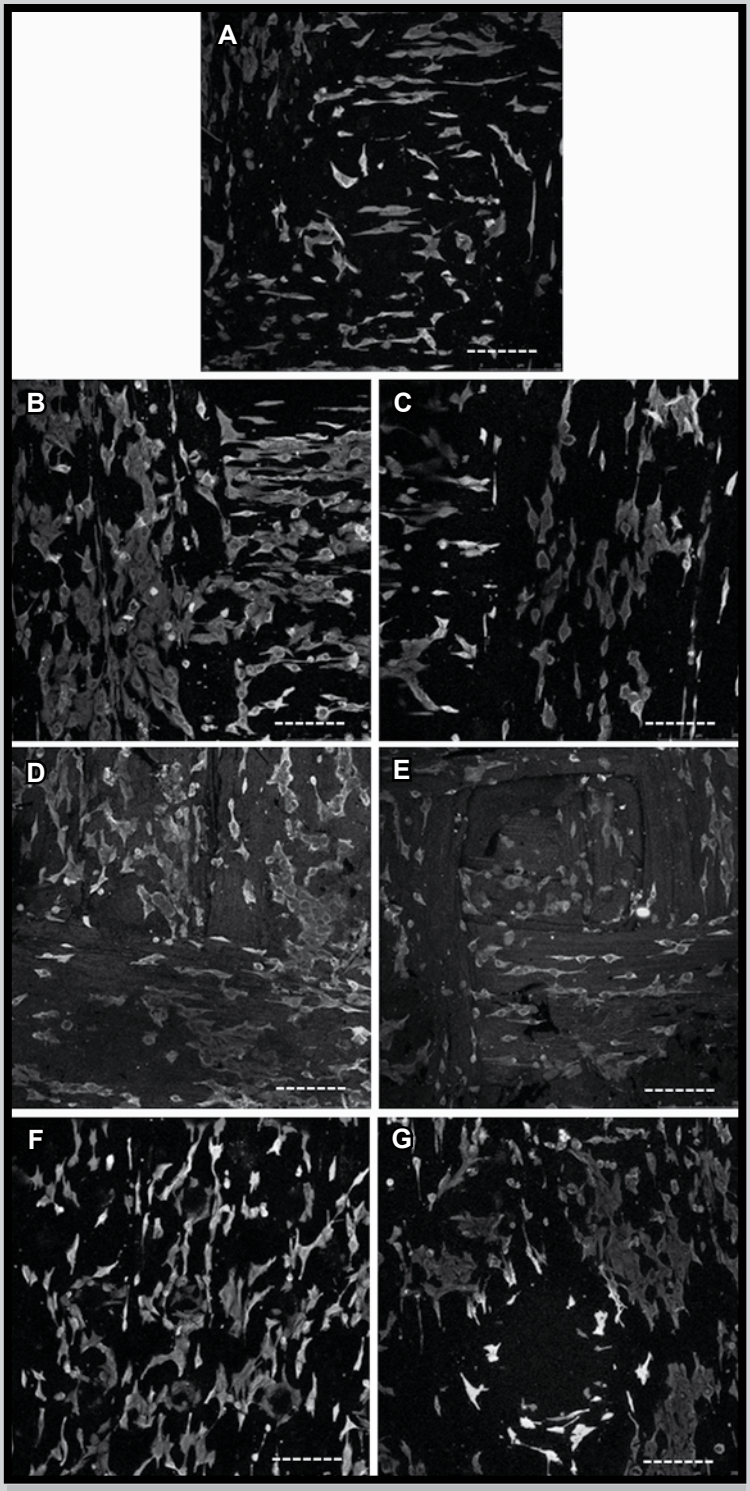

FIG. 3. Morphology of human osteoblast-like MG 63 cells on day 1 after seeding of unmodified CFRC composites (group 1, A), composites modified by grinding (group 2, B), laser irradiation (group 3, C), Type 1 amorphous hydrogenated diamond-like carbon (D) and 2 (E), laser perforation 1 (F) and laser perforation 2 (G). Leica DM 2500 confocal microscope, obj. 10x, bar = 300 µm. The cells are visualized in spectral colors (violet, blue, green, yellow and red from the material surface to the depth). A: summation of 18 optical sections performed to a depth of 170 µm; B, C: 8 sections, depth 70 µm, D: 6 sections, depth 50 µm, E: 25 sections, depth 240 µm F: 11 sections, depth 100 µm, G: 29 sections, depth 280 µm, The thickness of all sections was 10 µm.

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THE ADHESION AND GROWTH OF VASCULAR SMOOTH MUSCLE CELLS IN CULTURES ON CARBORANETHIOL-MODIFIED GOLD FILMS

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Abstract

*Metal surfaces have become important over the last decade for potential surgical implants, and within this context we present here a study of the cell growth on modified gold surfaces. Gold films, deposited on glass plates and annealed with a hydrogen flame, were modified with four different carboranethiol derivatives: 1-(HS)-1,2-$C_2B_{10}H_{11}$ (**A**), 1,2-$(HS)_2$-1,2-$C_2B_{10}H_{10}$ (**B**), 9,12-$(HS)_2$-1,2-$C_2B_{10}H_{10}$ (**C**) and 1,12-$(HS)_2$-1,12-$C_2B_{10}H_{10}$ (**D**). The materials engendered from these modifications were used to investigate the adhesion and growth of rat aortic smooth muscle cells cultured on these surfaces in a DMEM medium with 10% of fetal bovine serum. One day after seeding, the highest number of initially adhered cells was found on the surface of a bare gold film. However, three days after seeding, the number of cells on carboranethiol-modified gold samples **B**, **C** and **D** was significantly higher than the number on a bare gold film. After seven days, the number of cells on a bare gold film and on gold films modified with derivatives **A**, **B** and **D** was very similar, but the surface of a gold film modified with derivative **C** exhibited a significantly smaller number of cells. This may be explained by the exposure of the CH vertices of the carborane cluster, which are more acidic than the BH vertices exposed toward the cells in either **A** or **B**.*

Keywords: *Metal coating, gold film, carboranethiol, cell adhesion, cell spreading, cell proliferation, biomaterials, tissue engineering, surgical implants.*

[Engineering of Biomaterials, 75, (2008), 6-8]

Introduction

Artificial and nature-derived materials, including metals, have been intensively studied in medicine and in various biotechnologies. Examples are bio-imaging, bio-sensing, drug delivery, cell cultivation, and the construction of replacements of irreversibly damaged tissues and organs. A largely accepted concept in recent tissue engineering is of surfaces supporting and controlling cell colonization associated with successful integration of an implant within the organism. This concept is used for the construction of durable bone prostheses persisting in the patient for many years, and is being developed to make bio-artificial replacements of blood vessels, liver, pancreas or even nervous tissue (for a review, see [1-3]).