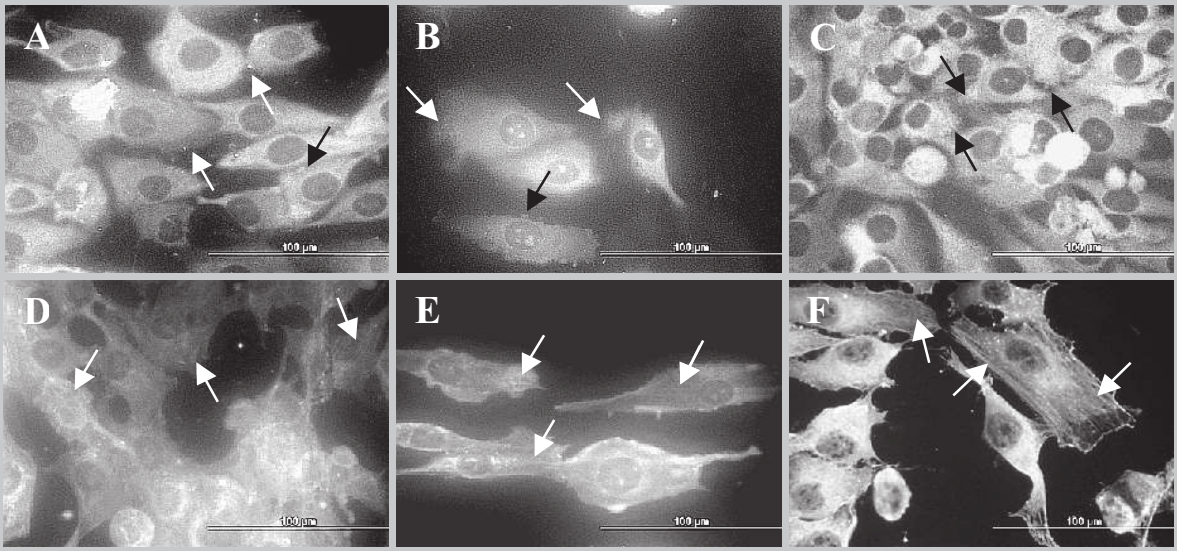

FIG. 4. Immunofluorescence staining of vinculin (A-C) and beta-actin (D-F) in MG 63 cells on day 2 after seeding on CFRC (A,D), fullerene-coated CFRC (B,E) or standard polystyrene tissue culture dish (C, F). Arrows indicate vinculin-containing focal adhesion plaques (A-C) or beta-actin filament bundles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

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

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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-

ciplinary field known as Tissue Engineering has been developed to design artificial biocompatible materials in order to substitute irreversibly damaged tissues and organs [1-6]. Nowadays it is very trendy to study carbon materials for potential biomedical use. Carbon is an important component of cells and tissues, so that these materials could be well tolerated by the surrounding tissue. In addition, their mechanical properties (e.g. the density, porosity, modulus of elasticity) can be tailored to be very close to the bone. Thus, these materials are very promising for the use in orthopaedic and dental surgery.

Our earlier studies showed that carbon-carbon composites (i.e., carbon fibre-reinforced carbon composites, referred as CFRC) supported adhesion and growth of osteogenic as well as vascular endothelial and smooth muscle cells in vitro, especially after modifications of the CFRC surface by grinding with metallographic paper, polishing with colloidal SiO_2 or diamond paste and deposition of various bioactive layers, such as pyrolytic graphite, Ti/C:H, Ag/C:H with low silver concentration or even fullerenes C60 [7-10]. Despite of these encouraging results, the CFRC manifested a relative high tendency to release carbon particles, which is mainly due to the brittleness of the carbon matrix [7,8]. Therefore, in the present study, we constructed carbon-polymer composites, i.e. materials in which the carbon matrix was replaced by a polymeric resin. These new-generation composites were expected to be more resistant to the material disintegration and supportive for colonization with osteogenic cells. Some composites were enriched with alginates, i.e. nature-derived polysaccharides containing uronic acids, which are considered as promising biomaterials for tissue engineering, such as construction of bioartificial cartilage [2,3], myocardium [4] or skeletal muscle [5]. Another perspective application of alginates is encapsulation of transplanted cells, such as hormone-secreting cells (parathyroid and pancreatic islet cells [2,11-13]) or genetically-engineered tumour-killing cells [1,14].

Material and methods

Carbon fibres in the form of plaited fabrics were obtained at the Department of Advanced Ceramics, University Science and Technology, Krakow, Poland. A special epoxide resin covering the fibres was removed in an oxidizing atmosphere 35 min/400^{0}C. In order to obtain short carbon fibres (diameter 7.05±0.23 μm, length 1.2±0.25 mm), the carbon fabric was cut in a special mechanical mill.

A terpolymer of polypropylene, polytetrafluorethylene and polyvinyldifluoride (PTFE/PVDF/PP, density 1.600, purchased from Aldrich Chemical Co., USA, Cat. No. 45 458-3) was dissolved in acetone (POCh SA. Gliwice, Poland, Cat. No. 102480111; 5g of PTFE/PVDF/PP polymer resin per 50 ml of acetone), and the following 7 groups of samples were prepared:

1-CP0. Pure PTFE/PVDF/PP polymer samples; the terpolymer solution was poured onto glass Petri dishes and left to freely evaporate for 2 days.

2-CP4. Terpolymer with short carbon fibres; the fibres suspended in acetone were distributed on Petri dishes, and then the terpolymer solution was poured onto them and left to freely evaporate for 2 days.

3-CP5. Terpolymer with plaited fabrics which had regular packing fibres; the terpolymer solution was poured onto the fabrics inserted in Petri dishes and left to freely evaporate for 2 days.

4-CP6. Terpolymer with short carbon fibres and alginate fibres; the latter were obtained at the Department of Man-Made Fibres, Faculty of Textile Engineering and Marketing, Technical University of Lodz, Poland. The content of acid's radicals in the alginate was 65-75% for guluronic acid and 25-35% for mannuronic acid. In the first step, both types of fibres in acetone were distributed on Petri dishes, and in the next step, the polymer solution was poured onto them and left to freely evaporate for 2 days. Then the alginate fibres were removed in hot water (85^{o}C/28h) in order to create gaps of the diameter of 5.2±0.25 mm. These gaps were probably open pores penetrating the whole thickness of the material membranes, i.e., 178 mm).

5-CP7. Terpolymer with short carbon fibres and alginate powder; the latter were purchased from the same company as the alginate fibres, and were of the same chemical composition. In the first step, both fibres and powder were suspended in acetone and distributed on Petri dishes, and in the next step, the polymer solution was poured onto them and left to freely evaporate for 2 days. Then the alginate powder was removed in hot water (85^{o}C/28h), and gaps of the size of 1.8±0.15 mm were created. Similarly to the sample CP6, the gaps probably penetrated through the entire thickness of the material sheet (which was thinner that in CP6), thus their depth was 168 mm.

6-CP8. Terpolymer containing short carbon fibres and alginate powder; both fibres and powder in acetone were distributed on Petri dishes, and then the polymer solution was poured onto them and left to freely evaporate for 2 days.

7-CP9. Terpolymer containing short carbon fibres and alginate fibres; both types of fibres were suspended in acetone, distributed on Petri dishes and then the polymer solution was poured onto them and left to freely evaporate for 2 days.

The surface characterization was accomplished by contact angle measurements. As the comparative liquid, twice distilled water was used (TABLE 1).

For cell cultivation, thin circular sheets of the materials (diameter 1.67 cm) were sterilized by H_2O_2 - plasma method (Sterrad 120, ASP, Johnson & Johnson), inserted in polystyrene 24-well-multidishes (TPP, Switzerland), seeded with human osteoblast-like cells of the line MG63 (European Collection of Cell Cultures, Salisbury, UK) at the initial density of 17000 cells/cm^2, and incubated in 1.5 ml of Dulbecco-modified Eagle Minimum Essential Medium (Sigma, U.S.A., Cat. No D5648) supplemented with 10% foetal bovine serum (Sebak GmbH, Aidenbach, Germany) in humidified air atmosphere containing 5% of CO_2. On day 1 and 3 after seeding, cells were fixed with 70% ethanol, stained with propidium iodide (5µg/ml), and their number and morphology were evaluated in a fluorescence microscope. On day 7, cells were harvested by trypsin-EDTA solution (Sigma, U.S.A.; exposure 10 min at 37^{o}C) and counted in a Bürker's haemocytometer.

Quantitative data were presented as means ± SEM (Standard Error of Mean) from 8-18 measurements obtained from 3 samples for each experimental group).

Sample name	Contact angle [^{o}]	Surface energy [mN/m^2]	Dispersion component [mN/m^2]	Polar component [mN/m^2]
CP0	99.7±2.5	20.31±1.62	18.31±1.31	2.00±0.31
CP4	87.2±2.5	24.00±0.76	23.59±0.69	0.40±0.07
CP5	99.6±2.66	20.15±0.52	18.25±0.27	1.86±0.38
CP6	78.4±3.25	20.28±0.61	17.52±0.43	2.76±0.17
CP7	67.8±2.94	21.52±1.74	18.35±0.86	2.29±0.73
CP8	83.5±2.86	26.25±0.98	23.52±0.82	2.73±0.18
CP9	88.8±4.25	31.18±1.15	25.92±0.77	5.26±0.54

TABLE 1. Results of contact angle measurements on carbon-polymer composites.

Results and discussion

On day 1 and 3 after seeding, the numbers of MG 63 cells on all carbon-polymer composites as well as the pure terpolymer were similar to the value obtained on the control polystyrene culture dish (PS). The latter values amounted to 2694±424 cells/cm^2 and 11349±1968 cells/cm^2 on day 1 and 3, respectively. However, on day 7 after seeding, the highest number of MG 63 cells (67002±6850 cells/cm^2) was found on the sample CP9 (i.e., the carbon fibre-reinforced terpolymer containing alginate fibres). This number was more than twice higher than that obtained on standard tissue culture polystyrene (FIG.1,2). This result could be due to a soft and sponge-like structure of the alginate, which allowed the cells to penetrate inside the material, and thus to use more space for their expansion. Also the relatively high surface energy, especially its polar component (TABLE 1) might contribute to the highest cell colonization of the CP9 sample [6,15]. Another explanation is direct mitogenic action of alginates due to the presence of various contaminants [2,11,16], their attractiveness for protein adsorption [12], as well as their stimulatory effects on production of growth-promoting substances in adjacent cells [1,11,13]. However, the supportive action of alginates on cell colonization could be associated with immune activation of these cells, manifested by increased expression of immunoglobulin and selectin adhesion molecules and synthesis of cytokines, followed by infiltration and encapsulation of the implant with inflammatory cells and fibroblasts [2,8,11-13,16]. From this point of view, the composites CP4 (carbon fibre-reinforced terpolymer) and CP5 (carbon fabric-reinforced terpolymer) seem to be more advantageous, because their chemical composition (i.e., absence of alginates) may be less immunogenic. Similarly to CP9, also on these composites relatively high cell numbers were found on day 7 after seeding (60911±5478 cells/cm^2 on CP4 and 54820±5625 cells/cm^2 on CP5; FIG.1 and 2 C, D). On CP 5, this number might be even higher, because as detected by propidium iodide staining, a considerable amount of cells were retained inside the carbon fabric even after cell harvesting by trypsinization. Similarly on CP4, some cells were localized below the carbon fibres, prominent on the material surface (FIG.2D). Interestingly, on CP5, cells were mainly of longitudinal shape and oriented along the fibres forming the carbon fabric (FIG.2C). Similar influence of the material surface topography on the cell morphology was also observed in the CFRC [7, 8].

The lowest number of MG63 cells was found on the pure terpolymer PTFE/PVDF/PP (sample CP0; 17766±3180 cells/cm^2). In addition, the cells on this material were less spread and distributed less homogeneously (FIG.2E). These findings could be attributed to a relatively high surface hydrophobia of the terpolymer (TABLE 1). It is known that cell adhesion on artificial materials is mediated by adsorption of extracellular matrix molecules, e.g. vitronectin, fibronectin, collagen and laminin, provided by the serum of the culture media or synthesized by cells [6,15]. If the material is too hydrophobic, these molecules are adsorbed in a rigid state and less appropriate spatial conformation, so that their specific binding sites, e.g. amino acid sequence RGD, are less accessible for adhesion receptors on cells [6,15]. The hydrophobicity of the PTFE/PVDF/PP terpolymer could be lowered by physical methods described earlier, such as bombardment with ions, irradiation with ultraviolet light or exposure to plasma discharge (for review see [6]). Also other physical and chemical properties of the material surface, such as electrical charge, roughness and topography, mechanical properties (rigidity or flexibility) or presence of certain chemical functional groups (amine or oxygen-containing groups), are known to influence markedly the protein adsorption and cell adhesion [1, 6-10,12-13, 15, 16].

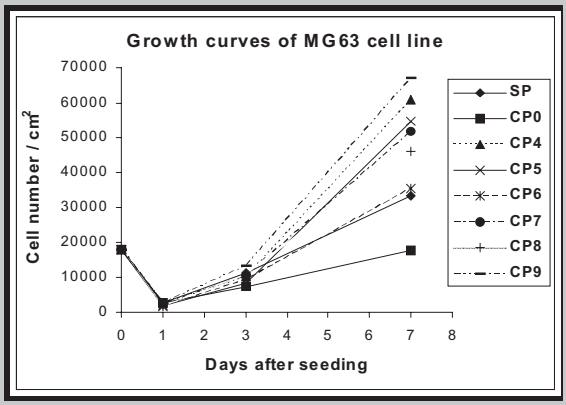

FIG. 1. Growth curves of human osteoblast-like MG63 cells on polystyrene culture dish (SP), terpolymer PTFE/PVDF/PP (CP0) and carbon-terpolymer composites (CP4-CP9; see the Material and methods).

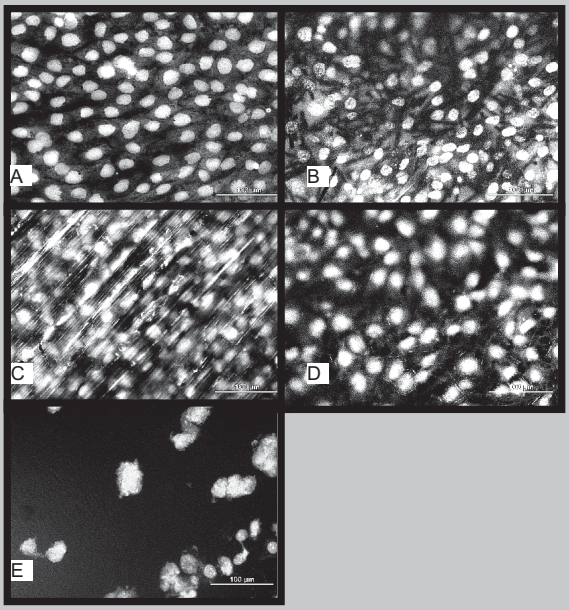

FIG. 2. Morphology of human osteoblast-like MG 63 cells on: Polystyrene culture dish (A), Sample CP9 (B),Sample CP5 (C),Sample CP4 (D) and Sample CP0 (E) (See the Material and methods).Day 7 after seeding, fixed with ethanol, stained with propidium iodide.

Conclusion

On day 7 after seeding, the lowest number of human osteoblast-like MG63 cells was found on the pure terpolymer of polypropylene, polytetrafluorethylene and polyvinyldifluoride, which was probably due to its relatively high surface hydrophobia. The cell colonization was markedly improved and the maximum cell number was achieved when the terpolymer was modified by addition of carbon and alginate fibres (sample CP9).

Acknowledgements

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ADHESION AND GROWTH OF VASCULAR SMOOTH MUSCLE CELLS ON PROTEIN ASSEMBLIES FOR BIOMATERIAL COATING

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Abstract

We investigated adhesion and growth of vascular smooth muscle cells (VSMC) on proteins assemblies prepared by coating of thin fibrin net with ultrathin layer of collagen I, fibronectin or laminin. The assemblies were deposited on polystyrene (PS) non-modified with plasma discharge, i.e. a treatment commonly used for creation of surfaces suitable for cell cultivation. All samples with fibrin assemblies improved initial attachment and spreading of VSMC as well as their subsequent growth in comparison with the pristine PS. The adhesion area of VSMC increased on fibrin coated with collagen I and fibronectin, while on laminin it remained similar as that on the pure fibrin. Thin 3D fibrin net markedly changed the morphology of VSMC from polygonal to star-like shape with protrusions. Cells all tested samples formed vinculin-containing focal adhesion plaques and α-actin cytoskeleton, an important marker of VSMC differentiation. The protein assemblies can be used for improvement of cell adhesion and growth on various artificial materials used in tissue engineering, e.g. for construction of bioartificial vascular prostheses.

[Engineering of Biomaterials, 47-53,(2005),9-12]

Introduction

Artificial implants into the human body are often made of bioinert materials not allowing colonization with cells, thus these materials cannot be considered as adequate and long lasting substitutes of the natural tissues. The latter concerns especially currently used vascular prostheses made of polyethylene terephtalate or polytetrafluoroethylene, i.e. highly hydrophobic polymers not supporting vascular tissue regeneration. Therefore, there is an effort to improve the cell adhesion and spreading by various physical and chemical modification of the material surface, e.g. by irradiation with ultraviolet light (Heitz et al. 2003), bombardment with C^+, O^+ and other ions (Bačáková et al. 2001), plasma discharge, covalent binding of ligands for cellular adhesion receptors, e.g. RGD, REDV (Bačáková et al. 2004, Benoit and Anseth 2005) or controlled deposition of layers containing extracellular matrix (ECM) molecules (Brodie et al. 2005).
In the present study, we evaluated the adhesion and growth of VSMC on fibrin assemblies on polystyrene, and the influence of coating these assemblies with collagen I, fibronectin or laminin, important components of the ECM in blood vessels.

Material and methods

Series of biomolecular assemblies on polystyrene surfaces (PS) were prepared at room temperature in wells of tissue culture plates non-treated with plasma discharge (Polystyrene Non-Tissue Treated Plate, 24 wells of diameter 1.5 cm, Falcon Multiwell TM). Five columns, each containing 4 wells, on a plate were coated with different assemblies; the sixth column was left uncoated as a reference sample. Fibrin (Fb) network was attached to PS surface and further coated with fibronectin (FN), laminin (La) or collagen I (CO I), or mixed with CO I. Therefore, we prepared the following samples: 1. PS- Fb, 2. PS- Fb/FN, 3. PS- Fb/La, 4. PS- Fb/CO I (i.e., collagen-coated Fb), 5. PS-(Fb+CO I), i.e. Fb mixed with collagen, 6. PS.
The preparation of assemblies on PS was started with adsorption of fibrinogen (Fbg) from TB (TRIS buffer, 50 mM