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Biological and biochemical properties of the carbon composite and polyethylene implant materials

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We studied the biocompatibility of the carbon composites and polyethylene materials with and without collagen or collagen and proteoglycan cover.

We used the *in vitro* technology to study the adhesion of model cells evalution, their metabolic activity and the production of TNF-α as a cytokine model. Under *in vivo* condition, the biocompatibility of tested polymers were studied in the implantation experiment, subcutaneously in the interscapular region in the laboratory rat.

We have found in the *in vitro* assay favorable proliferation and the smallest production of pro-inflammatory TNF-α cytokine in cells adherent to the hydrophobic polyethylene material coated with biological macromolecules.

Using *in vivo* tests performed by the implantation of materials to the rat we demonstrated that the materials are not cytotoxic. The tissue capsule surrounding the implants was not significantly influenced by the type of the implant and the pre-treatment by the biological molecules. However, the foreign-body giant multinucleated cells were observed only in the vicinity of the collagen – covered hydrophobic polyethylene implant. Interestingly, while the collagen coating improved the biocompatibility of tested polymers *in vitro*, the inflammatory reaction against this covered materials was higher under *in vivo* conditions. The pre-treatment of carbon composites by both types of biological macromolecules reduced the occurrence of carbon debris in the implantation site.

The tested carbon composites and polyethylene materials are not toxic. The pre-treatment of the materials by extracelular matrix components increased their biological tolerance *in vitro* and reduced implant wears in animal experiment, which can be important for the medical application.

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1. Introduction

Surface properties of an implant (for example, the surface free energy or the wettability of material) influence radically the biocompatibility of synthetic materials. These parameters are important for the adsorption of biologically active substances, such as fibronectin, fibrinogen, complement and subsequent colonization by cells including the leukocytes [1].

In our previous report we have shown the basic biological properties of carbon composite materials [2]. We continue studying this material, comprising the carbon composite with polyethylene. Both material types were covered with collagen or collagen and proteoglycans as an example of extracellular matrix components occurring in a connective tissue. The results observed under *in vitro* conditions were compared with implantation experiment with laboratory rat.

2. Materials and methods

2.1. Implant materials

Carbon–carbon composites (C/C) were prepared from plain-woven cloth (Torayca Carbon Fibers T800, Soficar,

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France) and phenolic resin (Umaform LE, Synpo Ltd., Pardubice, Czech Republic). The prepregs were stacked in the necessary number of layers, cured and carbonized in nitrogen at 1000 °C (Batch 1). Carbonized samples were impregnated in three steps with phenolic resin, infiltrated and covered with pyrolytic carbon in a tumbling bed reactor as described [3] (Batch 2).

Polyethylene material (PE) was manufactured commercially (Chirulen P, Hoechst AG Werk Ruhrchemie, Oberhausen, Germany). This material was used as a surface lamina of the polyethylene-based composite PE-C and PE-Si [4]. The high density PE material, with molecular weight in range $4–6 \times 10^6$ g/mol and with density 0.935 g/m^3 was formed under the temperature of 160 °C to the desired shape. Two types of the polyethylene materials were used: (a) with an untreated surface (HFO), (b) with the chemically treated surface, to get an increasing hydrophilic properties (oxide etching by 10% solution $KMnO_4$ in 96% H_2SO_4, 2 h) (HFI).

2.1.1. Preparation, coating and sterilization of materials

The plates (C/C composites 30 × 40 × 1 mm, Batches 1 and 2 and PE materials 36 × 40 × 1 mm) were prepared for the tissue culture assay, the implants (C/C 3 × 10 × 2 mm, Batch 2 and PE 5 × 10 × 1mm) for the animal experiment.

These materials were covered (a) with collagen (10% solution ISC_{40} from calf skin in 0.5 M acetic acid) [5], (b) with a mixture of collagen–proteoglycan (C + PGl): solubilized collagen ISC_{40} and 10% aqueous solution of calf articular cartilage proteoglycan (extracted by 2 M GuHCl) [6] were mixed in the weight ratio of both substances 1 : 1. The materials tested were treated by either the collagen or the mixture C + PGl and dried. A sterilization by ethylene oxide was performed before using.

2.2. Cell culture

Embryonic human lung fibroblasts LEP (22nd–24th passage, Sevac Prague, Czech Republic) were used under the standard conditions [2, 7].

2.3. Cytotoxicity tests *in vitro*

We tested the biological properties of materials: C/C composite, HFI and HFO polyethylene (all without cover and covered either by collagen or by C + PGl), using the evaluation of basic cell properties, such as the adherence and proliferation.

The materials were placed on the bottom of a dish (diameter 60 mm; GAMA, České Budějovice, Czech Republic) and the cells were inoculated to the system [2].

2.3.1. The evidence of a cell adhesion to the tested materials

The LEP cells at the density of 50 000/cm^2 were cultivated in 6 ml of minimal essential medium (MEM, Alseva, Prague, Czech Republic), supplemented with 10% bovine foetal serum (Veterinary University, Brno, Czech Republic), penicillin 100 U/ml and streptomycin 100 μg/ml (Alseva, Prague, Czech Republic). After three days, the materials with cells were transferred to a dish, fixed by paraformaldehyde (0.4%, 5 min), further treated by 0.2% TWEEN (Sigma-Aldrich, Prague, Czech Republic) and subsequently stained by a 1% aqueous solution of propidium iodide (Sigma-Aldrich, Prague, Czech Republic) in the medium (10 μl solution/6 ml MEM, overnight, 4 °C). The results were evaluated using a fluorescence microscope (Olympus).

2.3.2. The metabolic activity of the cells adherent to the tested materials and a cytokine production

LEP cells inoculated at the density of 80 000/cm^2 were cultivated in 6ml of MEM. The day after, the materials with cells were displaced to the blank dish and new medium was added, to exclude the cells adhering to the bottom of the dish. The metabolic activity was evaluated using MTT tests, such as described [2]. Medium of this cultivation experiment was also performed for cytokine TNF-α detection using the analyzer Immulite (DPC, Los Angeles, CA, USA). This system utilizes an assay-specific antibody or antigen-coated plastic beads as the solid phase, an alkaline phosphatase-labeled reagent and a chemiluminescent enzyme substrate. We used monoclonal anti-mouse TNF-α on the polystyrene beads and polyclonal anti-rabbit conjugate with alkaline phosphatase (Immulite, DPC, Los Angeles, CA, USA).

2.4. Biocompatibility test *in vivo*

The tested polymers were implanted subcutaneously in the interscapular region, under sterile conditions to 40 adult Wistar rats of both sexes (100 g), received from Anlab (Prague, Czech Republic) as described [2]. The animals were kept under standard conditions according to the rules for laboratory animal handling valid in the Czech Republic. Titanium alloy Ti–6Al–4V (Poldi, Kladno, Czech Republic) served as a well tolerated control material.

The implants (each type was tested in four animals) were evaluated 10 and 60 days after the surgery. The tissue surrounding the implants (Fig. 1) was investigated by the standard histological procedures (hematoxylin-eosin, Alcian Blue-8GX). The computer-assisted image analysis system (Lucia Laboratory Imaging, Prague, Czech Republic) was employed to quantify the thickness of a connective-tissue capsule surrounding the implant.

Fibronectin, chondroitin-sulfate and cytokines (TNF-α, IL-1α, IL-6, IL-8) were visualized by immunocytochemistry in frozen sections (as described [2]), using specific first-step antibodies (Sigma-Aldrich, Prague, Czech Republic). Because the first-step antibodies of mouse and rabbit origin were used, the second-step reagent of anti-mouse and rabbit specificity (SWAM-Px, SWAR-Px, Alseva Prague, Czech Republic) labeled with peroxidase and diaminobenzidinetetrachloride (Sigma-Aldrich, Prague, Czech Republic) as a chromogen substrate were employed to visualize the results of immunocytochemical reactions. The control experiments

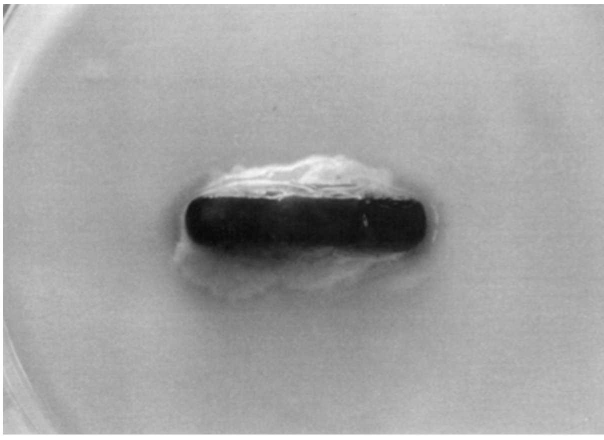


Figure 1 The C/C composite material with surrounding connective-tissue capsule, from the rat interscapular region, 60 days after implantation.

were performed by the omission of the 1st-step antibody or the replacement by the pre-immune serum.

3. Results and discussion
3.1. Tests *in vitro*
3.1.1. The evidence of a cell adhesion to the tested materials
The surface of C/C composite was well colonized with LEP cells (Fig. 2(a)–(c)). The pattern of cell adhesion respected the direction of carbon fibers, from which the material was prepared (Fig. 2(a)). The pre-treatment of C/C composite with both tested substances (i.e. collagen and collagen with proteoglycans) somewhat stimulated the attractivity of the studied composite for LEP cells. The data obtained by the evaluation of the metabolic activity of the studied cells (MTT test) are in agreement with the results of the morphological experiment (Fig. 3). The surface of the polyethylene was not colonized uniformly and the areas with a high cell density were usually in the neighborhood of poorly colonized districts. Pre-treatment by collagen significantly stimulated cell adhesion to the polyethylene with HFO surface. The polyethylene with HFI surface did not exhibit the similar tendency. The HFO polyethylene which was colonized with cells exhibited a chaotic appearance, which contrasted with cells growing on the HFI with a longitudinally oriented shape (Fig. 2(b), (c)).

Generally, the collagen pre-treatment of HFO polyethylene predominantly stimulated the growth of cells. A similar feature, but not so significant, was also observed in other studied materials. As the collagen is one of the fundamental components of the extracellular matrix (ECM) [8, 9, 10], this result is not surprising. Observations by others have shown that ECM is important for the binding and activation of growth factors [11, 12]. This mechanism can participate in the favorable properties of collagen covered with polymeric materials under *in vitro* conditions.

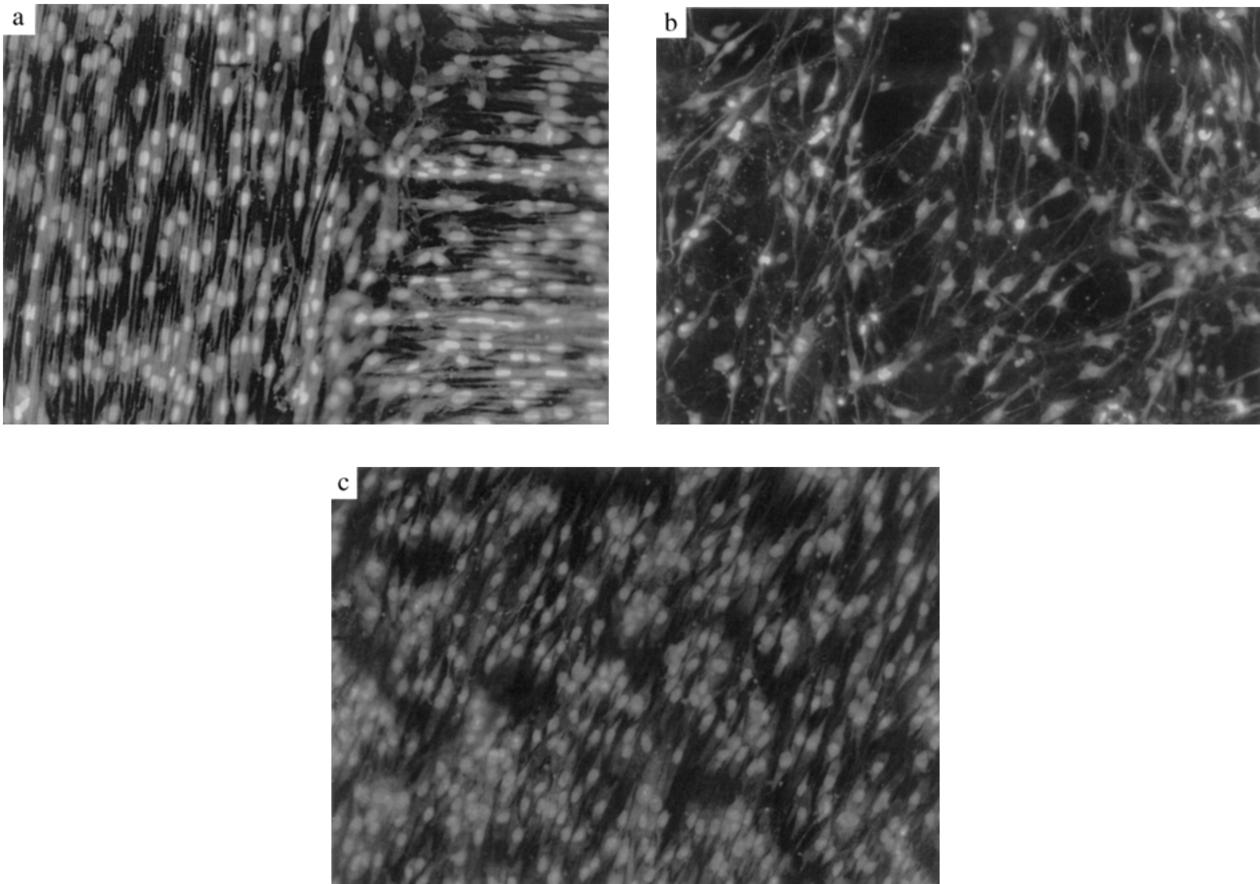


Figure 2 The LEP cells after 3 days cultivation on the C/C composite material (a), on the PE HFO (b) and on the PE HFI (c). Propidium iodide staining, magnification 160 × .

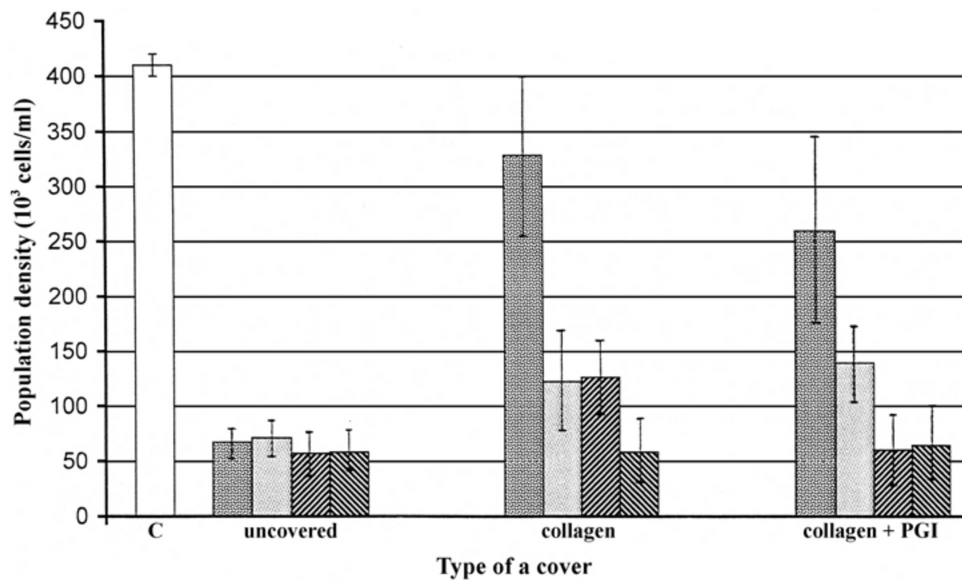


Figure 3 Metabolic activity of LEP cells, growing on the uncovered and covered tested materials. Type of supports: ☐ C-control: plastic dish, ▩ PE HFO, ☐ PE HFI, ▨ C/C composite (Batch 1), ▧ C/C composite (Batch 2). Each value represents the mean ± standard deviation of three samples.

3.1.2. Cytokine assay

In comparison with cells cultivated without tested materials, the introduction of these materials stimulated the production of the TNFα as a proinflammatory cytokine. There was only one exception, HFO polyethylene covered with collagen was well compatible according to the cytokine level, which was similar as in the control experiment. However, it can be generally stated that pre-treatment of all types of studied materials by both biological substances somewhat depressed the cytokine production response, although not to such an extent as measured in HFO polyethylene (Fig. 4).

The data observed *in vitro* have shown the biological significance of collagen on the growth of tested materials and the production of the proinflammatory cytokine. This observation underscores the advantage of the employment of the biological macromolecule (collagen) in the preparation of the potentional material for the integration into a living organism. Even the properties of polyethylene, which is known to be a nonadhesive material (HFO), were improved by the collagen pre-treatment. Of practical importance can be the decreased level of the production of proinflammatory TNF-α because it shows one of the ways, how to control the inflammatory response against the implant.

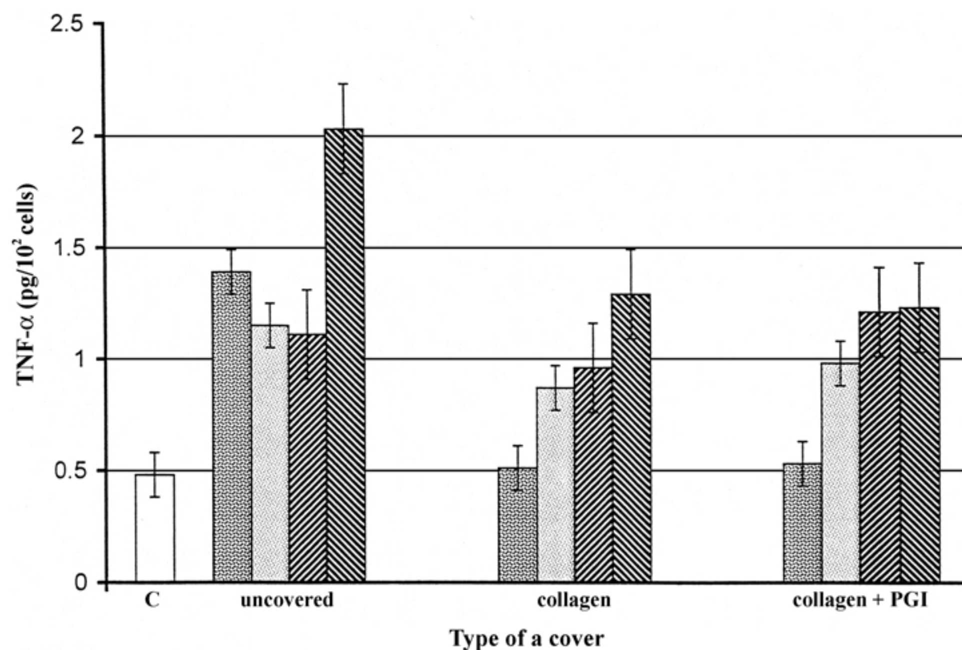


Figure 4 The production of TNF-α by LEP cells, growing on the uncovered and covered tested materials. Type of supports: ☐ C-control: plastic dish, ▩ PE HFO, ☐ PE HFI, ▨ C/C composite (Batch 1), ▧ C/C composite (Batch 2). Each value represents the mean ± standard deviation of three samples.

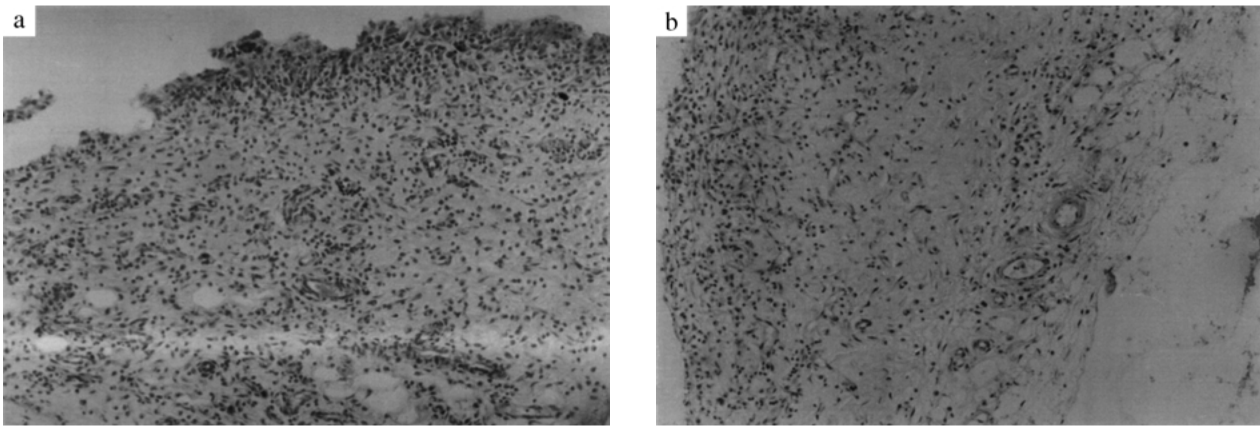


Figure 5 Cross section of the connective-tissue capsule surrounding the uncovered C/C composite (a) and C + PGl covered HFO polyethylene (b), 10 days after the surgery. Hematoxylin-eosin, magnification 130 × .

3.2. Biocompatibility tests *in vivo*

3.2.1. Histological evaluation

Although those materials are not toxic *in vitro*, the inflammatory cell infiltration was observed at the implantation site.

Ten days after the implantation, the vicinity of the implant was colonized by inflammatory cells without the effect of physicochemical properties of the implant (Fig. 5(a)). Only the cellularization around the PE covered with collagen and proteoglycans was insignificantly lower (Fig. 5(b)). These results suggest that biocompatibility of implants is more influenced by the surgical procedure than by the implant properties in course of the first 10 days after the surgery.

Sixty days after the implantation, the connective-tissue capsule surrounded all types of materials. The arrangement of this capsule can be characterized as hydrated or swollen in case of a C/C composite material without pretreatment by biological macromolecules (Fig. 6). Carbon debris were frequently observed around the carbon implant but the coating of C/C composite by collagen and glycosaminoglycans decreased probably the wear risk and the quantity of debris was reduced.

The foreign-body giant multinucleated cells were detected in the vicinity of the polyethylene implants HFO covered with collagen (Fig. 7). This observation is in contrast to the consequence of the lowest level of the TNF-α production induced by this material *in vitro* and needs further analyses. Because these multinucleated cells are known as a valuable marker of the chronic inflammatory response [13] the observed results indicated an improved toleration of these types of implant cover by the immune system.

The thickness of the connective-tissue capsule surrounding the implants was extremely variable, it varied even in different sides of one implant (Fig. 8). However, it can be stated that the biomolecules used for the synthetic material cover somewhat increased the capsule formation in contrast to nontreated specimens (Table I). This observation is in contrast to the result of the *in vitro* experiment. The biological substances stimulated the adhesion of fibroblasts and inhibited the TNF-α production. Because the thickness of the capsule is also evaluated as a compatibility factor, it seems that *in vivo* and *in vitro* results are in disagreement. The interpretation of these phenomena is hypothetical at this state of art but it is possible that biological substances are recognized by macrophages which produce cytokines stimulating the capsule formation.

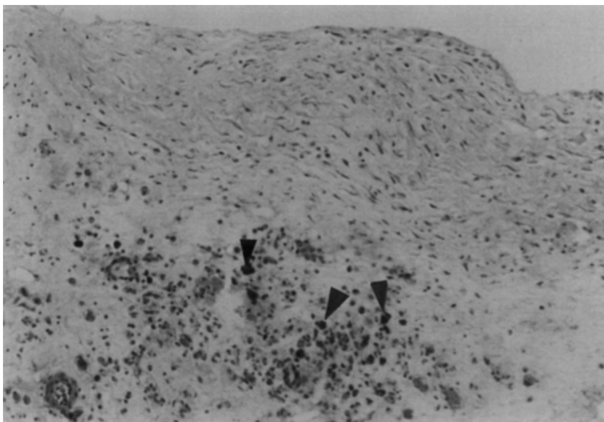


Figure 6 Cross section of the swollen connective-tissue capsule surrounding the implants prepared of C/C composite, arrows – the debris, 60 days after the surgery. Hematoxylin-eosin, magnification 130 × .

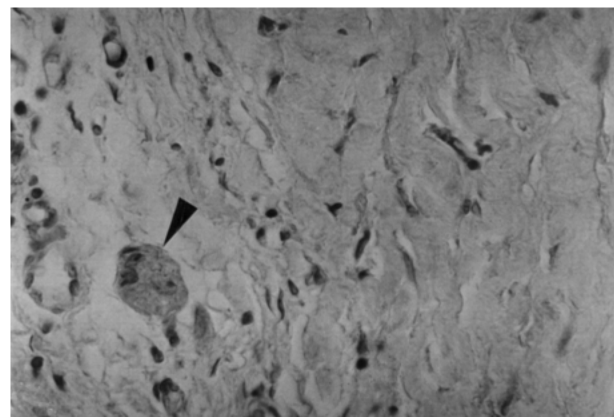


Figure 7 The foreign-body giant multinucleate cells (arrow) in the vicinity of PE HFO implant covered with collagen, 60 days after the surgery. Hematoxylin-eosin, magnification 370 × .

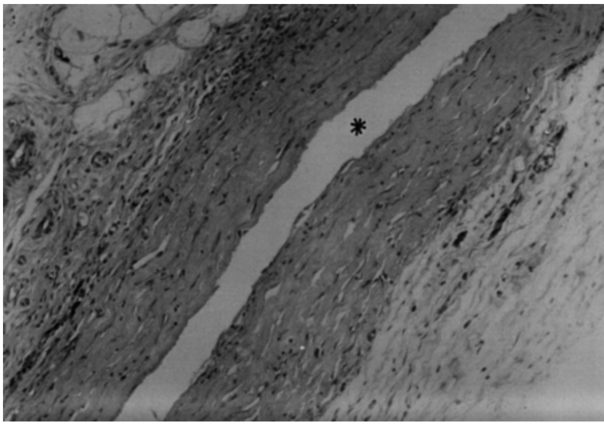


Figure 8 The thin, well differentiated connective-tissue capsule surrounding the PE HFI covered with C + PGl implant, 60 days after the surgery, * indicates the position of the removed implant. Hematoxylin-eosin, magnification 130 × .

The control experiment with titanium alloy demonstrated no strict difference from the materials studied.

3.2.2. Immunohistochemical evaluation

The cells with macrophage morphology exhibiting a positive indication for the expression of (TNF-α, IL-1α, IL-6, IL-13) were clearly observed in the tissue surrounding the implant.

The chondroitin-sulfate and fibronectin were visualized to better characterize the connective-tissue capsule. We have found the higher expression of chondroitin-sulfate by the all implant types 10 days after the implantation (Fig. 9). Comparing all tested materials 60 days after the implantation, the highest expression of fibronectin in the capsule surrounding the implant was found with the C/C composite (Fig. 10(a)). The pretreatment of materials by the biological substances exhibited only negligible influence on the reaction indication of both studied markers in capsular tissue. Maybe a little lower fibronectin positivity was showed by polyethylene materials (Fig. 10(b)). The intensity of the immunological reaction was very similar to the titanium alloy implant.

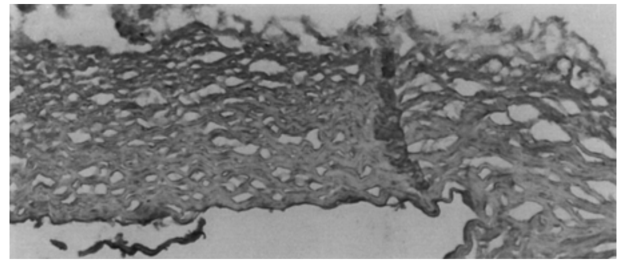
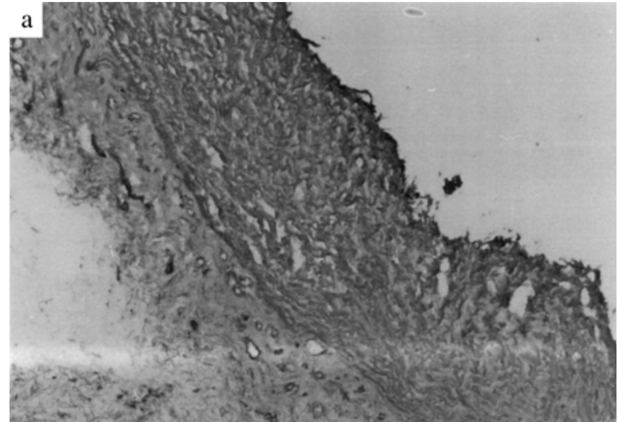


Figure 9 Detection of chondroitin-sulfate in the connective-tissue capsule surrounding the C/C composite implant, 10 days after the surgery. Magnification 220 × .



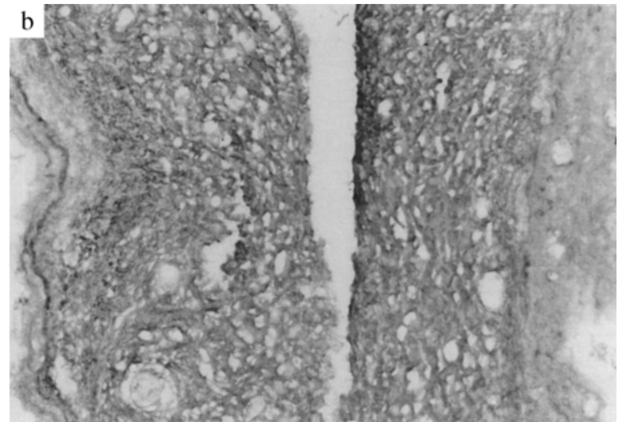


Figure 10 Detection of fibronectin in the rat connective-tissue capsule surrounding the implant: C/C composite without cover (a), PE HFO covered by collagen (b), 60 days after the surgery. Magnification 220 × .

TABLE I The thickness of connective-tissue capsule surrounding the implants, input subsutaneously in rat, 60 days after implantation

Type of material	Coating	Capsule thickness (μm)*	Std dev. σ
C/C composite	Without	209	49.2
	Collagen	254	71.7
	Collagen + PGI	337	101.0
HFO polyethylene	Without	283	102.0
	Collagen	254	66.5
	Collagen + PGI	256	46.3
HFI polyethylene	Without	169	30.9
	Collagen	216	52.4
	Collagen + PGI	189	42.3
Titanium alloy-control		188	44.3

* The average of five measurements from four implants.

4. Conclusions

The pre-treatment of the synthetic materials (PE, C/C) with extracelular protein components influenced their biological properties. The cells colonized better the surfaces covered with these macromolecules and the production of inflammatory cytokines was reduced. The biological macromolecules on C/C composite materials reduced significantly the wear of debris, which can be important for the employment of these materials in clinical practice.

However, the *in vitro* and *in vivo* results are slightly contradictory which needs a further research in this field.

Acknowledgment

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