Cells and Materials

Volume 9 | Number 2 Article 5

1999

In Vitro Comparative Biocompatibility Testing of Carbofilm Coated and Uncoated Polyetherimide for Cardiovascular Application

M. Bosetti
University of Novara

S. Mazzarelli
University of Novara

L. Nicolais
National Research Council, Naples, Italy

L. Ambrosio
National Research Council, Naples, Italy

M. Cannas
University of Novara

Cells and Materials Vol. 9, No. 2, 1999 (Pages 117-125) 1051-6794/99$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

IN VITRO COMPARATIVE BIOCOMPATIBILITY TESTING OF CARBOFILM COATED AND UNCOATED POLYETHERIMIDE FOR CARDIOVASCULAR APPLICATION

M. Bosetti[1*], S. Mazzarelli[1], L. Nicolais[2], L. Ambrosio[2], M. Cannas[1]

[1]Department of Medical Sciences, Human Anatomy, University of Novara
[2]Institute of Composite Materials Technology, National Research Council, Naples, Italy

(Received for publication July 24, 1998 and in revised form February 3, 1999)

Abstract

When blood contacts the surface of a material, several processes take place including the activation of coagulation and immune systems. The aim of this work is to study *in vitro* the biological reactions seen from the point of view of hemocompatibility and cytocompatibility of a new polymer suggested as an artificial surface for cardiovascular applications: Carbofilm® coated polyetherimide (C®PEI) in comparison to polyetherimide (PEI), the uncoated form. PEI and C®PEI showed no signs of acute cytotoxicity although following long term incubation with PEI cytotoxicity was somewhat increased; both materials supported good endothelial cell adhesion with a higher level of cell proliferation on the coated form. No significant difference was detected in the activation of the inflammatory response and in thrombogenicity tested by assay of prostaglandin E2 (PGE2) and tromboxane B2 (TXB2) respectively, following incubation of the biomaterials with platelet-rich plasma (PRP). Complement activation assessed by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE)/Western Blot analysis of both contacting plasma and protein which adsorbed on the surface of the polymers showed both materials to be activators of complement. In conclusion, Carbofilm® coating, showing lower cytotoxic activity and higher endothelial cell growth in comparison with uncoated material, seems to increase PEI compatibility.

Key Words: Polyetherimide, Carbofilm®, biocompatibility, *in vitro* test.

*Address for correspondence:
Mario Cannas
Department of Medical Sciences, Human Anatomy,
University of Novara
Via Solaroli 17, 28100 Novara, Italy
Telephone and FAX number: (39) 321-660632
E-mail: cannas@med.no.unipmn.it

Introduction

The use of synthetic materials for biomedical devices has increased considerably in recent years with the availability of new polymers having appropriate mechanical and physical properties for specific clinical applications (Remes and Williams, 1992). These applications depend on the complexity of biological reactions that occur at the interface between tissues and implant (Peluso *et al.*, 1994). To date, no synthetic material developed is entirely passive to blood. When blood contacts the surface of a material, several processes such as the activation of both coagulation and immune systems take place in order to protect the host against "not-self" components (Woffindin and Hoenich, 1988). Several humoral and cellular factors are recruited by a complex mechanism resulting in the recognition of foreign surfaces by the host (Montdargent *et al.*, 1992). Among these, the activation of the complement system is an important event resulting in the production of proteolytic fragments which are then recognized by different leukocyte subsets through specific receptors thus initiating the cell-mediated inflammatory response (Kazatchkine and Carreno, 1988; Labarre *et al.*, 1993). Platelet adhesion and secretion of intracellular components constitute another consequence of the contact of blood with foreign material surface (Kottke-Marchant *et al.*, 1989). Moreover platelets catalyze the interactions among plasma coagulation proteins, leading to thrombin formation (Leake *et al.*, 1989).

One of the most promising approaches to reducing the thrombogenicity of the blood contacting surface of synthetic polymers is to coat the surface with endothelial cells (ECs). Attempts were made either by seeding the polymer surface with low numbers of ECs at surgery and allowing gradual growth to confluence *in vivo* (Ortenwall *et al.*, 1988) or by sodding at high densities to produce a cell coated graft at implantation (Jarrell *et al.*, 1986).

It is generally accepted that the physicochemical composition of the substrate is a key determinant in the amount and conformation of adsorbed cell adhesion pro-

teins which mediate cell adhesion (Ratner, 1993). Besides fibronectin and extracellular matrix coating, pyrolytic carbon is widely used in prosthetic devices that come into contact with blood (Pacagnella *et al.*, 1986). Since pyrolytic carbon is one of the best artificial materials currently available in terms of chemical inertness and absence of thrombogenic reactions (Aebischer *et al.*, 1988), the aim of this work is to study, *in vitro*, the biological reactions, seen from the point of view of hemocompatibility and cytocompatibility of a new polymer proposed for cardiovascular application, that is, Carbofilm® coated Polyetherimide (C®PEI) in comparison with the uncoated form.

Materials and Methods

Biomaterials

Polyetherimide $(C_{38}H_{25}O_6N_2)_n$ (PEI, Litrex I; Petrochemie Danubia GmbH, Mannsworth, Austria) is a thermoplastic amorphous polymer with a glass transition temperature of about 217°C. The poly-(etherimide) resin is the latest synthesized polyimide (General Electric Co., Mt. Vernon, IN) and probably represents the most successful combination of polyimide performance and processability. This polymer is synthesized by reaction of dianhydrides containing ether links and diamines; the polyetherimide is characterized by high strength, rigidity and dimensional stability and good electrical insulation properties, which are largely maintained after prolonged exposure to temperatures up to about 180°C. This polymer is resistant to a wide range of solvents including alcohols and fully halogenated compounds.

Carbofilm® coated Polyetherimide (C®PEI, furnished by Tecnobiomedica, Rome, Italy) is made of the same material, PEI, modified by a pyrolytic carbon coating obtained by a physical vapor deposition (PVD) process, similar to that employed for valve prostheses. This technique is performed in a high vacuum and the transfer is obtained by bombarding the target with an ion beam at 40-200°C range (Pacagnella *et al.*, 1986).

Medical grade Silicone (Perthèse® LP 502-1, Laboratoire Perouse Implant, Bornel, France) and glass (microscope slide glass) were used as controls for hemocompatibility and cytocompatibility assessment.

All samples were squares of 1 cm x 0.5 cm, provided with siliconized edges to avoid direct contact of the material with the medium. The sterilization procedures were performed according to manufacturers' specifications: after three washes of 30 minutes each in distilled water and in 0.9% (volume/volume, v/v) NaCl solution, the materials were autoclaved at 121°C for 20 minutes.

Cytotoxicity evaluation

An *in vitro* screening test on cell cultures was carried out, to evaluate the acute cytotoxicity of the chemicals that could be released by the test materials into the culture medium. Evaluation was performed by neutral red vital stain (1 hour of exposure) uptake test {American Society for Testing of Materials (ASTM), 1985} and also by lactate dehydrogenase (LDH) concentration test on a monolayer of murine fibroblasts 3T3 (ATCC CRL 1658; American Type Culture Collection, Rockville, MD) cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma), penicillin (100 U ml^{-1}), streptomycin (100 g ml^{-1}) and 0.03% (v/v) L-glutamine. LDH is an enzyme released from the cell when the cell membrane is ruptured so the amount of LDH present in the media at the completion of the test correlates with the amount of cell death in the culture. In a 24-well plate, 1.3 x 10^4 cells/cm^2 were added onto the surface of PEI and C®PEI in triplicate; two additional cultures were used as negative controls and two others were treated with Triton X-100 providing positive controls for LDH analysis. After a 96 hour incubation, LDH activity (Sigma Kit) was measured at 340 nm, corresponding to the oxidation of NADH to NAD^+ in the presence of LDH and pyruvate.

To mimic the long-term effects of the body environment on the biomaterials, extracts were prepared in phosphate-buffered saline (PBS), corresponding to a surface area of 1 cm^2/ml, at 60°C for 10 days (artificial ageing extract) according to ASTM (1985) guidelines. The extracts were tested by the microculture tetrazolium assay using 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT assay). The assay is based on the observation that viable cells are able to metabolize a water-soluble tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT), into an insoluble formazan salt product by the action of mitochondrial succinate dehydrogenase (Kirkpatrick and Dekker, 1992; Smith *et al.*, 1992; Dekker *et al.*, 1994).

The extracts, obtained by maintaining the samples for 10 days at 60°C, were diluted with growth medium, containing double concentrated serum, to a ratio of 1:4. The dilution was added to the cell cultures; medium obtained in the same way without addition of polymers, provided the negative control, while 2% (v/v) dimethylsulfoxide (DMSO) in growth medium was used as positive control because of its inhibitory action on cell viability (Ignatius and Claes, 1995).

NIH 3T3 cells (8 x 10^3/well) were plated into 96 well microtitre plates; after 6 hours plating, the growth medium was replaced by 200 μl/well of diluted extracts. At least six replicates were prepared for each material and for positive and negative controls. The cells were exposed to the extracts for 4, 24, 48 hours at 37°C in a 5% CO_2 atmosphere. Then, 10 μl per well of an MTT

solution (5 mg.ml^{-1} PBS) were added and the plate was incubated for 4 hours. The medium was removed and the formazan crystals dissolved in dimethylsulfoxide (DMSO). The colorimetric staining was then evaluated on a multiwell plate reader (450 Microplate Reader, BioRad, Richmond, CA) at 540 nm.

Adhesion and proliferation test

Endothelial cells were obtained by isolation from human umbilical cord vein (HUVEC), collected from Caesarean deliveries according to the method described by Jaffe *et al.* (1973). Cells were cultured in tissue-culture grade polystyrene plates (Corning, Cambridge, MA) precoated with a 0.5% (v/v) gelatin (Sigma) solution in complete medium composed of M199 (Sigma) supplemented with 20% (v/v) FBS (Sigma), heparin (50 U/ml Sigma), 300 μg/ml endothelial cell growth supplement, produced according to Maciag *et al.* (1979), 0.03% (v/v) L-glutamine (Gibco, Paisley, UK), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma). Cells used for the experiments were between 2nd and 5th passage after the confluency of the culture.

Human umbilical vein endothelial cells were identified by immunofluorescent staining for von Willebrand factor (DAKO, Glostrup, Denmark). Briefly, after fixing with 3% (v/v) paraformaldehyde, endothelial cells were rinsed with phosphate buffered saline (PBS), permeabilized with 0.1% (v/v) Triton X-100 (1 minute) rinsed with PBS, incubated with primary antibody and stained with fluorescent goat anti-rabbit IgG. Negative controls were human fibroblasts (MRC5 cell line) and HUVEC not coated with primary antibody.

HUVEC were placed on the biomaterial surface at 5 x 10^3 cells/cm^2 in a 24 microplate well. Adhesion and proliferation tests were performed after 6 hours and 5 days, respectively. The materials, after rinsing in PBS, were fixed 20 minutes at 60°C and stained with a 0.025% (weight/volume, w/v) Acridine Orange solution, a nucleic acid staining dye (Kepner and Pratt, 1994). Cell number on each material was performed using a fluorescence microscope (Aristoplan; Leitz, Milano, Italy) and results are expressed as percent cell number with respect to plated cells (5 x 10^3 cells/cm^2), considered as 100%.

Hemocompatibility evaluation

Platelet rich plasma (PRP) was prepared using pooled venous blood from healthy donors collected in siliconized tubes containing 3.5% (w/v) sodium citrate as anticoagulant. PRP was obtained by centrifugation at 500 x *g* for 10 minutes at 4°C. Before using, it was analyzed using a flow cytofluorimeter (Becton Dickinson, Meylan, France) to evaluate the cellular population. PRP (1.8 ml) was added to each well of a 24-well microtitre plate (Corning) containing squares of 1 cm x 0.5 cm of PEI, C®PEI, silicone and glass as negative and positive control respectively (n = 16). The plate was then rotated for 30 minutes at room temperature for the contact between PRP and biomaterials. After the contact period, a platelet activity inhibiting solution, made of ethylene diaminotetracetic acid (EDTA) and indomethacin, was added to PRP specimens in the ratio of 10 parts of PRP and 1 part of solution. The PRP specimens were purified by solid/liquid extraction using Amprep minicolumns (Amersham International plc, Amersham, UK) before assaying arachidonic acid products. The concentrations of Prostaglandin E2 (PGE2) and Tromboxane B2 (TXB2) were determined with enzyme immunoassay (EIA) using PGE2 EIA Kit (Cayman Chemical Company, Ann Arbor, MI) and TXB2 EIA system (Amersham) respectively. Results are expressed as pg/ml.

Complement activation was studied through the analysis of complement components bound on the surface. A sample of PRP was inactivated by heating at 60°C for 30 minutes and used as additional control together with a sample of PRP inactivated with EDTA. Squares of biomaterials were incubated with plasma for 1 hour at room temperature. After removal of plasma, squares were washed four times in PBS. The squares were then transferred into Eppendorf tubes and incubated at 90°C for 10 minutes in a 2% (w/v) sodium dodecyl sulfate (SDS). Samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) and the gel was stained with a Silver Stain kit (BioRad). Western blot was performed using polyclonal anti-C3 antibody (DAKO) followed by horseradish peroxidase conjugated antibody. ECL Western blotting detection reagents (Amersham) were used for revelation.

Statistical analysis

Statistical analysis of the data was carried out using a PC-compatible equipped with SPSS for Windows software. Bonferroni evaluation was performed to compare cytotoxicity, adhesion and proliferation results; p value was obtained from the analysis of variance (ANOVA) table. The conventional 0.05 level was considered to reflect statistical significance.

Results

Cytotoxicity evaluation

Fibroblasts in contact with PEI and C®PEI showed no sign of damage such as morphological elongation, loss of neutral red, pycnotic nuclei, etc.. In contrast, cells cultured in contact with Triton X-100 (positive control) were scanty, rounded and had lost the vital stain (results not shown). These results were confirmed by

Table 1. MTT test expressed as negative control (in %)*.

Materials	4 hours	24 hours	48 hours
PEI	68.66 ± 6.97	73.78 ± 9.16	43.12 ± 3.22
Carbofilm PEI	68.45 ± 5.48	70.43 ± 6.71	52.37 ± 13.07
Positive Control**	46.74 ± 3.11	37.39 ± 2.16	23.55 ± 1.42

Relative MTT formazan formation by NIH 3T3 cells after incubation with extracts of the materials studied for 4, 24, 48 hours. The formazan formation is expressed as a percentage of the amount produced by cells incubated with fresh culture medium (means ± standard deviation of six replicates). Dilution factors of the primary extract = 4X.

* Negative control: Cells without polymer extracts in the culture medium.

** Positive control: Cells with 2% DMSO in the growth medium.

Table 2. Cytocompatibility results.

	Carbofilm PEI	PEI	Silicone	Glass
Cell Growth* (%)	453.14 ± 84.00	386.37 ± 70.00	121.80 ± 40.60	219.42 ± 83.00
Adhesion** (%)	77.37 ± 13.70	70.15 ± 10.90	54.28 ± 5.00	75.24 ± 12.00

* Day 5 cell count/plating cell density x100.

** Cell count at 6 hours incubation/plating cell density x100.

The materials with cells on the luminal surface were stained with Acridine Orange solution and cells were quantified using a fluorescent microscope. n = 4; values are mean ± standard deviation.

PEI = polyetherimide; CarbofilmPEI = Carbofilm coated polyetherimide.

Table 3. Arachidonic acid metabolism.

	Carbofilm PEI	PEI	Silicone	Glass
PGE2 (pg/ml)	58.94 ± 8.20	56.08 ± 6.70	51.25 ± 9.20	67.70 ± 6.40
TXB2 (pg/ml)	290.75 ± 111.50	209.10 ± 53.50	242.50 ± 49.30	299.30 ± 124.50

Arachidonic acid metabolites enzyme immunoassay results at 30 minutes contact between platelet rich plasma and biomaterials.

n = 16; values are mean ± standard deviation. Results are expressed as pg/ml.

LDH release assessment (Fig. 1) as a method for the evaluation of the cell membrane integrity.

MTT test results are expressed in Table 1 where the absorbance results showed the percentage of cell growth in relation to the negative control. Formazan salt production was significantly different between cells treated with DMSO (positive control) and both test materials at each incubation time. Both materials revealed a decrease in formazan salt production measured at 48 hours incubation in comparison with 4 and 24 hour incubation periods. This decrease was significant only for PEI (Bonferroni test with $\alpha = 0.05$).

Cytocompatibility

All surfaces showed great endothelial cell attachment with a significantly lower cell adhesion on silicone than on the other materials tested; no significant differences in adhesion were noted between PEI, C®PEI and

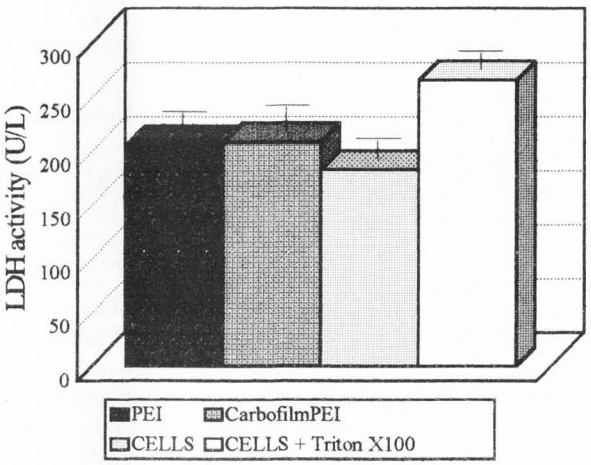

Figure 1. Results provided by assessment of cell membrane damage, are expressed as LDH activity (U/l) ± standard deviation at 340 nm.

---

glass although C®PEI supported the highest number of adherent cells (Bonferroni test with significance level 0.05). There was significantly more cell growth on PEI and C®PEI ($p < 0.05$) than on glass (positive control) or silicone; among all samples, C®PEI showed the greatest cell growth (Table 2).

Hemocompatibility

Arachidonic acid metabolite results are shown in Table 3. No significant differences in TXB2 concentration were observed among materials and values decreased as follows: glass, C®PEI, silicone, and PEI. PGE2 levels were higher in plasma which had contacted glass and no difference was revealed between the other materials.

The SDS-PAGE electrophoresis of proteins present on the surface of biomaterials, incubated with platelet rich plasma, is presented in Figure 2a. The predominant protein on all surfaces had molecular weight from 29 to 51 KDa. The surface of C®PEI appeared to have the least adsorbed protein.

The immunoblot, incubated with polyclonal anti-C3 antibody, showed C3 fragments both on the surface of every biomaterial and in the plasma after contact with the materials (Fig. 2b). The predominant polypeptides had molecular weights of ~ 70 KDa, and ~ 40 KDa, representing the β-chain of C3/C3b and fragments of iC3b respectively. These two bands have been found in the control PRP, in the incubation plasma used for the complement activation test and, at higher concentration, bound on the surface of the four materials. In Figure 2b, bands near 200 KDa, 120 KDa and 29 KDa were visible only for the biomaterials eluted proteins (lanes 2, 3, 4, and 5) and represented C3, α-chain of C3/C3b, and fragments of C3c, respectively. Generally, we have

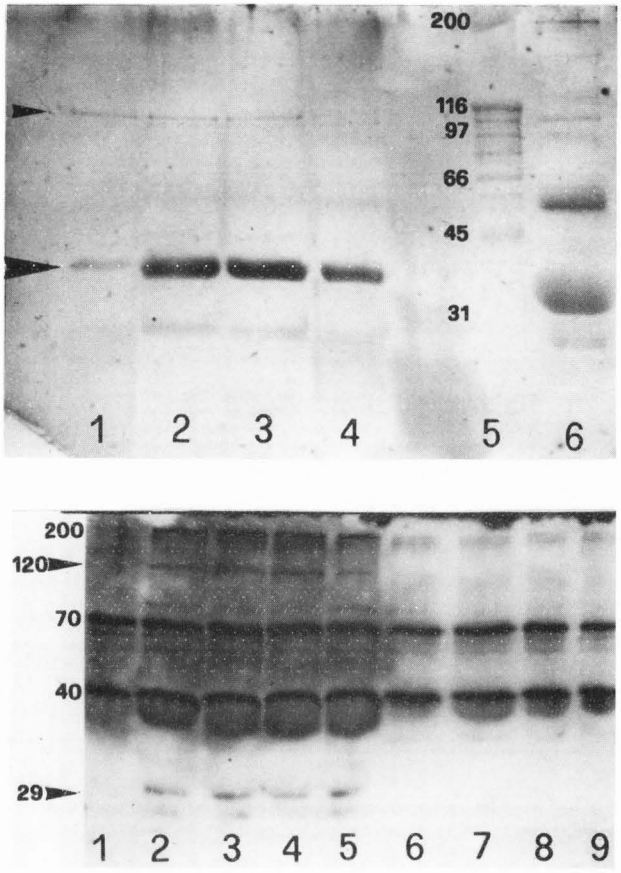

Figure 2. (a). SDS-PAGE patterns of protein layers adhered to biomaterials, after *in vitro* incubation with platelet rich plasma (PRP): (1) CarbofilmPEI, (2) PEI, (3) Glass, (4) Silicone, (5) Molecular Weight, (6) Control (PRP). (b). Immunoblot of C3 fragments present on the surface of biomaterials incubated with PRP. Lane 1: control PRP; Lane 2: CarbofilmPEI; Lane 3: PEI; Lane 4: Silicone; Lane 5: Glass; Lane 6: PRP after contact with CarbofilmPEI; Lane 7: PRP after contact with PEI; Lane 8: PRP after contact with silicone; Lane 9: PRP after contact with glass.

---

observed a reduced intensity band at 29 KDa and 75 KDa for silicone, and at 120 KDa and 70 KDa for glass compared to the other materials.

Discussion

To date, no synthetic material developed is entirely passive to blood. In particular, polymer surfaces developed for cardiovascular application cause platelet adhesion, followed by platelet release and aggregation. In addition, polymers are also able to stimulate coagulation, complement activation and leukocyte adhesion (Kottke-Marchant *et al.*, 1989).

Endothelial cells play a key role in the development and dissolution of thrombi, producing factors such as urokinase-type plasminogen activator, tissue type plasminogen activator, plasminogen activator inhibitor-1, platelet activating factor, prostacyclin, and nitric oxide (Vanhoutte, 1989). Attempts to form a complete endothelial cell layer on synthetic graft surfaces of small-diameter vascular prostheses have been made in order to overcome the thrombogenic properties of the exposed surface (Zhang *et al.*, 1995; Burmeister *et al.*, 1996; Poole-Warren *et al.*, 1996). With this in mind, the ability of endothelial cells to form a monolayer and to proliferate on a material, that was to be used for cardiovascular application, became an important and interesting parameter to study together with blood compatibility tests.

The aim of this work was to evaluate, *in vitro*, the biocompatibility of a new material, C®PEI in comparison with PEI. Cytotoxicity screening showed that both materials exerted no acute toxic effect on cultured fibroblasts as all the cultures showed a morphological appearance and LDH levels similar to that of negative control. The MTT test (demonstrating not only membrane integrity, but also intracellular mitochondrial succinate dehydrogenase activity) revealed a decrease in formazan production, an index of a toxic effect, for both the materials after a longer incubation period and this was particularly evident for PEI.

As complement activation plays an important role in the activation of leukocytes (Labarre *et al.*, 1993), the complement-activation induced by the materials has to be evaluated as part of its biocompatibility. Since deposition of C3b is believed to be the initiating event for efficient complement activation, a surface that does not bind C3b is expected to be a poor activator (Cheung *et al.*, 1990; Lin *et al.*, 1992). C3 deposition at the surface and C3 assay to detect the degree of complement activation in serum, that had been in contact with the surface of the materials, revealed that all the biomaterials were activators of complement. We can hypothesize that the large accumulation of C3 on the surface (200 KDa in the immunoblotting) was due to complement activation and not to spontaneous adsorption of C3, because, both the EDTA control involving heat-inactivated plasma were negative. The different immunoblot pattern of complement C3 fragment adsorbed on the tested surfaces in comparison with the one obtained from control plasma allows us to confirm a complement activation, this is also supported by the observation of fragments of iC3b at 40 KDa from the conversion of C3b at 70 KDa present at higher concentration bound to the surface rather than in plasma analyzed. PGE2 and TXB2 concentrations, markers of inflammatory phenomenon and platelet aggregation, evidenced no significant difference in the four materials considered: the higher result was for the positive control material, glass, and the lower value for the negative control material, silicone, while PEI and C®PEI were quite similar for PGE2 and TXB2 levels. C®PEI induced a TXB2 production not significantly higher than the values induced by PEI, indicating only a slightly higher activity on platelet aggregation.

In conclusion, on the basis of our results, neither material gave any sign of acute cytotoxicity and, at long term incubation, PEI was less biocompatible exhibiting lower cell vitality. No significant difference was detected in the activation of the inflammatory response or in complement activation. In addition the ability of endothelial cells to form a monolayer and to proliferate on both the materials was shown to be good and C®PEI showed higher endothelial cell growth, a sign of better endothelialization. Therefore, Carbofilm® coating of PEI seems to increase material biocompatibility when compared to uncoated PEI that itself is an excellent biocompatible material (Peluso *et al.*, 1994). The improvement of cytocompatibility and hemocompatibility of the coated material, however, needs more experiments to relate the obtained results, for example, to dynamic tests which approach *in vivo* conditions.

Acknowledgements

Supported by grants from the National Research Program: (theme 29) "Materiali compositi a base organica per applicazioni ad elevata criticità di prestazioni nel settore biomedico" line 2-1, between the University of Torino and Tecnobiomedica s.p.a.

References

Aebischer P, Goddard MB, Sasken HF, Hunter TJ, Galletti PM (1988) Tissue reaction to fabrics coated with turbostratic carbon: Subcutaneous versus vascular implants. Biomaterials **9**: 80-85.

ASTM (1985) Annual Book of ASTM Standards, Vol. 13.01, F619-79: Practice for extraction of medical plastics. Am Soc Testing Materials, Philadelphia. pp 176-180.

Burmeister JS, Vrany JD, Reichert WM, Truskey GA (1996) Effect of fibronectin amount and conformation on the strength of endothelial cell adhesion to HEMA/EMA copolymers. J Biomed Mat Res **30**: 13-22.

Cheung AK, Parker CJ, Wilcox LA, Janatova J (1990) Activation of complement by hemodialysis membranes: Polyacrylonitrile binds more C3a than cuprophane. Kidney Int **37**: 1055-1059.

Dekker A, Panfil C, Valdor M, Pennartz G, Richter H, Kirkpatrick CJ (1994) Quantitative methods for *in vitro* cytotoxicity testing of biomaterials. Cells Mater **4**:

101-112.

Ignatius AA, Claes LE (1996) *In vitro* biocompatibility of bioresorbable polymers: Poly(L, DL-lactide) and poly(L-lactide-coglycolide). Biomaterials **17**: 831-839.

Jaffe EA, Nachman RL, Becker CG, Minick CR (1973) Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest **52**: 2745-2756.

Jarrell BE, Williams SK, Stokes G, Hubbard FA, Carabasi RA, Koolpe E, Greener D, Pratt K, Moritz MJ, Radomski J (1986) Use of freshly isolated capillary endothelial cells for the immediate establishment of a monolayer on a vascular graft at surgery. Surgery **100**: 392-399.

Kazatchkine MD, Carreno MP (1988) Activation of the complement system at the interface between blood and artificial surfaces. Biomaterials **9**: 30-35.

Kepner RL, Pratt JR (1994) The use of fluorochromes for direct enumeration of total bacteria in environmental samples: Past and present. Microbiol Rev **58**: 603-615.

Kirkpatrick CJ, Dekker A (1992) Quantitative evaluation of cell interaction with biomaterials *in vitro*. Biomat Tissue Interface **10**: 31-41.

Kottke-Marchant K, Anderson JM, Umemura Y, Marchant RE (1989) Effect of albumin coating on the *in vitro* blood compatibility of Dacron arterial prostheses. Biomaterials **10**: 147-155.

Labarre D, Montdargent B, Carreno MP, Maillet F (1993) Strategy for *in vitro* evaluation of the interactions between biomaterials and complement system. J Appl Biomater **4**: 231-240.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**: 680-684.

Leake DL, Cenni E, Cavedagna D, Stea S, Ciapetti G, Pizzoferrato A (1989) Comparative study of the Dacron® combined with various polyurethanes. Biomaterials **10**: 441-444.

Lin YS, Hlady V, Janatova J (1992) Adsorption of complement proteins on surfaces with a hydrophobicity gradient. Biomaterials **13**: 497-504.

Maciag T, Cerundolo J, Ilsley S, Kelley PR, Forand R (1979) An endothelial cell growth factor from bovine hypothalamus: Identification and partial characterization. Proc Natl Acad Sci USA **76**: 5674-5678.

Montdargent B, Toufik J, Carreno MP, Labarre D, Jozefowicz M (1992) Complement activation and adsorption of protein fragments by functionalized polymer surfaces in human serum. Biomaterials **13**: 571-576.

Ortenwall P, Bylock A, Kjellström BT, Risberg B (1988) Seeding of ePTFE carotid interposition grafts in sheep and dog: Species-dependent results. Surgery **103**: 199-205.

Pacagnella A, Majni G, Ottaviani G, Arru P, Santi M, Vallana F (1986) Properties of a new carbon film for biomedical applications. J Art Organs **9**: 127-130.

Peluso G, Petillo O, Ambrosio L, Nicolais L (1994) Polyetherimide as biomaterial: preliminary *in vitro* and *in vivo* biocompatibility testing. J Mat Sci Mat Med **5**: 738-742.

Poole-Warren LA, Schindhelm K, Graham AR, Slowiaczek PR, Noble KR (1996) Performance of small diameter synthetic vascular prostheses with confluent autologous endothelial cell linings. J Biomed Mat Res **30**: 221-229.

Ratner BD (1993) New ideas in biomaterials science - A path to engineered biomaterials. J Biomed Mater Res **27**: 837-850.

Remes A, Williams DF (1992) Immune response in biocompatibility. Biomaterials **13**: 731-743.

Smith MD, Barbenel JC, Courtney JM, Grant MH (1992) Novel quantitative methods for the determination of biomaterials cytotoxicity. Int J Artif Organs **15**: 191-194.

Vanhoutte PM (1989) Endothelium and control of vascular function. Hypertension **13**: 658-667.

Woffindin C, Hoenich NA (1988) Blood-membrane interactions during haemodialysis with cellulose and synthetic membranes. Biomaterials **9**: 53-57.

Zhang JC, Wojta J, Binder BR (1995) Growth and fibrinolytic parameters of human umbilical vein endothelial cells seeded onto cardiovascular grafts. J Thorac Cardiovasc Surg **109**: 1059-1065.

Discussion with Reviewers

G. Laroche: It is not clear in my mind that the technique that is described as being used to coat the polyimide surface is PVD. Indeed, physical vapor deposition consists in vaporizing a coating substance on a substrate under vacuum. Most of the time, the coating media is heated, therefore, allowing its deposition onto the surface. However, coatings deposited in such a manner give rise to poor adhesion as the vaporized species do not have enough energy to be efficiently anchored onto the surface. To my point of view, the technique that is described by the authors (bombardment of a target with an ion beam) actually refers to sputtering deposition. This latter technique allows a better adhesion between the coating and the substrate as the coating molecules reach the surface with more energy.

Authors: We confirm that the technique used to coat the polyetherimide surface is a PVD process involving mass transfer from a pyrolytic carbon target to the substrates under high vacuum. The Carbofilm® adhesion properties, turbostratic structure and impurity content

have been reported by Paccagnella *et al.* (1986).

S.J. Northup: Based on the physical properties of PEI, this material is more likely to be considered as a substitute for pyrolytic carbon in heart valves or other soft tissue applications that require a rigid material (e.g., injection ports). Why was pyrolytic carbon excluded from the experiment?
Authors: In this work, we have studied if pyrolytic carbon coating lead to an increased hemocompatibility and cytocompatibility of PEI with the aim of obtaining a material with the physical properties of PEI and the biocompatibility properties of pyrolytic carbon. So we have studied pyrolytic carbon as PEI coating.

S.J. Northup: If silicone was one of the samples to be evaluated, why was the blood collected in siliconized test tubes?
Authors: Silicone was used as negative control for PGE2 and TXB2 because it was described in the literature as a non-activating material on the arachidonic acid cascade; so, to avoid contact of blood with glass or polypropylene when preparing PRP, we preferred to use siliconized test tubes.

S.J. Northup: Are there any qualitative or quantitative analytical data on the chemical residues in the extract evaluated in the MTT assay? Is the amount and identity of residual chemicals in an extract prepared at 60°C comparable to that found in an extract prepared at 37°C?
Authors: We have not done a qualitative or quantitative analytical study on the chemical residues in the material extracts evaluated in the MTT assay. ASTM (1985) Guidelines were followed to study long-term effects of the body environments on biomaterials cytotoxicity.

A. Dekker: The general conclusion of the MTT test is that cells in contact with extracts of the biomaterials tested formed less formazan salts than the cells incubated with fresh culture medium. In contrast, endothelial cells grew well on the biomaterials, without signs of cytotoxicity. This contrast is not surprising, since the osmolality of the diluted extracts is likely to be too high. Namely, the extracts of the biomaterials in PBS were diluted 1:4 with double concentrated culture medium, which contains twice the physiological salt concentration. PBS contains a physiological salt concentration. Therefore, the diluted extract will possess a salt concentration that is considerably higher than the physiological one. Such high osmolalities will impede cell growth. The usage of normal concentrated culture medium for dilution of the extracts would solve this problem. Please comment.
S.J. Northup: It is remarkable that an extract of PEI and C^{TM}PEI disrupted mitochondrial metabolism, yet the materials had no effect on lysosomal integrity (neutral red assay) or membrane integrity (LDH) assay), and supported enhanced cell proliferation. Please comment.
Authors: There is a misunderstanding regarding the methods to prepare material extracts for MTT assay: only serum is double-concentrated in the medium for dilution of the extracts. MTT results on biomaterial extracts should not be compared to neutral red, LDH and cell proliferation studies. MTT gives us information about long-term cytotoxicity, while the other methods evaluate acute cytotoxicity (neutral red and LDH) and cytocompatibility (proliferation).

A.Dekker: Essentially, in this study the biocompatibility of two quite different materials was tested, polyetherimide and pyrolytic carbon, respectively. The latter one was coated on polyetherimide. Is the biological performance of the carbon coated on polyetherimide, as you tested it, comparable with that of pyrolytic carbon coated on other materials, as described by other authors? How much does the substrate for the carbon coating influence the biocompatibility of the carbon surface?
Authors: Pyrolytic carbon was used in coating of different types of implant prostheses such as dental implants, percutaneous devices, tendon and tracheal replacements and, particularly, heart valve prostheses. Recently, it has been used as coating for vascular prostheses in Teflon® and Dacron® (Sbarbati *et al.*, 1991; Cenni *et al.*, 1995). In these studies, pyrolytic carbon coating shows a lower thrombogenicity and enhances *in vitro* endothelial cell attachment and growth when compared with uncoated materials.

S.J. Northup: Please comment on the risk of adverse health effects from circulating activated complement and activated complement that is bound to a material.
Authors: Complement activation has many clinical consequences on adverse health effects that correlate with production and release of inflammatory mediators, e.g., leukopenia and hypoxia associated with hemodialysis (Woffindin and Hoenich, 1988) and lung injury after cardiopulmonary bypass (Gillinov *et al.*, 1993).

The immobilized C3 on polymer surface can, most probably, function as receptors, e.g., neutrophils, potentially producing a local reaction, chemotaxis, increased vascular permeability, and vasodilatation. We believe that the absence of detectable C3 deposition on the surface does not exclude active complement products formed in the fluid in contact with the surface (Liu and Elwing, 1994) so that both the fluid phase activation and C3b binding to an activating surface are studied to evaluate biomaterial complement activation.

L.A. Culp: Regarding Figure 2b, how do the authors know that the respective bands on their gels can be definitively identified as C3 and C3 fragments? Another disturbing point about Figure 2b is the relative underloading of protein in lanes 6-9 compared to lanes 2-5; perhaps C3 and its fragments do appear in lanes 6-9 but the authors have not loaded a comparable amount of total protein.

W. van Oeveren: The **Results** on immunoblot (Fig. 2b) are not clear. Is Figure 2b only presenting proteins bound to the biomaterial surface, or is lane 6-9 presenting the C3 fragments in serum? An important conclusion from these qualitative complement tests could be that despite reduced protein binding to carbofilm, complement binding and activation is high, indicating poor biocompatibility to this aspect.

Authors: We have identified C3 and C3 fragments bands in the blot incubated with anti-C3 antibodies based on molecular weights (Berger *et al.*, 1994). In Figure 2b we have loaded the same amount of total protein in lanes 2-9. Lanes 6-9 present C3 fragments in platelet rich plasma, used as a control at a higher total protein concentration than in lane 1; lanes 2-5 present C3 fragments eluted from the materials. The proposed conclusion on our qualitative complement tests is interesting but needs more tests to be confirmed.

L.A. Culp: The authors should state whether their cultured 3T3 cells contained serum (type of serum?) in the medium when tested with biomaterials. This is critical to evaluate whether cell surface proteins are interacting with the material directly or indirectly via adsorbed serum proteins. The same comment applies to culture of the endothelial cells in **Materials and Methods**.

G. Laroche: Experiments on platelet and fibrinogen adhesion would have brought additional useful information on carbon-coated polyetherimide performances.

Authors: Our medium used to culture 3T3 and HUVEC cells on biomaterials contained fetal bovine serum (Sigma). In this study, we have not evaluated if cell interaction with the material is mediated by adsorbed serum proteins. We have only evaluated differences in cell adhesion and proliferation between coated and uncoated material. Biomedical carbon surfaces and their interactions with plasma proteins have been studied by Feng and Andrade (1994a,b, 1995).

Additional References

Berger M, Broxup B, Sefton MV (1994) Using ELISA to evaluate complement activation by reference biomaterials. J Mat Sci Mat Med **5**: 622-627.

Cenni E, Arciola CR, Ciapetti G, Granchi D, Savarino L, Stea S, Cavedagna D, Curti T, Falsone G, Pizzoferrato A (1995) A platelet and coagulation factor variations induced in vitro by polyethylene terephtalate (Dacron) coated with pyrolytic carbon. Biomaterials **16**: 973-976.

Feng L, Andrade JD (1994a) Protein adsorption on low temperature isotropic carbon. Isotherms, competitivity, desorption and exchange of human albumin and fibrinogen. Biomaterials **15**: 323-333.

Feng L, Andrade JD (1994b) Protein adsorption on low-temperature isotropic carbon. Protein conformational change probed by differential scanning calorimetry. J Biomed Mater Res **28**: 735-743.

Feng L, Andrade JD (1995) Protein adsorption on low temperature isotropic carbon. How is it related to its blood compatibility? J Biomater Sci Polym Ed **7**: 439-452.

Gillinov AM, DeValeria PA, Wilkenstein JA, Wison I, Curtis WE, Shaw D, Yeh CG, Rudolph AR, Baumgartner WA, Herskowitz A (1993) Complement inhibition with soluble complement receptor type 1 in cardiopulmonary bypass. Ann Thorac Surg **55**: 619-624.

Liu L, Elwing H (1994) Complement activation on solid surfaces as determined by C3 deposition and hemolytic consumption. J Biomed Mat Res **28**: 767-773.

Sbarbati R, Giannessi D, Cenni MC, Lazzerini G, Verni F, De Caterina R (1991) Pyrolytic carbon coating enhances Teflon and Dacron compatibility with endothelial cell growth. Int J Artif Organs **14**: 491-498.