JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 5 (1994) 738–742

Polyetherimide as biomaterial: preliminary *in vitro* and *in vivo* biocompatibility testing

G.PELUSO, O. PETILLO
Institute of Protein Biochemistry and Enzymology-CNR, via Toiano 6, Arco Felice 80072, Naples, Italy

L. AMBROSIO, L. NICOLAIS
Department of Materials and Production Engineering and Institute of Composite Materials Technology-CNR, University of Naples Federico II, Piazzale Tecchio 80, Naples 81025, Italy

During the last few years, on the basis of their physico-chemical characteristics, thermoplastic materials, already used in several advanced industries, have become very attractive candidates for biomedical applications as matrix for composite femoral stems and bone plates. In the present study, the biocompatibility of a thermoplastic material, polyetherimide (PEI), was investigated both *in vivo*, and *in vitro*. Our results show that PEI did not elicit any toxic response in cell culture testing. It was possible to grow on PEI surfaces not only 3T3 fibroblasts but also human endothelial cells (HEC) from human umbelical cord, with no apparent deleterious effects on the cell viability. The attachment, spreading, and growth of cells on PEI material was followed with respect to time. Both the number of adhering HEC, and the spreading area of individual cells, increased as a function of time. The hydrogen peroxide-sensitive dye DCFH-DA was used to examine the functional activity of cells adherent to PEI or control material implants. PEI material did not induce significant DCFH oxidation until day 14, when levels of DCFH oxidation were significantly higher than observed with PDMS negative control, but lower than those observed on Pellethane positive control.

1. Introduction

The use of synthetic materials for biomedical devices has increased considerably in recent years. The principal reason for this proliferation has been the availability of new polymers possessing appropriate mechanical and physical properties for specific clinical applications. However, these applications often remain inhibited by the lack of understanding of the complex biological reactions that can occur with foreign body implants.

On the basis of their physico-chemical characteristics, thermoplastic materials, already used in several advanced industries, have become very attractive candidates for biomedical applications, mainly in orthopaedic prostheses, as matrix for composite femoral stems and bone plates [1]. For this reason, a large number of commercially available thermoplastic polymers have been evaluated both *in vitro* and *in vivo*, but because of their peculiarities, many materials have been rejected. Moreover, other selected materials have demonstrated, at the same time, both positive and negative properties after *in vitro* and *in vivo* tests. For example, poly(etherether)ketone has excellent mechanical stability but critical processing conditions, due to its temperature-sensitive semicrystalline structure. Polysulfone has shown a reduction of mechanical properties following saturation in Ringer's solution [2]. Finally, thermosetting polymers, such as epoxy resins, can present, following the polymerization process, many unreacted monomers which strongly affect their biocompatibility [3, 4].

In the present study, the biocompatibility of a thermoplastic material, polyetherimide (PEI), was investigated both *in vivo*, and *in vitro*.

2. Materials and methods

Polyetherimide (PEI) (Litrex I; Petrochemie Danubia Ges.m.b.H.) is a thermoplastic amorphous polymer with a glass transition temperature of about 217 °C. To allow the evaporation of any solvent residue the material was treated at 180 °C under vacuum for 3 days.

2.1. Haemolysis

A modified version of ASTM standard F756-87 was used to assess the haemolytic capacity of the tested material. The spectrophotometer (Kontron, model Uvikon 930) was calibrated by measuring the absorbance at 540 nm of a haemoglobin reference standard (Sigma) at known concentrations of 0, 6, 12, and 18 g/dl. Citrated platelet-poor plasma was added to Drabkin's solution (Sigma) and the absorbance measured at 540 nm. The absorbance was then backed out of the calibration curve as the plasma-free haemoglobin level. These values were normalized to the unexposed control for that particular draw and the haemolytic index determined.

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2.2. Cell cultures

Cell culture testing was conducted growing human endothelial cells and 3T3 mouse fibroblasts to confluency in direct contact with PEI. The cells were cultured in complete DMEM with 10% fetal calf serum and antibiotics. Flat sheets of PEI were cut into 35 mm diameter circles and were tightly wedged into the culture wells, resulting in a flat, even-bottomed surface. To keep the flat discs of PEI from floating to the surface in the presence of medium, O-rings of silicon rubber tubing were used to wedge the polymer to the bottom. Appropriate cultures performed on the polystyrene of the culture plate itself were used as controls. The cytotoxicity was evaluated not only by microscopic examination but also by LDH concentration in the cell-conditioned media. Lactate dehydrogenase (LDH) is an enzyme that is released from the cell when the cell membrane is ruptured. Thus, the amount of LDH present in the media at the completion of the test correlates with the amount of cell death in the culture.

Four control cultures included in the experiments were also used as controls for LDH analysis. After 96 h incubation, two of the four control cultures were given 100 μl of Triton × 100 and held at 37 °C for 2 h. These cultures served as positive controls for LDH testing since Triton causes complete cell death. The other two control cultures served as negative controls for LDH analysis. The amount of LDH in each culture was measured in units, and was corrected for background activity by subtracting the average activity of the negative controls.

2.3. Human endothelial cell cultures

Endothelial cells were isolated from human umbilical cord veins according to the isolation method described by Jaffe *et al.* [5].

Cells were normally cultured in tissue culture polystyrene flasks (Falcon) precoated with a fibronectin (Sigma) solution for 1 h (a 2% solution, w/v, of fibronectin in complete medium). The serum-containing culture medium consisted of complete medium, an equal mixture of Medium M 199 and RPMI Medium 1640 (both from ICN), with 2 mM L-glutamine (ICN), 100 units/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml fungizone (all from ICN), which was supplemented with 20% fetal calf serum (FCS). Cells were used for experiments after the third passage when the culture had reached confluence. Harvesting of cells for subculturing or testing was performed with a trypsin solution (0.05% trypsin/0.02% EDTA, Gibco). Trypsin was subsequently inactivated with FCS.

2.4. Implant system

PEI (1 cm × 2.5 cm) was placed in stainless-steel wire mesh cages (3.5 cm in length and 1 cm in diameter) and then ethylene oxide-sterilized. Sterilized cages were implanted subcutaneously, using sterile techniques, in the backs at the level of the panniculus carnosus of 3-month-old, 250–300 g, female Sprague Dawley rats. At 4, 7, 14, 21 days post-implantation, the implanted cages were surgically removed and the different specimens were retrieved from the cage.

2.4.1. Intracellular hydrogen peroxide level measurements

The intact specimens were examined for the ability of the adherent leukocytes to produce hydrogen peroxide. To measure the intracellular hydrogen peroxide levels within adherent leukocytes, the oxidation of 2′,7′-dichlorofluorescein (DFCH-DA) non-fluorescent probe to the fluorescent compound DCF was monitored according to the method of Bass *et al.* [6, 7].

DCFH-DA is a stable, non-fluorescent, non-polar compound that readily diffuses into viable cells where it is hydrolyzed to the non-fluorescent polar derivative DCFH and is trapped within the cells. The intracellular molecule is rapidly oxidized to the highly fluorescent compound DCF in the presence of hydrogen peroxide, which is generated during respiratory burst activity by activated leukocytes. Thus, cellular fluorescence intensity is directly proportional to the levels of intracellular hydroperoxides and can be quantified by fluorimetry.

Once surgically removed, the materials were placed in Hank's balanced salt solution, rinsed, and incubated at 37 °C for 15 min with 5 μM DCFH-DA in a shaking water bath. The pieces of biomaterials were removed and Triton-X 100 (1%) was added to the reaction mixture to lyse the cells for complete release of the oxidized DCF. Fluorescence was measured using a Perkin-Elmer LS-5 fluorescence spectrometer set at 488 nm excitation per 525 nm emission. The data are expressed as fmol DCF oxidized on a per cell basis; thus, the data are normalized for differences in absolute numbers of cells adherent to the different material samples. The number of adherent cells was determined by a computerized image analysis system (Zeiss interactive digital analysis system) directly on the specimens before cell lysing.

As positive control a polyurethane (Pellethane 2363 80 A), and as negative control, a silicon material, PDMS, was utilized.

2.5. Surface analysis

For scanning electron microscopy (SEM) analysis the materials were rinsed in PBS and fixed in 2.5% glutaraldehyde solution containing 0.1 M cacodylate buffer, slowly dehydrated using increasing concentrations of ethanol (up to 100%) then treated with hexamethyldisilazane, mounted, sputter-coated with gold-palladium (2.5 nm thick) and analysed using a Hitachi scanning electron microscope.

SEM was used also to examine cell morphology and to determine cell spreading by measuring the surface area of adhering cells. This was carried out by projecting enlarged SEM photonegatives with adherent cells on a digitizer tablet (Apple). Data (μm^2) are expressed as the mean surface area of 45 randomly chosen cells (± standard error of the mean, s.d.) for each substrate after the time interval concerned.

3. Results and discussion

Polyimides are materials characterized by an imide linkage with high service temperatures and organic solvent resistance. While polyimides are used widely in the electronics industry and show many characteristics suitable for sensor encapsulation, little has been published with respect to their use as biomaterial. For this reason, only few studies have addressed the biocompatibility issue of polyimides [8]. For example Haggerty and Lusted [9] investigating the tissue reaction of polyimide sheated implants placed in cat cochlea, showed that the tissue reaction was minimal and the observed regions of inflammation were mild and confined to the immediate vicinity of the implant.

Our results show that PEI elicited a negligible toxic response in the cell culture testing. It was possible to grow on PEI surfaces not only 3T3 fibroblasts but also human endothelial cells from umbilical cord with no apparent deleterious effects on the cell viability.

Furthermore, the attachment, spreading, and growth of cells on PEI material was followed with respect to time. Both the number of adhering HEC, and the spreading area of individual cells, increased as a function of time. The first observation was made at 60 min after seeding and then periodically thereafter until the cell reached confluency (Fig. 1a, c). In the presence of medium containing FCS, endothelial cells (HEC) showed a gradual increase of spreading immediately after seeding on PEI, HEC reaching a mean surface area of approximately 1700 μm^2 at 60 min. Following further incubation, this cell spreading continued until a plateau value of 2400 μm^2 was reached. In the presence of culture medium without FCS cellular spreading on PEI occurred also, but a low level (800 μm^2) compared to endothelial cells cultured for the same time (60 min) in complete medium. By SEM analysis, it was possible to demonstrate that after 180 min many cells showed complete flattening in close contact with the substrate, while cytoplasmic processes had broadened (Fig. 1b, d). Finally, growth was monitored for 7 days and observations showed that the cells reached confluency within the first 5 days after the initial seeding. This indicates the effectiveness of PEI in supporting cell growth and spreading in the same way as the control polystyrene surface. When confluent, HEC shows the typical "cobblestone" monolayer pattern.

As expected from the results of the cell culture testing, the haemolytic capacity of PEI was effectively indistinguishable from the negative controls.

The polymer samples from the implanted cages were subjected to SEM and the majority of adherent cells were macrophages or foreign body giant cells (FBGCs). Qualitative observations showed that the surfaces of 4-day PEI material revealed areas of high cell density in which the adherent cells showed a variety of different shapes, morphologies, and degrees of cytoplasmic spreading. The morphologic changes occurring in the cells at 4–7 days were considerable in terms of an increase in adherent leukocytes having

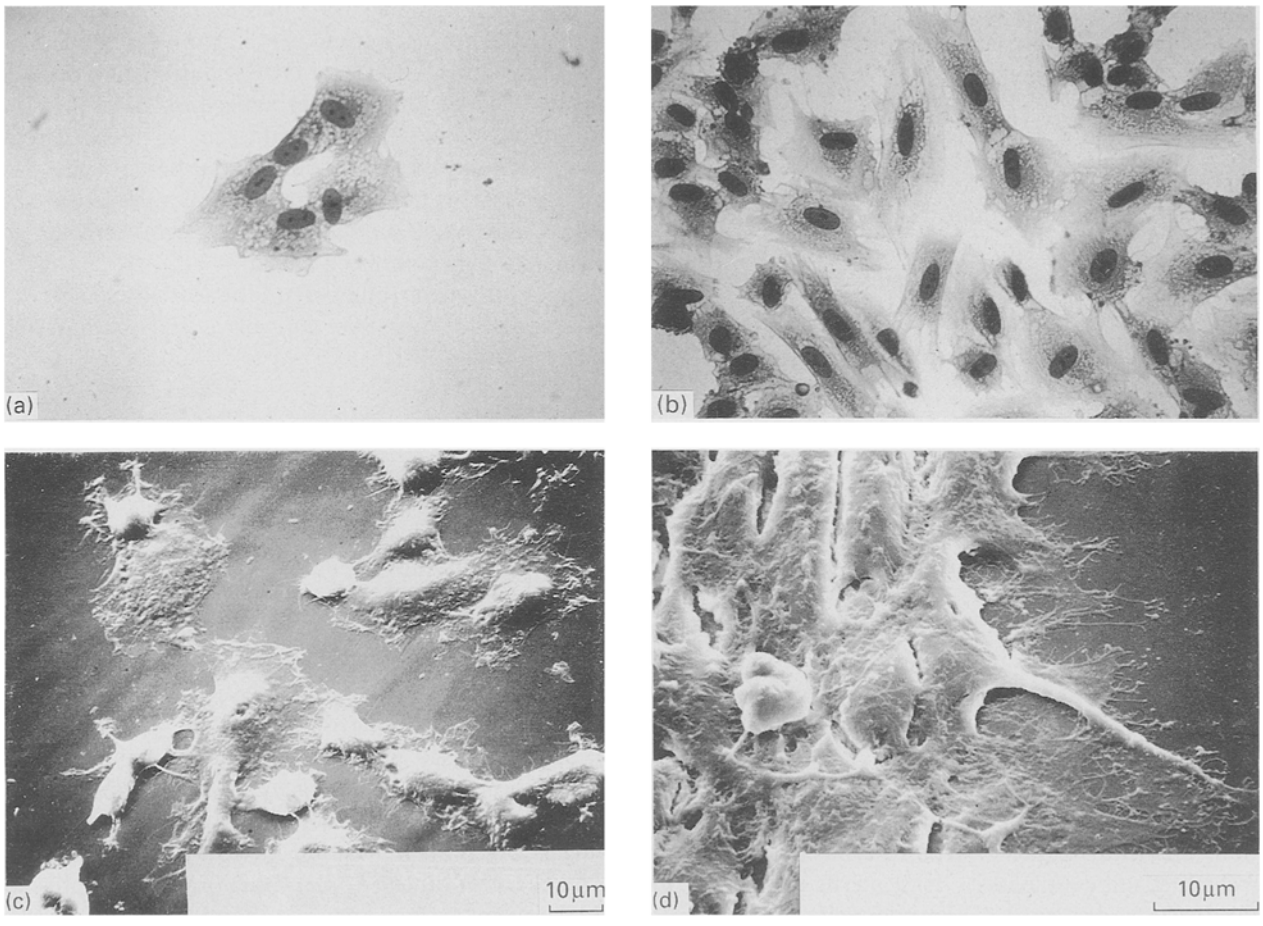
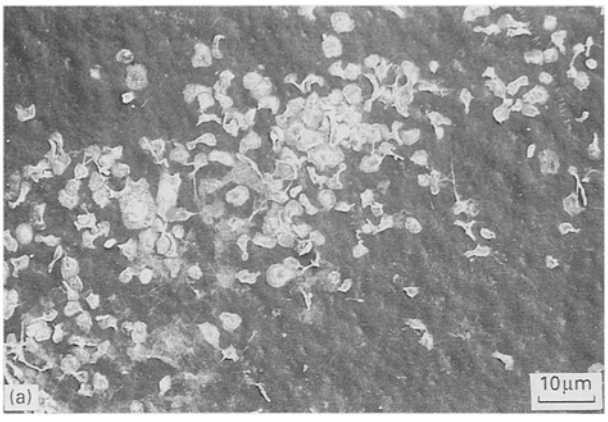
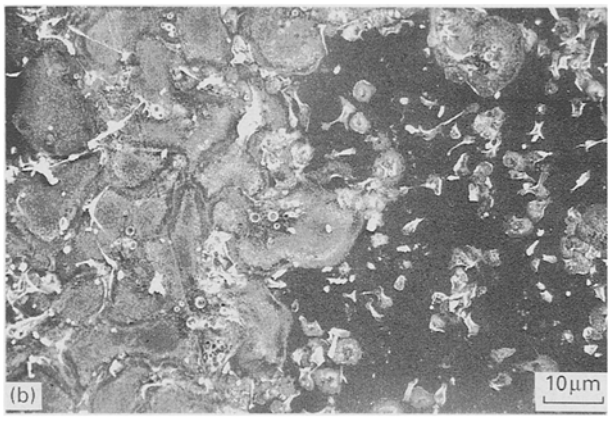


Figure 1 HEC adhesion and spreading on to PEI. Optical (25 × original magnification) and SEM micrographs at 60 min (a, c) and at 180 min (b, d) show increase in both the amount of adherent cells and their degree of spreading.





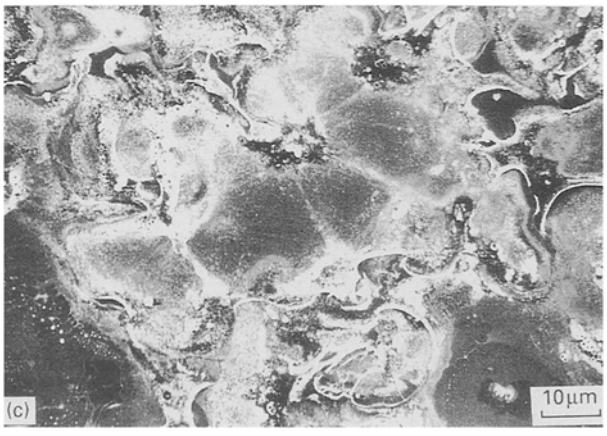


Figure 2 Leukocyte adhesion and spreading on to PEI. SEM pictures show a different cell morphology on PEI surface at different times after material cage implantation: (a) 3 days; (b) 14 days; (c) 21 days.

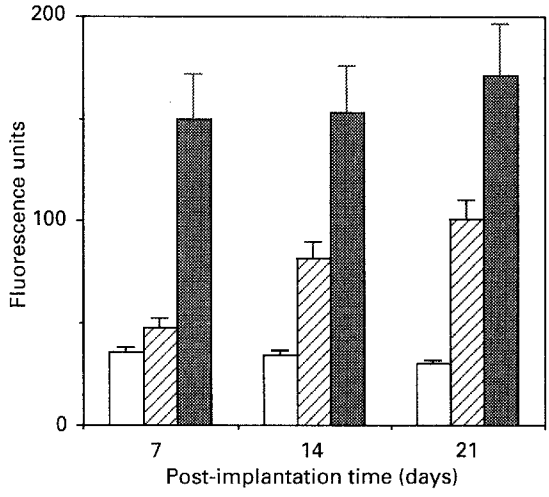


Figure 3 Hydrogen peroxide production by leukocyte adherent to PEI during postoperative period. (□ PDMS; ▨ PEI; ■ Pellethane).

extensive cytoplasmatic spreading (Fig. 2a). By day 14, the majority of the cells exhibited cytoplasmic spreading and well-developed FBGCs were observed on all the material implant surfaces (Fig. 2b). The differential counts of the cells adhered to the material surfaces revealed that the mononuclear cells, presumably macrophages, were the predominant cell type, although a certain number of polymorphonuclear cells were present during the first days of implantation. Indeed, only about 11% of the cells attached at day 4 were polymorphonuclear leukocytes, with the percentage dropping to nearly zero at all subsequent time points. At day 21, a considerable proportion of the cells on the PEI surface were FBGCs showing cytoplasmic spreading and vacuolation, which is a sign of cellular activation (Fig. 2c). The formation of FBGCs is believed to occur through the cytoplasmic fusion of macrophages after they have simultaneously attempted to engulf the same particle. This fusion process seems to be regulated by several cytokines, molecular components of the physiological environment of *in vivo* monocyte activation [10]. The cytokines, produced in large amounts during the inflammatory processes, would promote the formation of FBCGs leading to an increased number of FBCGs or an increase in the size of the FBCGs already present on the material surface. The observation that only at 21 days following cage implantation was it possible to show a significant increase in FBCG numbers on the PEI surface may be evidence for the presence of mild inflammatory response to the material implant. In fact, previous studies have demonstrated that the inflammatory stimulus, due to the presence of reactive material in a tissue, is an important factor controlling FBCG formation.

The hydrogen peroxide-sensitive dye DCFH-DA was used to examine the functional activity of cells adherent to PEI or control material implants. Historically, a number of models have been used to examine the leukocyte activation induced by cell–biomaterial interactions, including the direct incubation of immunocompetent cells with non-phagocytizable materials, such as nylon wool fibres [11, 12]. Many investigators have found that leukocytes undergo "frustrated phagocytosis" in response to biomaterials or foreign bodies [13, 14]. Thus, these cells increase their ability to produce hydrogen peroxide and superoxide anion, displaying decreased bactericidal and phagocytic activities, and enhanced degranulation. Release of granule content and reactive oxygen intermediates could be considered as an exhaustion of leukocyte functions, resulting in membrane damage and defective bactericidal activity [14].

Our data demonstrated that Pellethane induced a greater amount of DCFH oxidation than observed

with either PDMS or PEI. PDMS, which is considered relatively inert, stimulated a low amount of respiratory burst activity that did not vary over the time period examined (Fig. 3). Beginning at postoperative day 3, implantation of Pellethane was associated with a significant increase in DCFH oxidation that remained increased at postoperative day 8 and was further elevated at postoperative day 14. Our positive control Pellethane appeared, therefore, to have the greatest ability to induce DCFH oxidation by adherent cells. In contrast, PEI material did not induce significant DCFH oxidation until day 14, when levels of DCFH oxidation were significantly higher than observed with PDMS. These results are in accordance with the PEI morphological surface analysis whereas the presence of FBCG was evident only after 14 days following cage implantation.

4. Conclusions

This *in vitro* and *in vivo* study shows that PEI is, in general, an excellent substrate for cell spreading and growth, eliciting no cytotoxic response or haemolysis. While it induces FBCG formation *in vivo*, it seems to determine only a moderate degree of hydrogen peroxide production by adherent leukocytes. Coupled with both its good film forming and sterilizability, these data suggest that PEI could be an attractive biomaterial, either alone, or, in particular, as matrix for a composite structure.

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