# *In vitro* biocompatibility of EPM and EPDM rubbers

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The *in vitro* toxicity of two EPDM rubbers (K 778 and K 4802) and one EPM rubber (K 740) was tested using human fibroblasts. The modulus of elasticity of each rubber was varied by exposure to different amounts of electron-beam radiation (0, 5 and 10 Mrad). The short-term *in vitro* toxicity was tested by culturing cells on polymer films. The long-term effect of ageing was simulated by growing fibroblasts in nutrient media prepared from extracts of heat-exposed materials. Cell cultures were studied both quantitatively and (ultra) structurally. Growth curves obtained in the toxicity test did not differ significantly from control values at any day of observation, and also showed that electron-beam radiation did not alter the biocompatibility. The same results were found for all but one material in the artificial ageing test. The number of cells in the K4802/10 Mrad extraction medium was decreased. Ultrastructurally no gross deviations from normal morphology were observed, either in the direct contact test or in the artificial ageing test. The most characteristic feature was a somewhat dilated endoplasmic reticulum. In summary, the *in vitro* biocompatibility of EPDM-rubbers as observed in this study is satisfactory and motivates further investigation of their biocompatibility in animal experiments.

## 1. Introduction

In the last three decades many attempts have been made to develop a synthetic trileaflet valve for replacement of diseased heart valves [1–6]. So far, these types of valves are only used in short-term applications such as artificial blood pumps, heart assist devices and artificial hearts [7–13]. The main obstacle to long-term application is their thrombogenicity and the occurrence of calcification, which initially leads to leaflet stiffening, valve stenosis, leaflet tears and ultimately to valve dysfunction [6, 14].

The reported research is part of a larger project founded upon the idea that detailed analysis of the structure and function of the natural aortic valve may lead to improved prosthetic valve design. In earlier studies the hydrodynamical [15], mechanical [16], and kinematical [17] aspects of the natural aortic valve were studied. Since it is suggested in the literature that prosthetic valve failure may be related to its stress–strain distribution during the cardiac cycle [14, 18], a study was undertaken to formulate mechanical specifications for a valve which optimizes the stress–strain distribution. This led to the concept of a valve with flexible, fibre-reinforced leaflets [19, 20]. High flexibility is required for easy and full opening of the valve, whereas fibre-reinforcement should provide the tensile strength needed in the closed position.

For the fabrication of valve leaflets, polyurethane is the most commonly used elastomer [6, 12, 21, 22]. However, recent reports indicate that polyurethanes are unsuitable, because they undergo enzymatic and oxidative degradation [24, 25], and are susceptible to calcification [14, 18, 21] and thromboembolism [21].

As a possible alternative to polyurethanes ethylene propylene monomer (EPM) and ethylene propylene diene monomer (EPDM) rubbers are currently being studied. These rubbers are known for their high flex life, good abrasion resistance and low oxygen reactivity. Using electron-beam radiation the modulus of elasticity can be modified. As far as we know EPM and EPDM rubbers have never been used in a biomedical application before. Therefore, prior to using the material in a prototype a biocompatibility evaluation is required. This paper reports the *in vitro* toxicity of three EP(D)M rubbers and the influence of electron-beam radiation on their biological properties.

## 2. Material and methods

### 2.1. Polymers

As candidate materials for the construction of artificial valve leaflets three types of EP(D) M rubber were selected. EPM and EPDM are copolymers of ethylene and propylene. In the case of EPDM a diene is present in the backbone as a third monomer. The backbones of these polymers are fully saturated, whereas the small side chains are unsaturated. The composition of the three types studied in this paper, Keltan 740, 778 and 4802 (DSM, The Netherlands), is given in Table I.

Due to the saturated carbon backbone EPDM rubbers exhibit a high flex life and good abrasion

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TABLE I Chemical composition of selected EPDM rubbers

| | K740 | K778 | K4802 |
|---|---|---|---|
| Termonomer type | – | ENB | ENB |
| content (wt %) | | 4.0 | 4.0 |
| Ethylene content (wt %) | 60 | 65 | 55 |
| MW distribution | small | medium | medium |

ENB = ethylidene norbornene

TABLE II Ion concentrations (in meq/1) in PECF

| Ion | Concentration |
|---|---|
| $Na^+$ | 154.5 |
| $K^+$ | 5.4 |
| $Cl^-$ | 118.5 |
| $HCO_3^-$ | 44 |

resistance. Furthermore, they are not very sensitive to oxidation. Young's modulus for the polymer could be varied between 1 and 3 MPa by introducing cross links formed during electron-beam (EB) radiation. Each type of rubber was exposed to, respectively, 0, 5 and 10 Mrad of EB radiation.

Films were cast from polymer solutions (15 wt %) in xylene. The solvent was allowed to evaporate gradually at atmospheric pressure over 1 week. Xylene residues were extracted by subjecting the sheets to vacuum at 60 °C for 2 days. To remove possible contaminants the materials were kept in 99% alcohol for 5 days and flushed in running tap water for 24 h. Prior to cell seeding the films were UV sterilized overnight.

### 2.2. Cell culture

The cells used in the various toxicity tests were human fibroblasts obtained from nasal septum explants in the authors' laboratory. Cells were cultured in Dulbecco's Modified Eagle's Medium and Ham's F12 in a ratio of 3:1 supplemented with foetal calf serum (5%), hydrocortisone (0.4 g/ml), isoproterenol ($10^{-6}$ M), penicillin (100 U/ml) and streptomycin (100 g/ml). Epidermal growth factor was added after 3 days of culture. The cells were cultured in 10% carbon dioxide at 37 °C. The medium was refreshed twice a week.

### 2.3. Direct contact test

To determine the *in vitro* toxicity of the materials, fibroblasts were grown on thin films placed in 3.5 cm diameter tissue culture polystyrene (TCPS) dishes. Control values were obtained from cultures in TCPS dishes without rubber. The initial density was 12 500 cells/cm$^2$. Cell number and morphology were determined after 1, 4, 6, 10 and 14 days of growth. Cells were harvested from the culture dishes by trypsinization. For each experimental condition three dishes were used. The cells were counted using a Coulter counter.

### 2.4. Extraction test

An important effect of ageing of a material is the release of additives and low molecular weight compounds. This ageing effect was simulated by heat-exposing a material in a pseudo-extracellular fluid (PECF) at 115 °C for 48 h, according to an extraction test previously described [23, 26]. Ion concentrations present in the saline are given in Table II. The surface area of the polymer films was 0.5 cm/ml PECF. Aliquots of PECF served as a basis for preparing the nutrient media. Heat-exposed PECF without material was used as a basis for the control medium. A positive (toxic) control medium was prepared from PECF containing heat-exposed PVC.

The initial density was 12 500 cells/cm$^2$. The test media were added only after 3 days of growth in routine medium. Cell number and morphology were determined after 1, 3, 4, 6, 10 and 14 days of growth.

### 2.5. Preparation for morphological study

Cell cultures were fixed in 1.5% glutaraldehyde in 0.14 M sodium cacodylate buffer (pH 7.4, 4 °C, 1 h). After rinsing in phosphate-buffered saline the cells were postfixed in 1% osmium tetroxide at room temperature for 30 min. The cells were then rinsed again and photographed using a phase-contrast light microscope. Specimens of days 6 and 14 were dehydrated in graded alcohol series up to 100%, critical point dried and gold sputter coated for scanning electron microscopy (Philips S525). Cultures of day 14 were also dehydrated, embedded in Epon and cut into ultrathin sections for transmission electron microscopy (Philips EM 201).

### 2.6. Statistical analysis

Growth curve differences between materials were tested statistically using the Kruskal–Wallis one-way analysis of variance by ranks. When the obtained $p$-value of this test was less than 5%, then multiple comparisons were carried out using Dunn's test.

## 3. Results

### 3.1. Direct contact test

Growth curves for cell proliferation on rubbers K740, K778 and K4802 are shown in Fig. 1a–c. In each panel control values are drawn as well (thick solid line). The curves display the normal, roughly sigmoid shape, exhibiting the highest growth rate between days 4 and 10. Analysis of variance revealed that none of the growth curves differed significantly from control values at any day of observation. This also indicates that electron-beam radiation of the materials did not alter their biocompatibility as evaluated in a direct contact test.

Light microscopically, the fibroblasts were seen to form clusters at day 4, reaching confluency at day 10. The proliferation of cells cultured on rubber films was

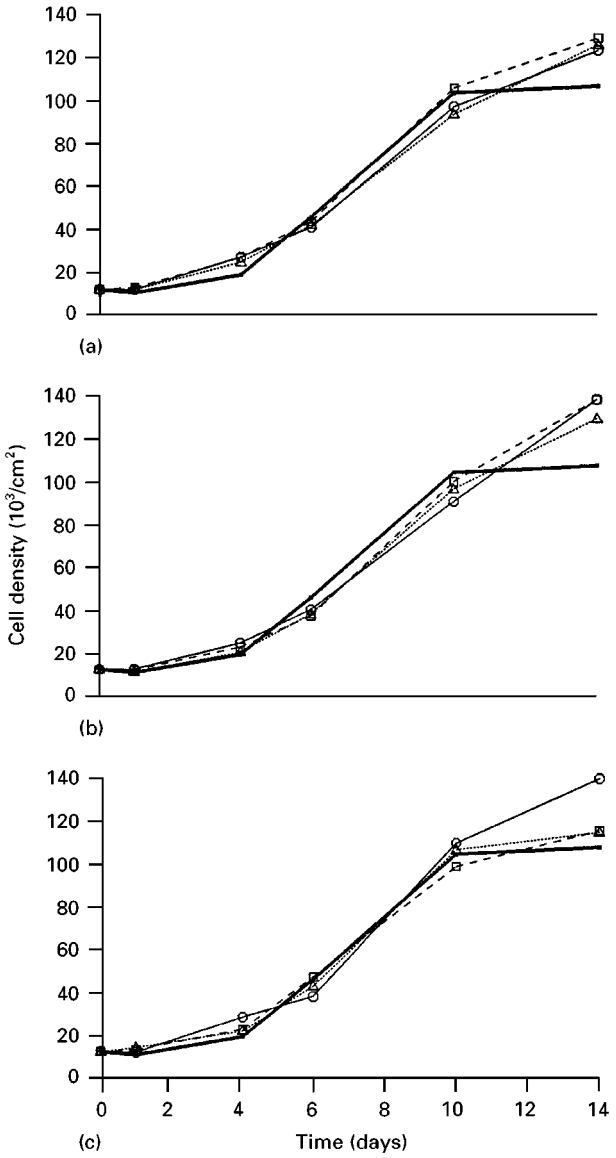


*Figure 1* Growth curves of human fibroblasts cultured on EP(D)M rubber sheets. In each panel the same control curve is drawn (thick solid line): (a) K740; (b) K778; (c) K4802. —■— control; —○— 0 Mrad; – –△– – 5 Mrad; – –□– – 10 Mrad.

not different from control cultures. Scanning electron microscopy showed that cell proliferation resulted in one or more cellular layers (Fig. 2). Ultrastructurally, the cells cultured on the films did not appear to deviate from controls. An example of a section of a culture on K740/10 Mrad is given in the electron micrograph of Fig. 3. The long, extended cells had a normal nucleus, a somewhat dilated endoplasmic reticulum, and well-developed ribosomes. The mitochondria had a normal morphology with intact cristae. The Golgi apparatus was abundantly present. The plasma membranes showed many fusing vesicles, which points to high exo- or endocytotic activity.

### 3.2. Extraction test

Growth curves for cell proliferation in extracts of K740, K778, K4802 are shown in Fig. 4a–c. In each panel the same control curves are drawn as well (thick solid line). Statistical analysis revealed that none of the

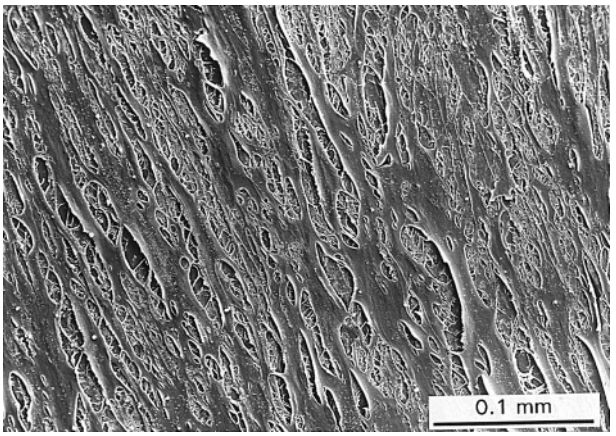


*Figure 2* Scanning electron micrograph of fibroblasts (control culture) after 14 days of growth.

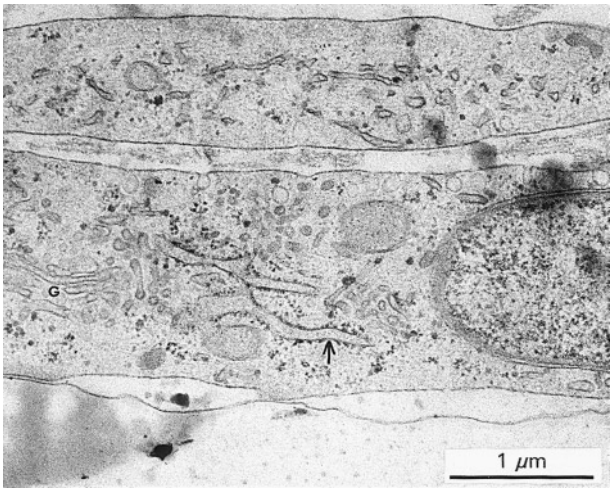


*Figure 3* Transmission electron micrograph of fibroblasts cultured on K740/10 Mrad. Note the dilated rough endoplasmic reticulum (arrow) and the well-developed Golgi apparatus (G).

growth curves in panels 4a and 4b differed significantly from control values at any day of observation. An effect of EB radiation was seen in cultures in K4802 extract media. Radiation of the material with 10 Mrad resulted in a significantly reduced rate of cell division, which became significant at day 14. Growth curves for the positive (toxic) control PVC and negative control are given in Fig. 4d. In PVC-derived extract media significant growth inhibition was observed at day 14.

Cells cultured in extract media prepared from the EP(D)M rubbers had a good overall appearance. In some preparations (K740/5 and K778/5) lipid vacuoles were found. Cells cultured in positive control medium (PVC) showed an abnormal morphology, in the sense that they showed damaged cell membranes and narrowed rough endoplasmic reticulum.

## 4. Discussion

From the viewpoint of hemodynamics and blood compatibility, preserved natural tissue is superior to synthetic materials to construct a leaflet valve. The long-term durability of preserved tissues, however,

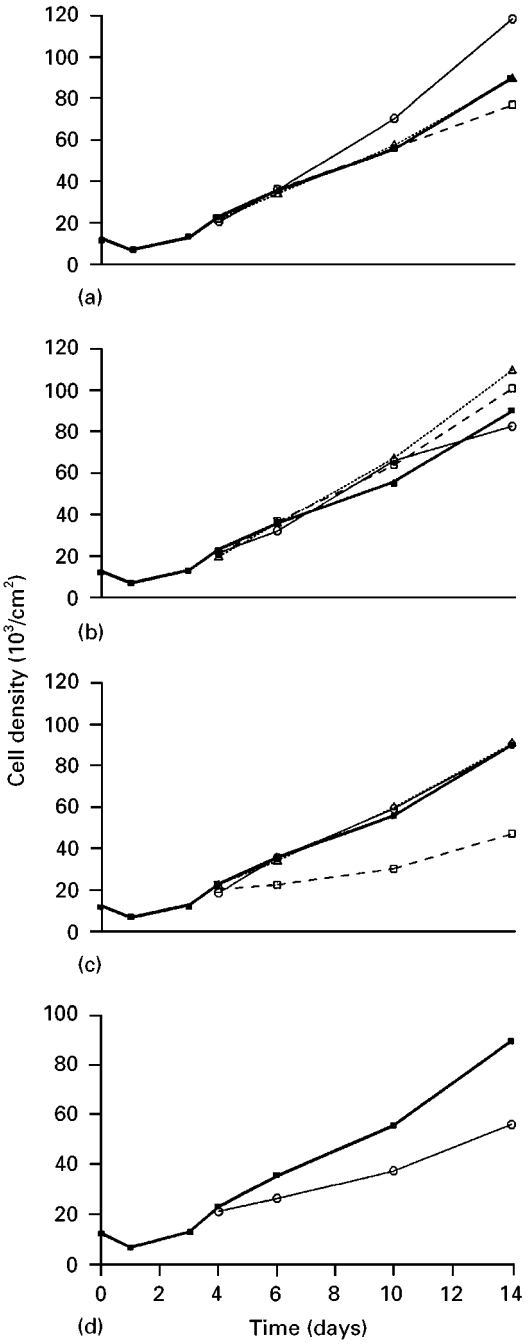


*Figure 4* Growth curves of human fibroblasts cultured in extracts of K740, K778, K4802 and PVC. In each panel the same control curve is drawn (thick solid line): (a) K740; (b) K778; (c) K4802; (d) PVC. (a), (b), (c) —■— control; —○— 0 Mrad; – – △ – – 5 Mrad; —□— 10 Mrad. (d) —■— control; —○— PVC.

appears less advantageous. The success of fabricating a synthetic, trileaflet valve to replace diseased heart valves depends, among other factors, on the development of both strong and flexible composites. In this study EPM and EPDM rubber has been presented as a new matrix material that has never been used in a biomedical application before. EP(D)M has been shown to be durable, especially in flexure, and can be processed into virtually any shape. Moreover, this material can be combined with polyethylene fibres. Before using the polymer in a valve prototype the *in vitro* toxicity was evaluated using a direct contact test as well as an extraction test.

Quantitative analysis of cell cultures on three types of EP(D)M rubber showed no differences in cell proliferation with respect to control at any day of observation. Neither was there an effect of electron-beam radiation on cell number. On morphological examination an abounding presence of Golgi apparatus was found, together with a dilated endoplasmic reticulum. This is a sign of high cell metabolism, indicating the vitality of the cells.

In the extraction test only one significant effect was noticed. The number of cells in the K4802/10 Mrad extraction medium had decreased. The morphological appearance of the cell culture, however, was normal.

PVC was selected as a positive (cytotoxic) control material. The growth inhibition observed was significant, but less than expected. This is most probably due to the fact that cytotoxic degradation products of PVC are volatile, and will to a large extent escape from the medium. This renders PVC suitable as a positive control in direct contact tests, but not optimal for the preparation of positive nutrient media.

Several studies demonstrated a correlation between *in vitro* and *in vivo* toxicity [23, 27, 28]. *In vitro* tests have the advantages of speed and low cost, and are sometimes more sensitive than *in vivo* tests. Results of *in vitro* biocompatibility studies are therefore frequently used to obtain an impression of *in vivo* biocompatibility, and may offer a basis for selection or rejection of materials in an early screening phase. Some criticism on this method, however, seems to be justified. Because of the complexity of the *in vivo* behaviour an *in vitro* test may not sufficiently imitate *in vivo* conditions. Under *in vitro* conditions a substance released by a material may become harmful due to its accumulation in the medium, whereas in the whole organism it may be removed from the *in vivo* milieu by metabolism or excretion. Therefore a material found to be toxic *in vitro* may not appear to be so *in vivo*. On the other hand, establishing *in vitro* biocompatibility of a material is not enough to infer its applicability as an implant. Because the polymers tested in this study are meant to be used in a cardiovascular implant, final evaluation of the biomaterial also requires *in vivo* testing, involving the evaluation of hemocompatibility and mechanical behaviour.

In conclusion, the *in vitro* bicompatibility of EPM and EPDM rubbers is promising and motivates further investigation of their biocompatibility in animal experiments.

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*Received 10 October 1995*
*and accepted 22 March 1996*