JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 6 (1995) 510–517

Degradation and calcification of a PEO/PBT copolymer series

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In this study the influence of polyethylene oxide content (70, 60, 55, 40 and 30%) on degradation and calcification of dense polyethylene oxide (PEO)/polybutyleneterephthalate (PBT) copolymer cylinders was evaluated at subcutaneous sites in goats. Histologically, the 30/70 and 40/60 PEO/PBT implants revealed no signs of calcification and of degradation, although slight surface alterations were evident after 1 year. In contrast, peripheral fragmentation and intermediate calcification were observed for 55/45 and 60/40 PEO/PBT specimens. After subsidence of the inflammatory reaction to the surgical intervention, the tissue reaction to these four PEO/PBT proportions was characterized by a fibrous capsule, although macrophages were frequently seen bordering 55/45 and 60/40 fragments. More extensive degradation and calcification were encountered for 70/30 PEO/PBT samples. Peripheral crack formation occurred from 4 weeks on and at 12 weeks, surface fragmentation was observed for this material. Fragments were usually surrounded by macrophages and, in addition, phagocytes with a foamy appearance were found. Fragmentation and phagocytotic activity increased during the course of the experiment. Using confocal Raman microspectroscopy, it was demonstrated that phagocytes contained 70/30 PEO/PBT fragments in their cytoplasm, as intracellular fragments were chemically composed of PEO and PBT. Systemic effects of the fragmentation were not observed in axial lymph nodes. It can be concluded from the data obtained in this model that a PEO/PBT copolymer series was well tolerated. This study showed that PEO content in PEO/PBT copolymers is directly related to degradation and calcification of these materials and that the extent of these parameters may vary under different experimental conditions. The application of confocal Raman microspectroscopy enables analysis of the chemical composition of intracellular fragments and may therefore be a valuable instrument in the prediction of long-term implant performances.

1. Introduction

Currently, an array of degradable polymers has found application in reconstructive surgery [1–3]. In view of the increasing use of such biomaterials, research has focused on obtaining more knowledge on the degradation kinetics in relation to cell and tissue response [4, 5]. The extent of the degradation process is an important determinant for the tissue response, as they are mutually related [4–7]. Several mechanisms, such as hydrolysis, enzyme/cell mediated processes, mechanical stress and surface or bulk erosion, have been reported to be involved in implant degradation [5, 7]. In addition to the chemical composition and physical aspects of the implant, experimental factors including implant geometry, implant site and choice of animal, influence the response of the recipient tissue [5, 8–10]. Since the success rate of degradable implants is ultimately based on this cell and tissue response, many investigations have concentrated on the assessment of these reactions and the governing parameters. Despite these efforts, knowledge of the degradation kinetics and subsequent tissue response is considered incomplete [4, 5, 8, 9].

Recently, a new degradable polymer (Polyactive^{R}) has been introduced as a promising skin substitute and as the tympanic membrane component of a total artificial middle ear [5, 11]. This material is a segmented polyether polyester copolymer, composed of a soft block, polyethylene oxide (PEO), and a hard block, polybutylene terephthalate (PBT). The mechanism of degradation of this PEO/PBT copolymer is not completely resolved as yet, although several studies suggest that hydrolysis plays an important role [5, 12, 13]. Another feature of PEO/PBT copolymers is the calcification behaviour occurring at the near surface. It was demonstrated that calcification of the

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material surface is a critical determinant for the occurrence of bone-bonding [14, 16]. Indeed, a direct relation between calcification of the material surface and bone-bonding has been established [15, 16]. By variation of the two individual components, a range of PEO/PBT proportions, with different mechanical behaviour and biological characteristics, can be obtained. Previous studies with such a range of PEO/PBT proportions showed that PEO content affected the degradation behaviour and calcification rate [14]. It should be emphasized that the data on degradation and calcification were predominantly obtained after implantation of relatively small (1.5 × 1.5 × 1.5 mm) blocks and porous films in rats.

We implanted large PEO/PBT cylinders in goats to obtain a comprehensive understanding of degradation and calcification and especially of the effect of variation in experimental conditions. The host response at 1, 4, 12, 26 and 52 weeks was morphologically assessed and the influence of PEO content (30–70%) on degradation and calcification of a range of PEO/PBT copolymers was studied. Furthermore, the composition of material fragments was investigated using confocal Raman microspectroscopy. Hence, this technique was monitored as a possible tool for obtaining information on degradation products, which may give an indication of long-term implant performance.

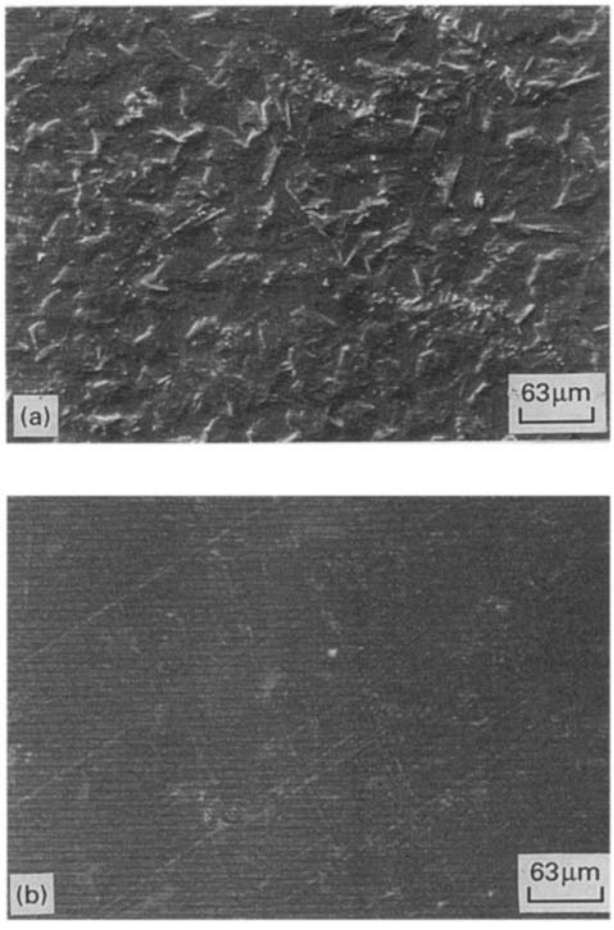


Figure 1 The surface morphology of 70/30 (a) and 30/70 (b) cylinders.

2. Materials and methods

2.1. Implants

Dense PEO (MW = 1000 D)/PBT cylinders (HC Implants bv, Leiden, The Netherlands) were prepared by injection moulding granular starting material. The cylinders were 7 mm in length and 5 mm in diameter. The following range of polyethylene oxide (PEO)/polybutylene terephthalate (PBT) proportions was studied: 70/30, 60/40, 55/45, 40/60 and 30/70 (based on weight in starting chemicals). The implants were checked macroscopically for irregularities, and samples of each implant type were examined using scanning electron microscopy (SEM, Phillips S525) for surface topographical assessment (Fig. 1). Roughness measurements showed a smooth surface for the whole range of materials (R_A-values 0.03–0.5) [15]. The implants were then cleaned in ethanol, dried at 50 °C and gamma-sterilized (2.5 MRad), prior to implantation.

2.2. Surgical procedure

Twenty mature female Dutch goats (CAE/CL-arthritis negative) weighing 40–60 kg were obtained from a professional stock-breeder. The animals were quarantined for at least 4 weeks and examined by a veterinary surgeon. To insert the subcutaneous specimens, 2–3 cm incisions were made in the shaven skin over the left midline of the spinal cord. Five subcutaneous pockets were created per goat and two cylinders of one implant type were implanted in each pocket (a total of 200 implants). For each subcutaneous site, the different implant types were allocated to five positions according to the method of Latin squares. This procedure ensured a randomization of goat number, position and implant type. The animals were fed *ad libitum* with water, hay and pelleted food (Kasper Faunafood, Lelystad, The Netherlands) and were transferred to a stable with grassland, at the fifth post-operative day. Four goats were sacrificed per follow-up period of 1, 4, 12, 26 and 52 weeks by an overdose of Nembutal^R and Pavulon^R. Details concerning the surgical procedure and animal care during the experiment have been described elsewhere [15].

2.3. Microscopy

The subcutaneous samples were fixed at 4 °C in 1.5% glutaraldehyde fixative in sodium–cacodylate buffer (pH = 7.4) and dehydrated in a graded series of ethanol. In the first mode of evaluation, the specimens were embedded in methyl methacrylate (MMA). Light microscopical sections (1–3 μm) were cut, orthogonal to the long axis of the specimens, on a Reichert Jung 2050 microtome and stained with toluidine blue. A second mode of evaluation was performed after artificial removal of the implants. The surrounding tissue was embedded in glycol methacrylate (GMA) and investigated according to the above described

method for MMA. Selected GMA-sections (70/30, 52 weeks) were collected on quartz glass and studied in confocal Raman microspectroscopy (CRM). The removed implants were air dried, gold coated and investigated in SEM for surface topographical alterations. Unstained histological blocks were polished with diamond paste, carbon coated and subjected to backscatter electron microscopy (BSE, Phillips S525) and X-ray microanalysis (XRMA, Tracor

2.4. Lymph nodes

Axillary nodes from animals sacrificed at 12 and 52 weeks were obtained from locations adjacent to the created subcutaneous pockets. All glands were fixed in 1.5% glutaraldehyde, dehydrated, embedded in MMA and light microscopical sections were prepared according to the above described regime for subcutaneous implants.

3. Results

3.1. Animals

One animal broke a limb three weeks post-operatively and was removed from the protocol (4-week survival time). The other goats showed no post-surgical complications and considerable weight changes during the experiment were not observed.

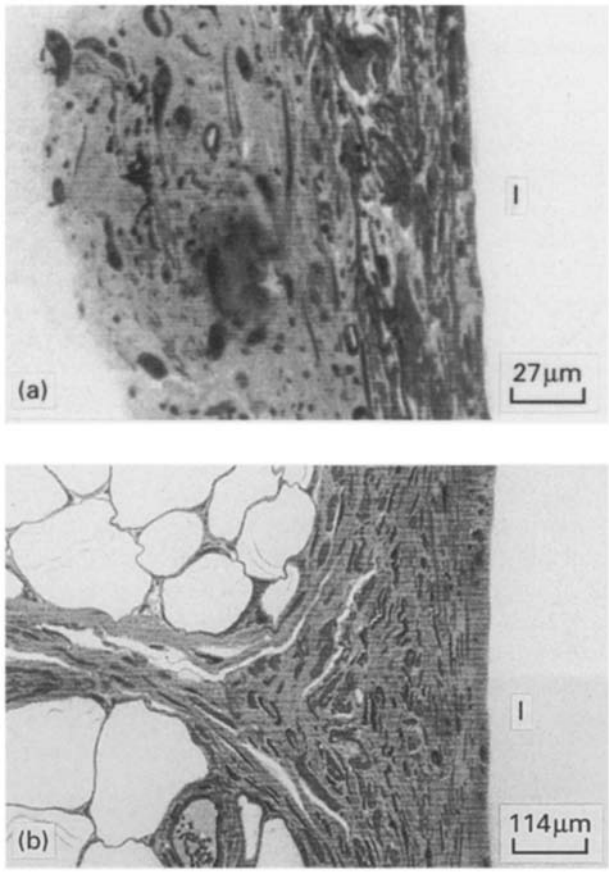


Figure 2 Cellular reaction to the insertion of PEO/PBT implants (I) at 1 week, resulting in various types of cells at and in the vicinity of the interface: (a) = 70/30; (b) = 30/70.

3.2. Cell and tissue response

Generally, negative effects of the implantation procedure (two cylinders per pocket) were not observed, largely because the implants were encapsulated separately. After 1 week of implantation, a similar reaction to all subcutaneous implants was observed by light microscopy (Fig. 2a, b). A blood clot, combined with a cellular infiltrate, surrounded the cylinders. At a distance from the interface, loosely organized fibrous and fat tissue was seen. Four weeks post-operatively, a fluid space, containing mainly erythrocytes but also lymphocytes and macrophages, was occasionally apposed to the subcutaneous implants (Fig. 3). Most surfaces, however, were covered by a 1–5 cells thick layer of macrophages and fibroblasts. On top of that layer, away from the interface, a zone consisting of collagen and fibroblasts was evident (Fig. 3). It should be stressed at this point that the histological description of the interface as described below largely refers to 30/70 and 40/60 ratios and to a somewhat lesser extent to 55/45 and 60/40 PEO/PBT proportions. At later survival times, lymphocytes had disappeared, the amount of macrophages at the interface had decreased and large areas were solely covered by a capsule of elongated fibroblasts, which were few in number, and collagen fibres, which ran mostly parallel to the implant surface (Fig. 4a, b). The tissue response to PEO/PBT proportions, which showed an obvious alteration in the course of the experiment, is described next.

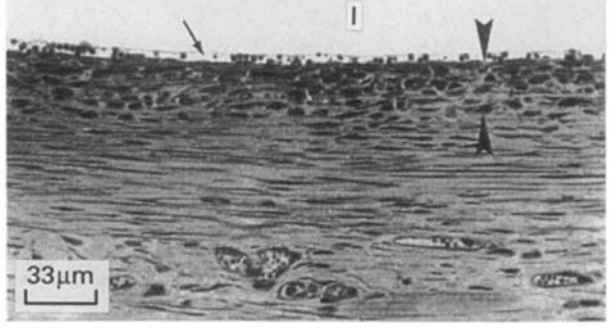


Figure 3 Four weeks postoperatively, a fluid space (arrow), containing erythrocytes and macrophages is observed at the interface. A second zone of a few cell layers thick is mainly composed of macrophages and fibroblasts (arrowheads): I = 30/70.

3.3. Implant degradation and consequences for tissue response

The first signs of material degradation, peripheral crack formation, were histologically seen at the 4-week survival time for the 70/30 samples. From 12 weeks on, relatively large fragments (20–100 µm) had completely detached from the bulk of these implants. These fragments were embedded in fibrous tissue and were frequently bordered by macrophages, which were preferentially aligned to these loose fragments. Fragmentation occurred only at the peripheral part of the implant, resulting in an irregular appearance of the surface compared to the bulk part, which remained

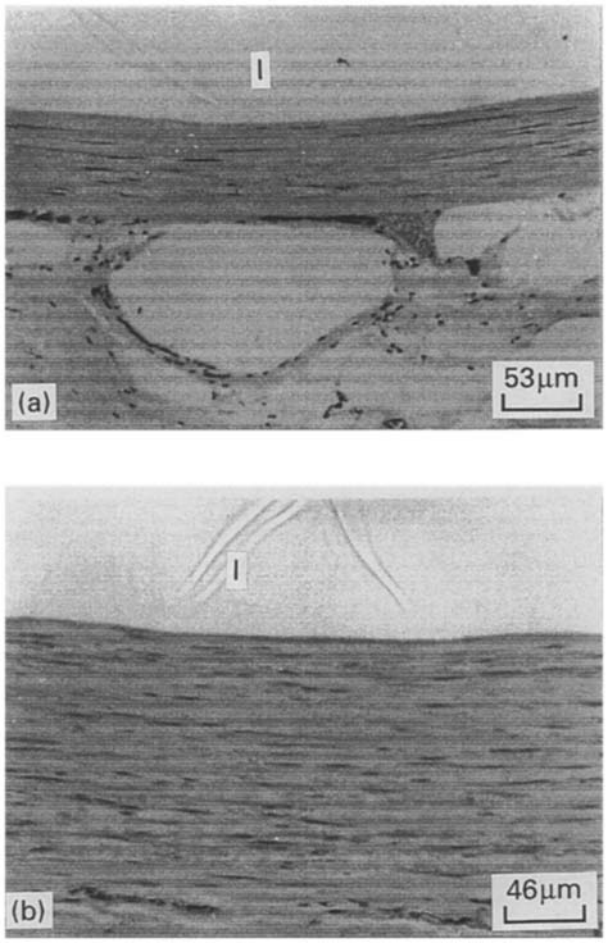


Figure 4 The tissue response, at 1 year, to non-degrading areas of 60/40 PEO/PBT (a) and non-degrading 40/60 PEO/PBT (b) copolymers. The implants (I) are surrounded by a fibrous capsule of collagen and elongated fibroblasts.

morphologically unaltered. In addition, phagocytic cells with a 'foamy' appearance, containing fragments in their cytoplasm, were present along the bulk 70/30 surface (Fig. 5a).

At 52 weeks, the tissue covering 70/30 implants was organized into three separate layers with variable thickness. Away from the interface, an area of loosely organized fibrous and fat tissue, an intermediate layer characterized by collagen and elongated fibroblasts and adjacent to the implant, a zone consisting of phagocytic 'foam' cells which were more abundantly present compared with the 12 and 26-week observations (Fig. 5b). Occasionally, fragments were found at extracellular locations in these tissue layers. Chemical analysis, using CRM, of the fragments within these phagocytes showed (after subtraction of cellular background and embedding media) characteristic PEO and PBT peaks. The pattern of peaks is highly comparable to spectra obtained from 70/30 cylinders 1 week postoperative [17] (Fig. 5c). Despite the high rate of peripheral fragmentation of 70/30 implants, the bulk and thus the contour of the implants, remained largely intact. Upon artificial removal of the implants from the surrounding tissues at 52 weeks, however, a decrease of their preoperative mechanical properties was evident. A clearly altered morphology of the surface of the removed 70/30 specimen was revealed by SEM. Especially at lower magnifications, the implant surface was dominated by multiple cracks. Between these cracks, the preoperative surface topography had disappeared, and small fragments ($< 10\,\mu m$) were observed loosely attached to the bulk (Fig. 5d; compare with 1a).

Degradation of 60/40 and 55/45 PEO/PBT proportions was observed from 12 weeks on. Fragmentation occurred at the periphery of the implants and detached fragments appeared within the surrounding fibrous capsule (Fig. 6a). Macrophages were often appositioned to these fragments, but cells with polymer fragments within their cytoplasm were not seen. The amount of fragmentation of these proportions did not increase markedly up to the 1-year survival time and cells displaying phagocytic activity were not found at 60/40 and 55/45 surfaces. A considerable number of cracks in the implant surface was seen with SEM, although the preoperative contours of the implants were intact. At the 1 year survival time, the surface of these PEO/PBT ratios appeared similar to the 70/30 specimen: generally smooth with, at some locations, distinct surfacial fragmentation. In the histological specimens degradation of the 40/60 and 30/70 PEO/PBT proportions was not observed, although SEM did suggest an altered surface morphology compared to the preoperative topography. After 1 year a surface, which vaguely showed the signs of the preoperative topography, and peripheral crack formation was apparent (Fig. 6b; compare with 1b). It should be emphasized that the processing of PEO/PBT implants for SEM might induce crack formation. However, this will then be representative for each ratio and is therefore useful for comparative observations within the PEO/PBT range.

3.4. Lymph nodes

The histological analysis of axial lymph nodes did not reveal signs of a deviating morphology after 12 weeks and after 1 year. Axial nodes consisted largely of lymphocytes, monocytes, macrophages and, occasionally, mast cells and material particles were not seen.

3.5. Subcutaneous calcification

From 4 weeks on, spots with a granular appearance were histologically observed within the surface of the 70/30 implants (Fig. 7a). The bulk of these spots was often denser when compared to the periphery, where individual granules were frequently observed. These spots appeared white in BSE, suggestive of a higher density of hard material (Fig. 7b). Elemental analysis (XRMA) demonstrated the presence of calcium and phosphorus, while quantitative analysis occasionally showed Ca/P ratios approximating 1.67, but the results varied greatly and were usually between 1.5 and 1.6. The amount of calcification for the 70/30 increased up to 12 weeks post-operatively and, restricted mainly to peripheral areas, remained then at the same level up to 1 year. Calcification, with a composition similar to that described above for 70/30 samples, was

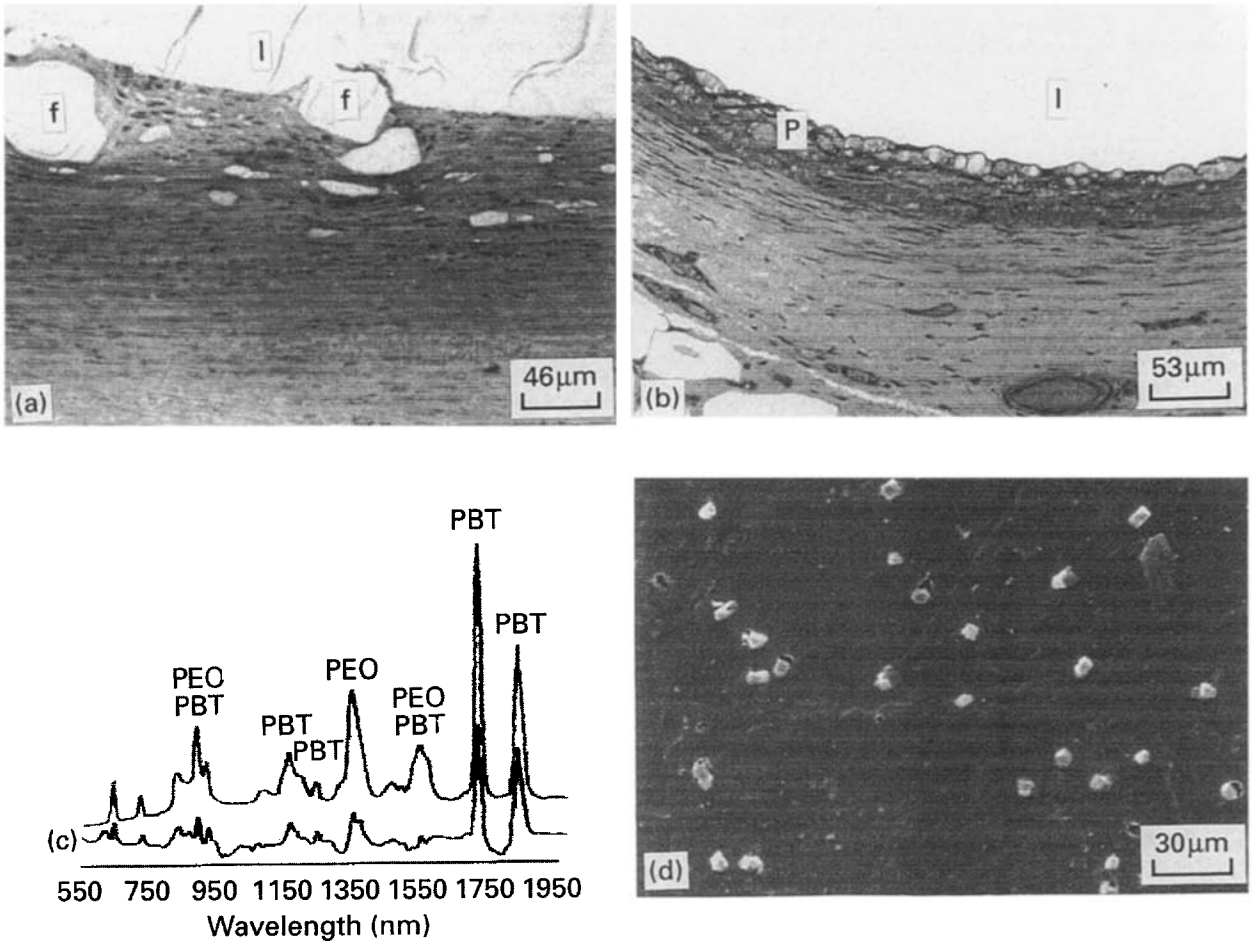


Figure 5 (a) Twenty-six week histological appearance of a 70/30 implant (I). Fragments (f), partially detached from the bulk are found in fibrous tissue. (b) LM picture of a 70/30 cylinder (I) 1 year postoperatively. Phagocytic cells (P) containing polymer fragments dominate the interface zone although an inflammatory reaction is absent. (c) CRM-spectrum of the intracellular fragments shown in 5b. Distinct peaks are seen for PEO and PBT (after subtraction of cellular background and embedding media). Although the (upper) spectrum is highly comparable to the lower spectrum obtained after 1 week of the bulk of a 70/30 implant, indicative of the same origin, small differences point to chemical changes before or after phagocytosis. (d) The surface morphology of a 70/30 implant after 1 year. Note the disappearance of the preoperative surface topography and the small (< 10 μm) fragments which seem to be in the process of detaching indicative of fragmentation.

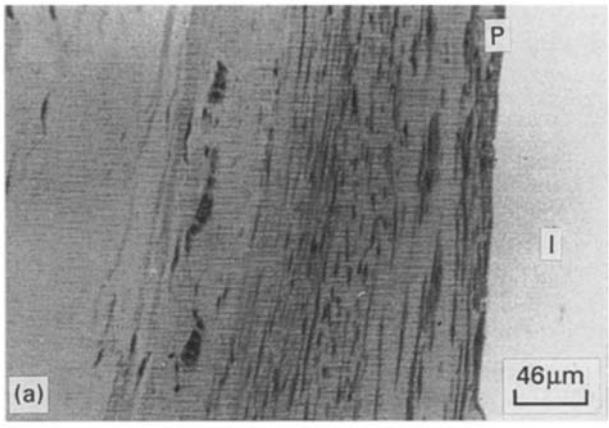
observed for 60/40 and 55/45 specimens although to a much lesser extent. In contrast, calcification was not encountered in 40/60 and 30/70 implants.

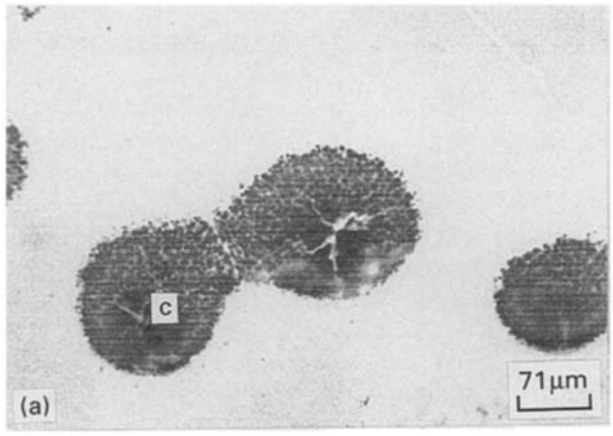
4. Discussion

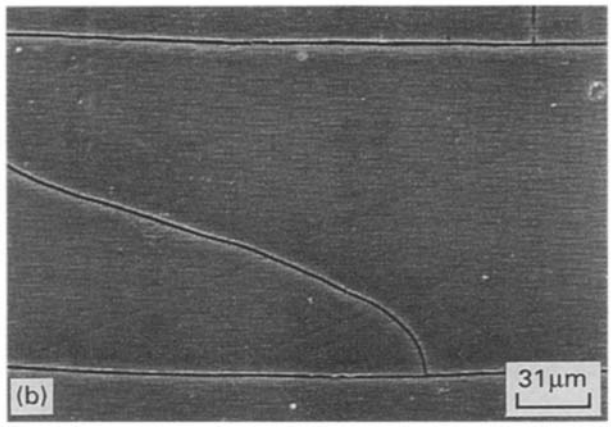
Information on degradation and calcification of a range of PEO/PBT copolymers has mainly been acquired on porous films or small blocks in rats [5, 14]. To obtain additional information and to explore the influence of different experimental conditions, we wished to employ larger implants in goats. Dense and smooth injection moulded cylinders were chosen to exclude geometrical effects such as surface texture and porosity [5, 8, 9]. Furthermore, to remain distant from the edges, all samples were analysed in a standardized central area perpendicular to the longitudinal axis of the implants.

The early post-operative results showed an inflammatory response resembling the wound reaction that is induced by surgical intervention [9]. It was therefore impossible to discriminate between early tissue reactions to the implantation trauma or to the presence of a foreign body response. At 4 weeks, macrophages surrounded by fibrous tissue covered the implant surface. Mast cells and foreign body giant cells lining the implant, as reported by others, were not seen [5, 10]. At later survival times, PEO/PBT proportions that revealed no morphological signs of degradation were surrounded by a capsule of fibroblasts and collagen, and sometimes macrophages. The thickness of this capsule varied considerably, but this seems rather insignificant since it was previously demonstrated that capsule thickness is a time- and implant-independent parameter [18].

PEO content in PEO/PBT copolymers clearly influenced degradation of a range of bulk non-porous cylinders. Fragmentation was observed around 70/30 specimens at the 12-week survival time and to a much lesser extent for 60/40 and 55/45 implants, whereas 40/60 and 30/70 samples only showed slight surface alterations after 1 year. Fragmentation of 70/30 cylinders resulted in phagocytosis of particles by cells that had the morphological appearance of foam macrophages. Elsewhere, it was shown by transmission electron microscopy, that such fragments were indeed







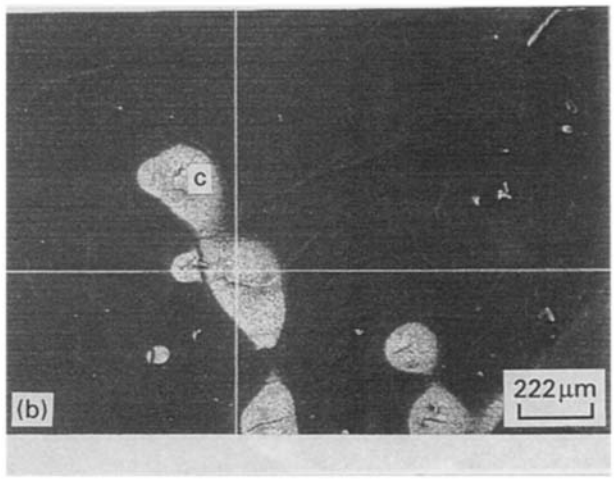


Figure 6 (a) Peripheral fragmentation of a 60/40 implant (I). These fragments (P) were embedded in fibrous tissue. LM, 1 year. (b) Crack formation as a sign of an altered surface morphology in a 30/70 cylinder, 1 year postoperatively (compare with Fig. 1b).

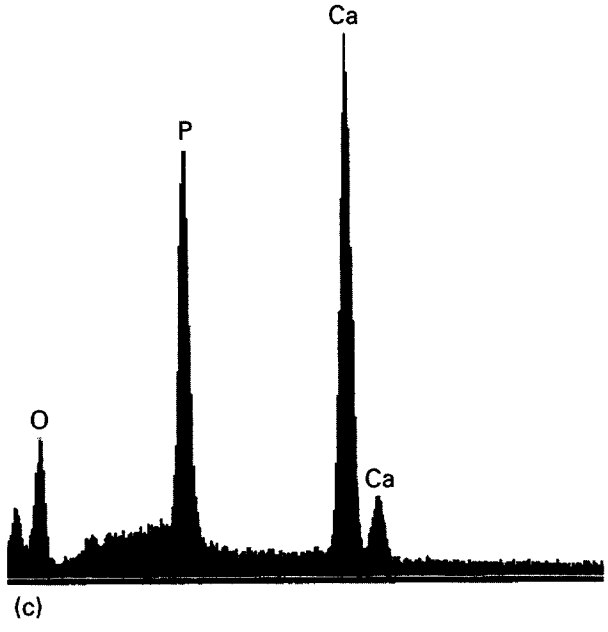


Figure 7 (a) The granular appearance of calcification (c) spots in a 70/30 cylinder. (b) Similar view as in 7a, obtained by BSE. The white calcification (c) spots are composed of calcium and phosphorus as shown in the XRMA spot analysis (at centre of cross).

located intracellularly and often rounded-off after phagocytosis [4, 14], indicative of a persisting degradation process after phagocytosis [5, 14]. The presence of these intracellular fragments, as well as the observed surface smoothness, were suggestive of implant resorption. In this study, fragmentation was limited to the periphery of the implants. In contrast, in previous experiments in rats, fragmentation of smaller blocks extended to the bulk, whereas porous films were barely retrievable at later survival times [5, 14]. In general, it seems that degradation of larger bulk implants was slower than expected based on results on smaller test samples. Despite the limitation of fragmentation to the periphery of the implants, phagocytotic activity was clearly observable. The morphology of phagocytes remained intact and no local inflammatory response was revealed, confirming previous studies in this respect [14]. This result is in contrast with several reports on other degradable polymeric biomaterials, which described an extensive inflammatory reaction to degradation products [1, 6, 8, 19, 22]. The surface area occupied by phagocytotic cells containing 70/30 particles remained constant in time, possibly caused by transport [19]. However, evaluation of axial lymph nodes did not reveal any systemic effect up to 1 year, supporting investigations on deep inquinal nodes after femoral implantation of PEO/PBT copolymers (unpublished results, AM Radder and CA van Blitterswijk). This is significant because poly-L-lactic particulate remnants, diagnosed as sinus histiocytosis, were found in the

latter glands [22]. The absence of systemic effects, after implantation of PEO/PBT proportions, is in concordance with *in vivo* experiments that did not detect accumulation of polymeric fragments in any of the major organs [5]. This is corroborated by experimental data in the literature that did not indicate any adverse effects of PEO/PBT components [5, 14, 23–25]. The application of confocal Raman microspectroscopy is of interest in this respect. In this study, this technique confirmed that phagocytozed particles were composed both of PEO and PBT and did, therefore, originate from the implant. In addition to the determination of chemical composition, confocal Raman microspectroscopy is useful to assess changes in the composition. It is clear from comparing 1-year to 1-week CRM measurements that intracellular fragments had undergone chemical changes before or after phagocytosis [17].

Although variation of PEO content clearly influenced the degradation characteristics of PEO/PBT copolymers, the underlying mechanisms have not fully been clarified. Polymer degradation is considered to occur, chiefly, by hydrolysis *in vivo* [5–7, 12–14, 19]. Experiments involving polyethylene oxide/polyethylene terephthalate copolymers reported that the preferential hydrolytic cleavage site is the ester bond present in the soft segment [12, 13]. An increase in PEO content will facilitate hydrolytic processes in two ways: first, because of the hydrogel properties of PEO/PBT copolymers, ester bonds are easily accessable for water molecules and secondly, the amount of available ester bonds will increase with higher PEO amounts. Other factors that are involved in degradation rate may be oxidation and surface erosion, presumably under the influence of mechanical factors such as stress and strain [7]. Fragmentation was initiated at the periphery of PEO/PBT proportions and most probably proceeds towards the bulk part of the implants. This process will result in a higher surface area and possibly induce cell-mediated degradation. Although an involvement of enzymes in polymer degradation has been reported for polyethylene terephthalate, *in vivo* enzyme catalysed bulk degradation has not yet been demonstrated [5, 7, 26].

Variation of PEO content in PEO/PBT copolymers also influenced the calcification rate. Calcification is a relatively common feature for polymers and some studies, usually designed to inhibit calcification, showed that the soft PEO segment is able to absorb calcium [27]. Additionally, PEO homopolymers with a molecular weight 1000 D, as used in this study, revealed the highest calcium uptake [27]. This study supported a relation between PEO content and calcification rate by demonstrating increased calcification with higher PEO contents. Further, it underlined earlier experiments with porous films and small blocks in rats [5, 14], suggesting that the relation between PEO content in PEO/PBT copolymers and calcification can be sustained after variation of experimental conditions. However, calcification was less abundant in larger cylinders and did not decrease in time as was reported for porous films [14]. These differences indicate that the extent of calcification may be dependable on factors such as implant geometry, implantation site and choice of animal.

It can be concluded from this study that the biocompatibility of a range of PEO/PBT copolymers is satisfactory. Although no linear relation was established, variation of PEO content did influence degradation characteristics and calcification rate; the higher the amount of PEO in PEO/PBT proportions, the higher degradation and calcification rate. Furthermore, it was shown that the extent of calcification and degradation rates differ with variations in experimental conditions. To our knowledge, this is the first time that confocal Raman microspectroscopy has been applied in the biomaterials field. We feel that this technique provides a valuable additional instrument in the screening of degradable polymers and in the prediction of the long-term biocompatibility of these biomaterials.

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Received 16 August
and accepted 26 November 1994