Tissue reactions of subcutaneously implanted mixture of ε-caprolactone-lactide copolymer and tricalcium phosphate. An electron microscopic evaluation in sheep

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Biodegradable polymers, mainly derivates of α-hydroxy acids, are widely used today in oral- and maxillofacial surgery, orthopedics, and other fields of surgery. These biomaterials are well tolerated by living tissue and fracture fixation devices made of polylactic or polyglycolic acid are clinically widely used today. Still, there are some problems in application of biodegradable polymers. Abacterial inflammatory reactions have been noticed after the clinical introduction of these devices. Both swelling and pain at the site of implantation have also been reported. The etiology of this inflammatory reaction is still unknown, despite the numerous studies. Therefore, the aim of the present study was to further characterize this inflammatory reaction in detail, by electronmicroscopy. We prepared a mixture of ε-caprolactone–lactide copolymer and tricalcium phosphate and placed it in the dermis in 12 sheep. Follow-up times were 9, 14, 24, and 52 weeks. We found that the mixture caused a mild inflammatory reaction. There were no signs of cell damage. Fibroblasts, macrophages, and eosinophils were found adjacent to the copolymer. The mixture is easy to handle and can be moulded into different shapes in room temperature. The results encourage us to continue our studies to develop a filling material for bone defects.

Introduction

Fracture fixation devices made of biodegradable polymers, mainly derivates of α-hydroxy acids, are widely used today in oral- and maxillofacial surgery, orthopedics, and other fields of surgery. Polymers most often used are polydioxanone, polylactide, polyglycolide, and their co- or stereopolymers. D,L-lactide and ε-caprolactone are such derivatives and can be copolymerized to form ε-caprolactone–lactide copolymer, which in different forms has been used in experimental studies. Numerous studies have been done using copolymer of lactide and ε-caprolactone as nerve guide [1–8], suture [9], barrier preventing tissue ingrowth [10], material substituting dura mater [11], repairing meniscus [12] and treating furcations in molars [13]. Tissue reactions reported in these studies have been minimal.

In general, all of the above mentioned biodegradable materials are well tolerated by living tissue and fracture fixation devices made of polylactic or polyglycolic acid are clinically widely used today. Still, there are some problems in application of biodegradable polymers. The small particles generated during biodegradation of an implant are usually cleared off by tissue macrophages by phagocytosis. When biodegradation is excessive, the particles accumulate and the local defense processes foster the onset of an inflammatory reaction. In our previous histological studies, we found that ε-caprolactone–lactide copolymer in paste form provoked a severe inflammation reaction in soft tissue and a moderate one in bone [14]. Therefore, the aim of the present study was to further characterize this inflammatory reaction in detail, by electronmicroscopy.

Materials and methods

Experimental animals and implant material

The animal handling and the procedures were performed according to local ethical rules (Helsinki Declaration) and the experiments were approved by local ethical committee. Twelve adult Finnish landrace sheep of both sexes, weighing from 52 to 83 kg, were used as experimental animals. The raw material used was a copolymer of ε-caprolactone and D,L-lactide acid (P(ε-CL/DL-LA)) polymerized in the Laboratory of Polymer Technology, Helsinki University of Technology, Espoo, Finland. Polymerization has been explained in detail by Hiljanen-Vainio *et al*. [15]. The initial monomer ratio was 60/40 w/w, and the weight average molecular weight (M_w) of the copolymer was 17 900. It was in paste form. The implanted material was a 27/73 w/w mixture of P(ε-CL/DL-LA) and β-tricalcium phosphate (β-tricalcium phosphate®, Fluka Chemie AG, Buchs, Switzerland). It was prepared in the Institute of Biomaterials, Tampere University of Technology, Tampere, Finland by heating the copolymer slightly and mixing it mechanically with TCP powder. The mixture of P(ε-CL/DL-LA) and TCP was sterilized by means of gamma radiation (Kolmi-Set Oy, Ilomantsi, Finland) with a minimum dose of 25 Gy.

Operative procedure

Food was withheld for 48 h prior to surgery, but sheep had free access to drinking water. The sheep were free of any clinical signs of disease. A 0.5 mg dose of atropin (Atropin® 1 mg/ml injekt, Orion, Espoo, Finland) was administered subcutaneously (sc) half an hour before induction of anesthesia. Medetomidine (Domitor® 1 mg/ml, Orion-Farmos Co, Turku, Finland) 20 μg/kg body weight (bwt) was given by intravenous (iv) injection. Anesthesia was induced by iv propofol (Diprivan® 10 mg/ml, Zeneca Ltd, Macclesfield, England) 3 mg/kg bwt. The sheep were intubated and positioned in sternal recumbency with the head extended and fixed in a cushion. Anesthesia was maintained with 2.5% halothane (Trothane®, I.S.C. Chemicals Ltd, Bristol, England). The saphenous vein was cannulated. During the operation, 1000 ml of isotonic fluid (Ringersteril®, Medipolar, Oulu, Finland), metronidatzole (Flagyl® 5 mg/ml, Rhone-Poulenc Rorer A/S, Birkerod, Denmark) 11 mg/kg and benzylpenicillin sodium (Geepenil®, Orion-Farmos, Turku, Finland) 35 000 IU/kg were administered iv. Both sides of each mandible were shaved and scrubbed with antiseptic solution, chlorhexidine gluconate (Klorhexol® 5 mg/ml, Leiras, Finland). An extraoral incision was made on the right cheek, 1 cm from the commissure. About 0.3 mg of the material was carefully placed subcutaneously and the incision was closed on the skin of the cheek using an absorbable suture (Dexon®). The material was also implanted into the mandibular bone for bone growth studies, which are not presented here.

Postoperative procedure

Postoperatively, benzylpenicilliumprocaine (Ethacillin® vet injekt 30 000 IU/ml, Intervent, Boxmeer, Holland) 35 000 IU/kg sc and phenylbutazone (Reumuzol® 200 mg/ml vet injekt, Lääkefarmos, Turku, Finland) 8 mg/kg iv were administered once a day (SID) for three days. Animals were free to move in their boxes. The recovery was uneventful.

Follow-up times and specimens

The sheep were killed at 9, 14, 24, and 52 weeks postoperatively in groups of three. After careful dissection samples for electron microscopy were taken from the connective tissue encircling the subcutaneously implanted material. Immediately after removal, the specimens were prefixed in cold (+4 °C) phosphate-buffered glutaraldehyde (2.5%) for 2 h, washed with sodium phosphate buffer (0.1 M; pH 7.4) and postfixed in buffered 1% osmium tetroxide for 90 min. Samples were cut in semithin sections (0.5 μm) and stained with toluidine blue. They were evaluated under light microscope and a correct place for electron microscopy was chosen. The specimen were then treated with a mixture containing equal parts of propylene oxide and Epon LX 112 for 2–3 h and then embedded in Epon LX 112. Ultrathin sections were cut with Reichert Ultracut Ultramicrotome. The specimens were polymerized for the first 12 h at 40 °C, then for 20 h at 60 °C and finally 24 h at 70 °C. The sections were stained with uranyl acetate and lead citrate (Leica EM Stain) and examined with Jeol JEM 1200EX transmission electron microscope at the Institute of Biotechnology, Electron Microscopy, University of Helsinki. Four sections from each specimen were viewed in the electron microscope.

Results

Nine-week follow-up

The structure of connective tissue as well as the structure of collagen and fibroblasts was normal. TCP-particles were abundantly visible in all samples studied. The particles were distributed in groups. They were either rods or angular particles in shape with average size of 1–2 μm. The TCP-particles were found both intra- and extracellularly, but mostly they were seen within cells (Fig. 1). Mononuclear inflammatory cells were seen adjacent to TCP-particles as well as a few multinucleated giant cells, with intracellular TCP-particles (Fig. 2). Also one mast cell was seen, but there were no copolymer or TCP-particles in the vicinity of this cell.

Fourteen-week follow-up

TCP-particles were still abundant, but now their edges were more rounded and their shape was more elongated compared with the first follow-up point. However, the size of the particles (1–2 μm) was unchanged compared with previous follow-up. The TCP-particles were now found mostly intracellularly. TCP and copolymer were separated from each other in this follow-up. The copolymer without TCP was seen as a liquid filled space without any visible particles (Fig. 3). The copolymer did not elicit any inflammatory reaction, but there seemed to be mononuclear inflammatory cells, probably macrophages, adjacent to TCP-particles (Fig. 4).

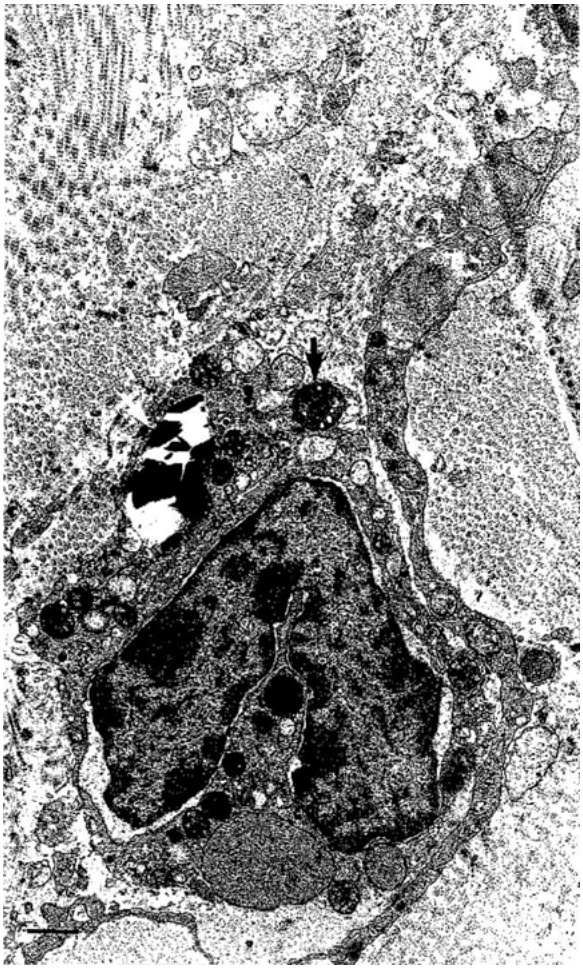

Figure 1 A macrophage with intracellular TCP-particles (white arrow) is seen. Lysosomes are found encircling the TCP-particles and in one lysosome remnants of TCP (arrow) is seen. Original magnification × 5000, bar 1 μm.

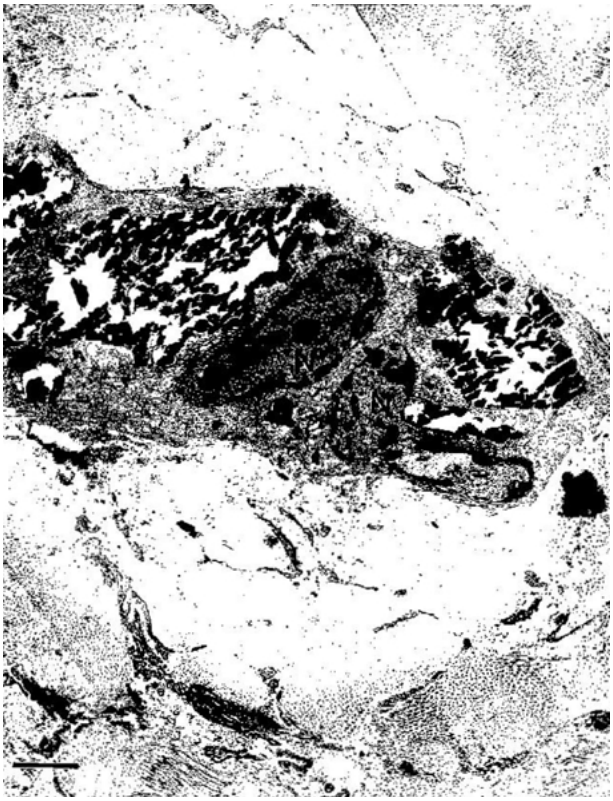

Figure 2 A giant cell, which has TCP-particles intracellularly is seen. Also two nuclei (N) can be observed. Original magnification × 3000, bar 2 μm.

Twenty-four-week follow-up

TCP-particles were distributed in groups similar to those at the earlier follow-up points. However, the shape of TCP-particles did not markedly change. Remnants of TCP-particles were found intracellularly in some macrophages, in which resorption of the TCP-particles had progressed further than in others. They were seen as dark, round inclusions. Copolymer devoid of TCP was seen in one sample. Fibroblast cell extension was encircling the liquid filled polymer cavity, a process not found in earlier follow-up. Fibroblasts and mononuclear inflammatory cells, probably macrophages were found adjacent to the copolymer (Fig. 5).

Fifty-two-week follow-up

TCP-particles were still found in all samples and their shape had changed further. The particles were round, dark and small, with size of approximately 0.7 μm. They were found mostly inside the macrophages and in some samples these TCP-particles were partially disintegrated and occasionally seen as small granules inside the lysosomes (Fig. 6). Cell extension was still lining the cavity of the copolymer. In contrast to the previous

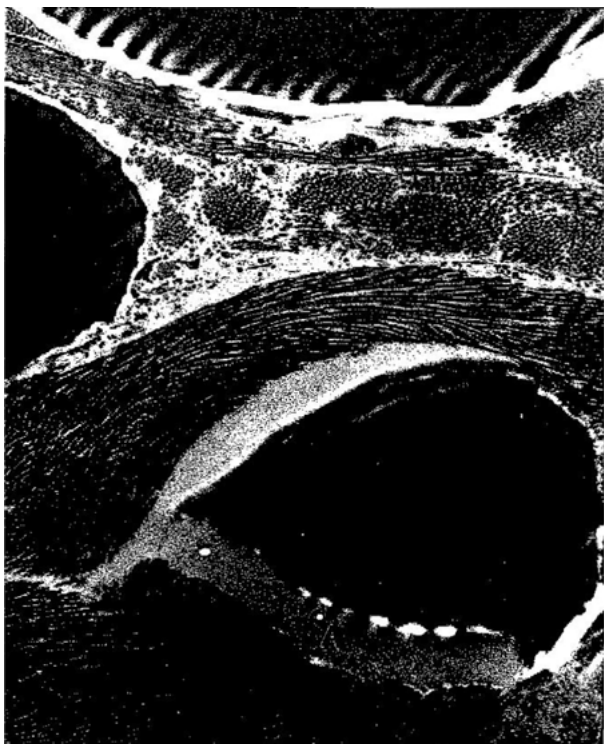

Figure 3 Copolymer is found without TCP. No inflammatory reaction is seen. The empty space between the collagen fibers (CF) and the copolymer is probably an artefact (A). Original magnification × 4000, bar 2 μm.

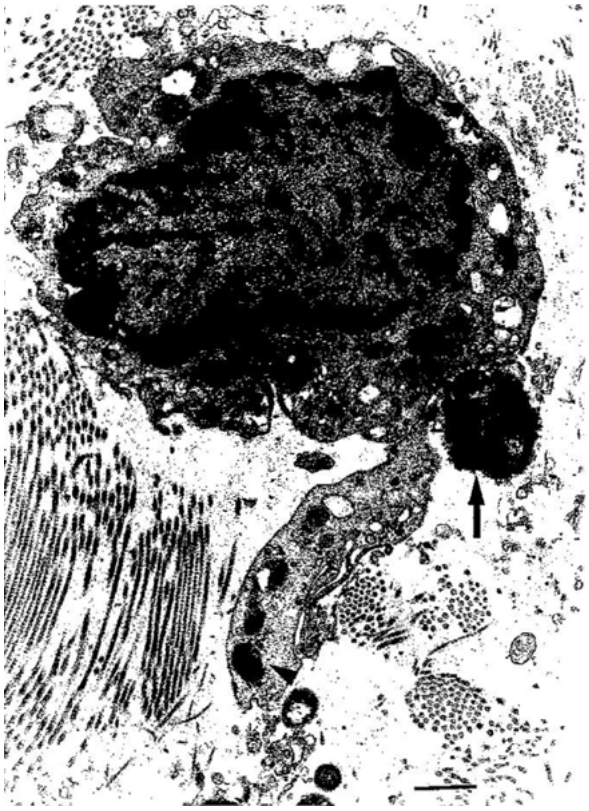

Figure 4 A macrophage with nucleus and an extension of possibly macrophage are seen. An extracellular TCP-particle (arrow) is in close contact with the macrophage. TCP-particles with varying degree of degradation are also seen inside of macrophages (arrowheads). Original magnification × 6000, bar 1 μm.

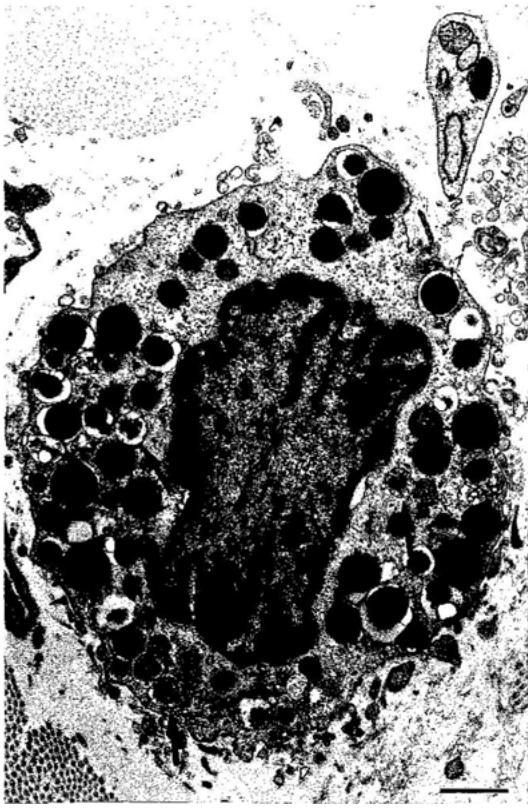

Figure 6 TCP-particles can be seen inside of mononuclear inflammatory cell, probably a macrophage. The shape of the particles is round possibly due to progression of the degradation. Degraded particles are also observed in the lysosomes (arrows). Original magnification × 6000, bar 1 μm.

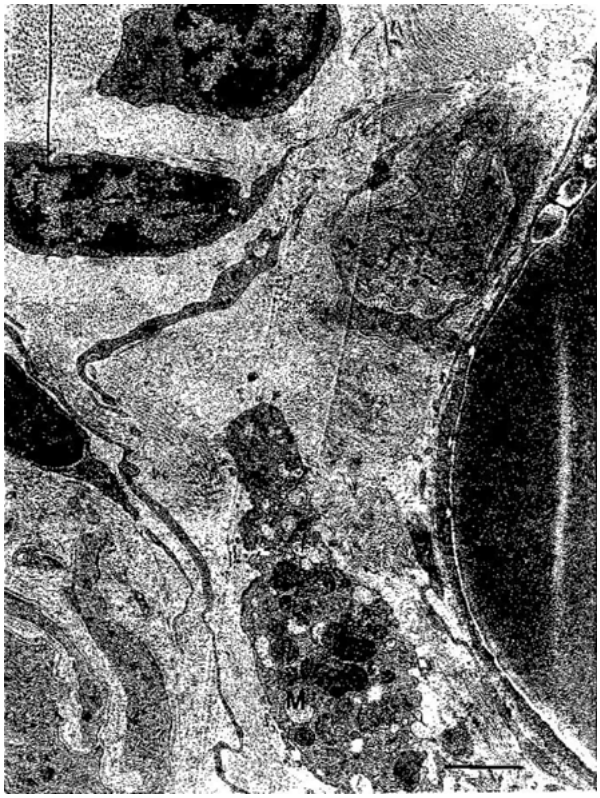

Figure 5 Copolymer is seen on the right side with cell extension lining it. Fibroblasts (F) and possibly extension of macrophages (M) are adjacent to the copolymer cavity. Lysosomes and TCP-particles are seen inside the macrophage. Original magnification × 4000, bar 2 μm.

follow up points eosinophilic leukocytes were now occasionally seen for the first time, located in the vicinity of the copolymer material (Fig. 7).

Discussion

This study was carried out to evaluate the transmission electron microscopical (TEM) findings during resorption of copolymer of 27/73 w/w mixture of P(ε-CL/DL-LA) and TCP. Very few TEM-studies of biodegradable materials have been published in the English literature earlier. Bergsma *et al*. [16] implanted poly-L-lactide capsules subcutaneously in rats and studied their degradation by transmission electron microscope. They found that the phagocyting macrophages and endoplasmic reticulum had a normal appearance. There were no signs of cell damage, which is in agreement with our earlier studies of composites of P(ε-CL/DL-LA) and TCP. In our study the ultrastructure of connective tissue was normal during the whole observation period. The copolymer did not elicit any inflammatory reaction during the first 14 weeks of observation. Fibroblasts and mononuclear inflammatory cells, probably macrophages, were found adjacent to the copolymer after 24 weeks of follow up. One of the main findings of the present study was that eosinophils were found in the vicinity of the copolymer material. Previously, we have been able to find eosinophilic leukocytes from connective tissue nearby the implanted copolymer both from the dermal

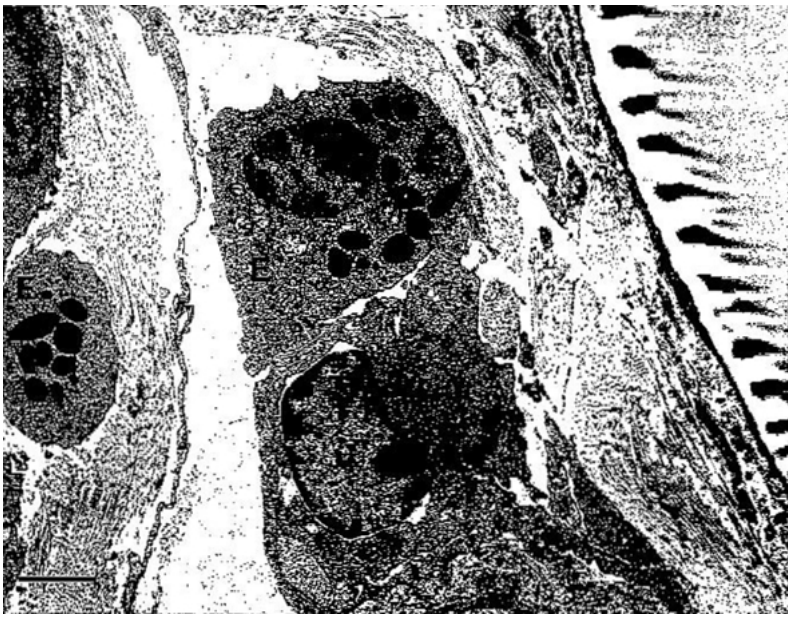

Figure 7 A cell extension is lining the copolymer cavity, no TCP-particles are visible, eosinophilic leukocytes (E) are in the vicinity of the copolymer. Original magnification × 3000, bar 2 μm.

specimens of rabbits and from muscle specimens of rats also in our former studies [14, 17]. Eosinophilic leukocytes were present from the second week until one year of follow up in the muscle specimens of rats. In the dermal specimens of rabbits we have been able to find them from the second week until the 18th week of observation. This finding suggests a role of eosinophils in the inflammatory reaction in the vicinity of the implanted material and is in agreement with previous studies [9, 18]. The inflammatory reaction in sheep soft tissue was mild. Mononuclear inflammatory cells, macrophages, multinucleated giant cells and fibroblasts were found adjacent to the implanted material, which is in line with former studies [1–6, 8–10]. TCP-particles and the copolymer were found to be separated from each other after the 14-week follow up time. The shape of TCP-particles, which were found mostly intracellularly, changed from angular or rod shape to round inclusions, but it seemed that the morphology of copolymer did not change at all during the follow up of 52 weeks. According to the present study, the inflammatory reaction seemed to be very mild and the copolymer with TCP well tolerated.

Eosinophils were found from the 52nd week of follow-up, located in the vicinity of the copolymer material. Eosinophils are granulated leukocytes, which usually have a protective role against infections in host immunity. An eosinophil consists of major basic protein, eosinophil peroxidase, eosinophil cationic protein and eosinophil-derived neurotoxin. Eosinophils are also a source of many growth factors and cytokines. These proteins, which are stored in granules, are cytotoxic for many cells and can initiate and sustain an inflammatory reaction in host tissue. It is a well known fact that the number of eosinophils is increased in many allergic diseases, especially in asthma [19].

Today several fractures and osteotomies can be fixed with biodegradable devices instead of metallic devices. The polymers most often used are poly (lactic acid) (PLA) and poly (glycolic acid) (PGA). However, abacterial inflammatory reactions have been noticed after the clinical introduction of these devices. Both swelling and pain at the site of implantation have also been reported. The etiology of this inflammatory reaction is still unknown, despite of numerous studies [20–25]. The presence of eosinophils, described in this study, in the vicinity of the biodegradable material and their cytotoxic granule proteins might explain some of the undesired reaction.

In oral- and maxillofacial-surgery there are multiple different causes for loss of bone including cancer, benign tumor, cysts of jaws, trauma, congenital deformities and periodontal lesions in teeth. Bone autotransplantations are normally used to fill bony defects. However, there are some disadvantages in their use, including donor site morbidity, need for a second operative site, increased risk for infection and limited graft supply, especially in children. Problems connected with use of allografts include transmission of viral infection and poor immunological compatibility. For the oral and maxillofacial surgeon, a synthetic biomaterial would be ideal to fill the bony defect. A copolymer of ε-caprolactone–lactide with TCP in paste form is very easy to handle and can be molded into different shapes in room temperature. The supply is not limited either. The results presented here encourage us to continue our studies to develop a filling material for bone defects.

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