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Applying a calcium phosphate layer on PEO/PBT copolymers affects bone formation *in vivo*

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Recently a copolymer (Polyactive^R) has been introduced that combines elastomeric and bone-bonding properties. Since calcification of the copolymer is a prerequisite for bone bonding, Polyactive was precalcified *in vitro* in order to increase the bone-bonding rate. Precalcification was performed by subsequent incubation in Ca and P solutions and resulted in formation of a hydroxyapatite layer on the surface of the implant. Within one week after implantation this layer had disappeared from the surface and a new calcification zone was formed under the surface of the copolymer. Longer implantation periods showed that in precalcified implants bone was apposited along the walls of the pores, while in control implants new bone was first formed in the centre of the pores. Consequently, the percentage of bone contact was increased in precalcified implants, however, the amount of bone ingrowth was equal in both control and precalcified implants. Transmission electron microscopy showed the presence of an electron-dense layer at the bone implant interface, which was indicative for bone-bonding. It is concluded from these experiments that precalcification of PEO/PBT copolymers affected the direction of bone apposition and increased the bone-bonding rate.

1. Introduction

Polyactive is a bone-bonding copolymer that is composed of a soft poly(ethylene oxide) (PEO) and a hard poly(butylene terephthalate) (PBT) segment [1, 2]. A series of copolymers with different mechanical and biological characteristics can be synthesized by varying the PEO/PBT ratio and the molecular weight of the PEO segment. Calcification is one of the most important properties of PEO/PBT copolymers. A positive relation was observed between increasing PEO/PBT ratios and/or the use of PEO with higher molecular weight and the calcification rate [3, 4]. Studies by Okumura *et al.* demonstrated that bone formation according to the osteogenesis theory was restricted to calcified areas in the copolymer [5]. Other implantation studies in rat and goat cortical bone showed, using transmission electron microscopical (TEM) techniques, the presence of an electron-dense layer at the interface between PEO/PBT 55/45 (PEO-MW 1000D) copolymers, which is indicative for bone-bonding [3, 4].

The exact bone-bonding mechanism of these copolymers is not fully understood as yet, but it resembles the bone-bonding mechanism of conventional bioactive materials [6, 7], although initially no calcium or other ions are present in the copolymer. The hydrogel characteristics of the PEO/PBT copolymers allow absorption of body fluids and calcium ions are specifically complexed within the helical structure of the PEO segment [8, 9] and can precipitate with phosphate ions into calcium phosphate crystals. This calcification phenomena was also shown for other hydrogel polymers like poly(HEMA) [10, 11] and Polyurethanes [8, 9, 12–16]. It is assumed that calcium is released from the calcified areas and precipitates with phosphate ions into an apatite-like layer at the interface. A continuity between newly formed bone and the calcified areas in the PEO/PBT copolymer is thus created.

Since calcification of the copolymer is a prerequisite for bone bonding this will be the rate-limiting step in the bone-bonding process. The aim of this study was to improve the bone-bonding characteristics of a PEO/PBT 55/45 (PEO-MW 1000D) copolymer by calcification of the implants prior to implantation. Precalcification was performed by subsequent incubation of the copolymer in calcium and phosphate solutions. In this study the effect of precalcification of the PEO/PBT 55/45 (PEO-MW 1000D) copolymer on bone formation *in vivo* was tested.

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2. Materials and methods

2.1. Precalcification procedure

Dense pressed plates or porous implants of PEO/PBT 55/45 (PEO-MW 1000D) (HC Implants, Leiden, The Netherlands) copolymers were incubated in a calcium solution (1 M $CaCl_2$) for 3 days at room temperature. Then the copolymers were briefly rinsed with distilled water and dried at 37° C. Precalcification was achieved by subsequent incubation in a phosphate solution (1 M Na_2HPO_4) for 3 days at room temperature. Control PEO/PBT 55/45 (PEO-MW 1000D) copolymers were incubated in distilled water according to the same incubation schedule. Finally, all copolymers were briefly rinsed in distilled water, dried and sterilized by gamma irradiation.

The presence of calcified areas in the copolymers after the precalcification procedure was detected by calcium-specific Alizarin red staining on sections of copolymers embedded in glycol methacrylate (GMA). Porous implants were checked for the presence of the calcium phosphate layer with backscattered electron microscopy. Elemental analysis of the crystals was performed by X-ray microanalysis on carbon-coated samples.

Small parts of precalcified copolymers plates were rinsed in 0.5 M NH_4Cl in phosphate-buffered saline (PBS), dehydrated through a graded series of dimethyl formamide (DMF) and embedded in Lowicryl (KM 4) resin under U.V. light exposure at room temperature for 3 days. Selected area diffraction patterns of the calcium phosphate crystals were obtained by studying unstained sections in a JEOL Jem 100 CX transmission electron microscope working at 100 keV and 46 cm camera length. After calibration of the microscope using evaporated aluminium, the lattice spacings (d) of the crystal were calculated and compared with X-ray diffraction patterns of known calcium phosphate crystals.

2.2. Implantation procedure

Male Wistar rats (300 g) were anaesthetized using Hypnorm (0.1 ml per 100 g body weight) and one cylindrical hole of 2.1 mm diameter was drilled in each femur. Per survival time six porous plugs (diameter 2 mm) of precalcified and control PEO/PBT 55/45 (PEO-MW 1000D) copolymers were implanted. The animals were killed after 1, 2, 3 and 4 weeks and the implants were fixed in 1.5% glutaraldehyde in 0.14 M cacodylate buffer and dehydrated through a graded series of alcohol. Per material and survival time four implants were embedded in methyl methacrylate (MMA). Bone ingrowth, bone contact and calcification in the porous implants were studied on backscattered images of carbon-coated polished MMA embedded implants. A VIDAS image analysis system was used to determine the percentage of bone ingrowth and bone contact in the porous implants. The other two femur implants were decalcified in 10% EDTA, dehydrated and embedded in Lowicryl for transmission electron microscopical evaluation of the interface between the copolymer and bone.

3. Results

3.1. Precalcified implants

Light microscopy (Fig. 1) of dense plates of precalcified PEO/PBT 55/45 (PEO-MW 1000D) copolymers stained with Alizarin red showed the presence of a calcium-containing layer on top of the surface. This layer seemed to be in close contact with the smooth surface of the copolymer and had an irregular outer surface. The layer had a variable thickness of approximately 2–20 μm. Backscattered images of porous precalcified implants showed that a thin, mostly continuous, coating was present on the surface of the implant pores. Large precipitates, not continuous with the coating, were found in some pores in the centre of the implant (Fig. 2). X-ray microanalysis performed on different spots on the coating and the precipitates revealed the presence of calcium, phosphorus and a small amount of potassium (Fig. 3). Transmission electron microscopy on unstained ultrathin sections of dense precalcified copolymers showed that the calcium phosphate layer was composed of small electron-dense crystals (Fig. 4). Table I shows that there is reasonable agreement between the two sets of d-

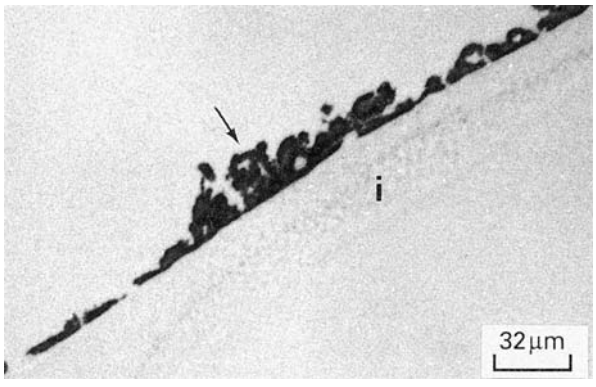

Figure 1 Light micrograph of a section of dense plates of precalcified PEO/PBT 55/45 (PEO-MW 1000D) copolymer. Note the Alizarin-red positive calcium-containing layer (arrow) on top of the implant surface (i).

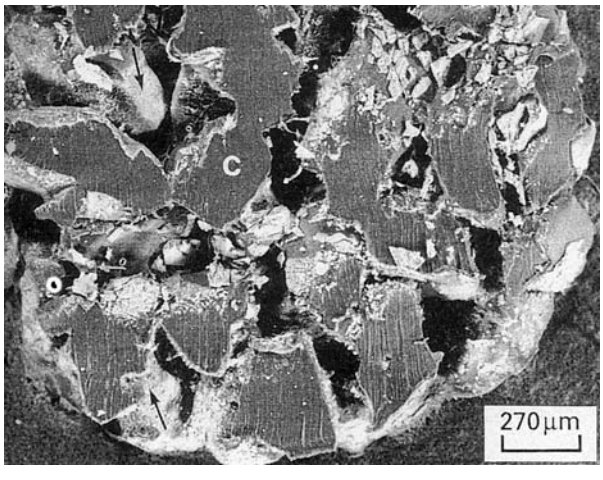

Figure 2 Backscattered electron (BSE) image showing a precalcified porous implant on cross-section. Arrows indicate the calcium phosphate coating on the surface of the copolymer (c). Arrowheads mark the irregular formed calcium phosphate deposits in the centre of the pores.

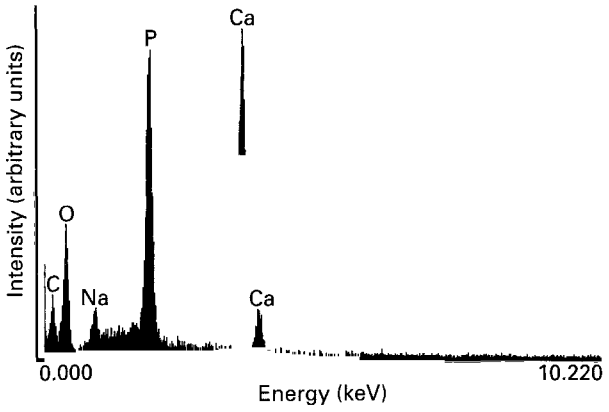

Figure 3 X-ray microanalysis of the calcium phosphate coating and deposits in the precalcified implant shown in Fig. 2.

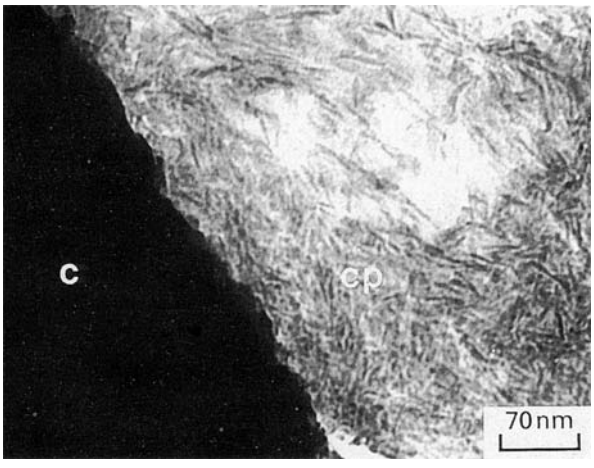

Figure 4 Transmission electron micrograph of the calcium phosphate layer on top of the copolymer (c) showing the presence of electron dense calcium phosphate crystals (cp).

TABLE I Lattice *d*-spacings (nm) measured from the selection area diffraction pattern of the calcium phosphate layer on top of the surface of a precalcified PEO/PBT 55/45 copolymer (see Fig. 4). A reference of *d*-spacings of HA has also been included for comparison

Measured *d* (nm)	HA *d* (nm)
–	0.817
0.375	0.388
0.344	0.344
0.307	0.308
0.245	0.253
0.217	0.215
0.198	0.200
0.189	0.189
0.161	0.161
0.138	–

spacings, confirming that the calcium phosphate crystals in this layer were highly similar to hydroxyapatite.

3.2. Bone reactions

Backscattered images of both control and precalcified PEO/PBT copolymers showed that after 1 week of implantation new bone was growing in the pores of the implants. In precalcified implants the initial present calcium phosphate coating on top of the surface and the precipitates in the pores had disappeared after 1 week of implantation. Instead, an approximately 15 μm thick calcium phosphate layer was formed under the surface of the copolymer (Fig. 5). After longer implantation periods bone was mainly apposited along the walls of the pores without the presence of an intervening fibrous tissue layer (Fig. 6a and 6b).

In control implants small calcification areas were formed under the surface of the pores after 3 weeks of implantation. New bone was first formed in the centre of the pores and usually a fibrous tissue layer was observed between the implant and bone, although some areas of bone contact were observed from 2 weeks implantation on (Fig. 7a and 7b). Longer implantation periods resulted in an increase in bone-contacting areas.

On first sight implantation of precalcified copolymers induced more bone contact than control implants, although the amount of bone ingrowth in the pores was almost equal for both implants. These observations were supported by quantitative analysis of the bone ingrowth (Fig. 8) and bone contact percentages (Fig. 9) in the implants. These findings indicate that precalcification influences the direction, but not the amount of bone ingrowth in the pores.

Transmission electron microscopy (Fig. 10) of decalcified sections showed that mineralized bone matrix was in close contact with the copolymer surface. A single electron-dense layer was observed at approximately 0.6 μm under the surface of the copolymer.

4. Discussion

In vitro calcification experiments with polyurethanes demonstrate their ability to complex calcium ions from solutions within the helical structure formed by the PEO segment [8, 12–16]. Thoma *et al.* indicated that hydrophobic anions, like phosphate, are easily extractable by the complexed calcium ions and can form calcium phosphate precipitates [17]. Based on

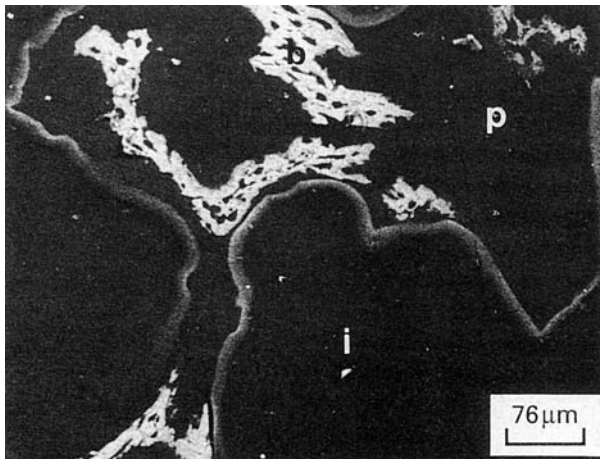

Figure 5 BSE image showing the approximately 15 μm calcium phosphate layer (arrow) under the surface of the precalcified implant (i) after 1 week implantation in bone. New bone (b) is growing in the centre of the pores (p).

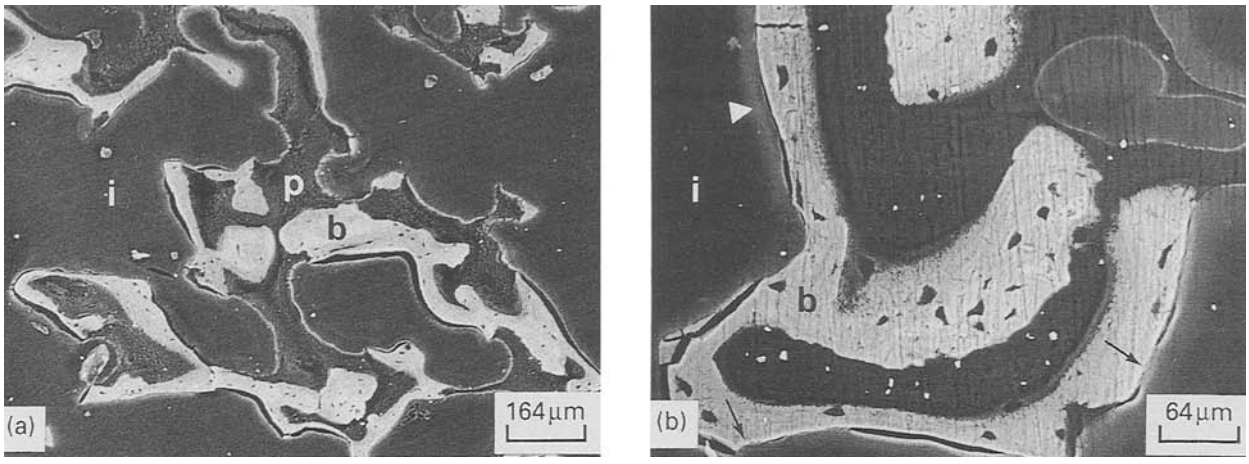

Figure 6 (a) BSE image showing bone ingrowth in precalcified implants (i) at 3 weeks. Note that bone (b) is mainly apposited at the walls of the implant pores (p). (b) Higher magnification showing bone apposition along the walls of the pores. Arrows mark the intimate contact between bone (b) and the implant (i) surface. Arrowheads indicate the calcification zone under the surface of the implant.

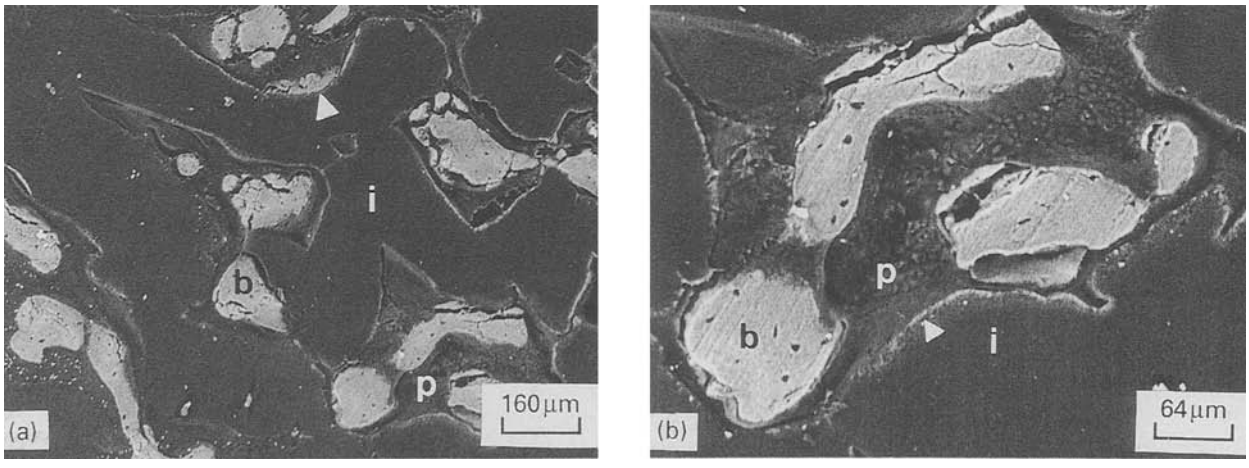

Figure 7 (a) BSE image showing bone ingrowth in control implants (i) at 3 weeks. Note that bone (b) is generally formed in the centre of the pores (p). Arrowheads mark contact area between bone and the implant. (b) Higher magnification showing bone (b) apposition in the centre of the pores (p) without contact with the implant surface (i). Arrowheads indicate calcification areas in the implant.

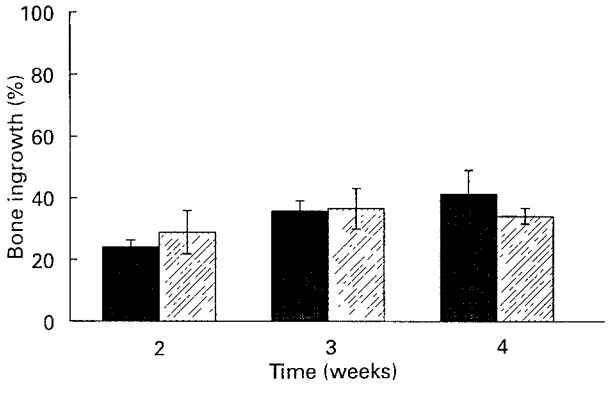

Figure 8 Quantitative diagram showing the percentage of bone ingrowth in the pores of (■) control and (▨) precalcified implants at 2, 3 and 4 weeks.

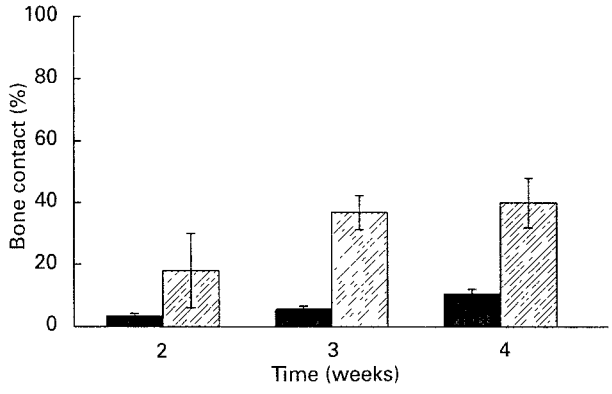

Figure 9 Quantitative diagram showing the percentage of bone contact with the implant surface of (■) control and (▨) precalcified implants at 2, 3 and 4 weeks.

these experiments PEO/PBT copolymers are expected to complex calcium ions from solutions and to form calcium phosphate deposits after subsequent incubation in phosphate solutions. Previous studies showed that, depending on the pH of the phosphate solution, calcium phosphate was precipitated on top (high pH) or approximately 10 μm under (low pH) the surface of the copolymer, which was probably due to differences in precipitation rate of calcium phosphate crystals in an acid or basic environment [18, 19]. In the study described here PEO/PBT 55/45 (PEO-MW 1000D) copolymers were used with calcium phosphate pre-

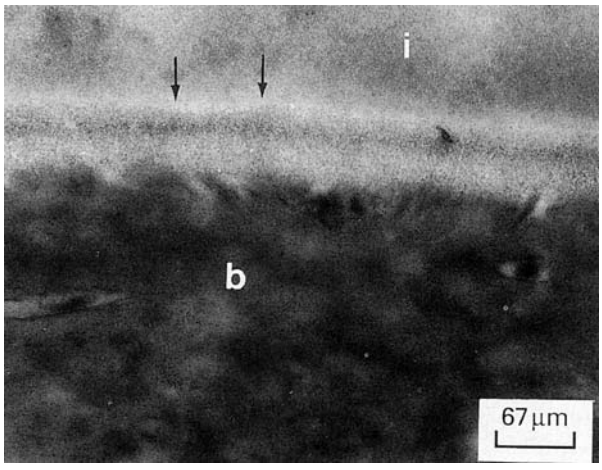

Figure 10 Transmission electron micrograph of a decalcified section of the interface of a precalcified implant at 2 weeks. Bone (b) is present at the surface of the implant (i). Arrows mark the electron dense layer under the surface of the implant.

cipitation on top of the surface. Selected area diffraction analysis showed that this calcium phosphate layer was composed of hydroxyapatite crystals.

Pollock *et al.* demonstrated that the presence of calcium in polyurethanes prior to implantation resulted in an accelerated calcification during *in vivo* implantation [20]. The present study showed that the calcium phosphate depositions on the surface of the precalcified implants disappeared after one week and a calcified zone under the surface of the copolymer was formed. These findings indicate that the calcification rate of PEO/PBT copolymers *in vivo* was increased by the initial presence of a calcium phosphate layer on the surface of the implant. This calcium phosphate layer will probably dissolve causing an initial increase of the local Ca and P concentrations to supersaturation levels, and is followed by reprecipitation of calcium phosphate crystals, which is a general phenomenon described for bioactive materials [6].

Implantation of precalcified and control PEO/PBT 55/45 copolymers in rat cortical bone in this study showed an equal amount of bone ingrowth. However, the percentage of bone contact was much higher for precalcified implants. This indicates that the presence of a calcium phosphate layer on the surface of the implant affects the direction of bone apposition. Studies by Okumura *et al.*, using an ectopic bone formation model, showed that implantation of PEO/PBT 55/45 copolymers showed first signs of bonding osteogenesis after 4 weeks of implantation [5]. Results in the present study indicate that centripetal bone apposition started after only 2 weeks of implantation in precalcified PEO/PBT 55/45 copolymers.

In general we can conclude from these experiments that PEO/PBT 55/45 (PEO-MW 1000D) copolymers are bone-bonding biomaterials, although initially no calcium or other ions were present. Applying a calcium phosphate layer on PEO/PBT copolymers prior to implantation changes the direction of bone apposition and enhances the bone-bonding rate.

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