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Pre-operative addition of calcium ions or calcium phosphate crystals to PEO/PBT copolymers (Polyactive™) stimulates bone mineralization *in vitro*

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The effect of the pre-operative addition of calcium ions or calcium phosphate to bioactive PEO/PBT copolymers (Polyactive™) on bone-bonding characteristics was evaluated *in vitro*. Atomic absorption spectroscopy was used to determine variations in the calcium concentration in the culture medium due to calcium release from the pretreated copolymers. Bone marrow cells were cultured on the copolymers for 2 and 3 weeks. Differences in formation rate and mineralization of bone nodules were analysed using LM and TEM techniques. Formation of bone nodules was observed after 2 weeks on non-treated copolymers. However, no mineralization in these bone nodules could be detected during the 3 weeks of this experiment. Addition of calcium ions to the copolymers initially increased the calcium concentration of the culture medium and led to formation of mineralized nodules after 2 weeks. The two precalcified copolymers had different effects on the calcium concentration of the culture medium. Instead of nodules, two or three continuous cell layers were formed and mineralization was observed after 2 weeks. Longer culture periods resulted in more mineralization in close contact with the copolymer surface. Based on the results of this experiment we conclude that the pre-operative addition of calcium ions or calcium phosphate to PEO/PBT copolymers stimulates mineralization in bone marrow cell cultures, which is an indication for better bone-bonding characteristics *in vitro*

1. Introduction

It is known that most bone-bonding biomaterials contain calcium ions and that the release of these ions plays a crucial role in the bone-bonding process. Calcium release from these implants is followed by reprecipitation with phosphate ions that are present in the body fluids, and leads to formation of an apatite-like layer at the interface [1–5]. This layer acts as a continuity between the calcium phosphate crystals in the implant and in bone. This bone-bonding mechanism has been described for several calcium phosphate ceramics [3, 6–8], glasses [9, 10] and glass ceramics [5, 9, 11–13]. Recent reports describe a similar bone-bonding mechanism for the elastomeric copolymer Polyactive™ [14–17].

Polyactive™ is a poly(ethylene oxide) (PEO)/poly(butylene terephthalate) (PBT) segmented block copolymer (Polyactive™) which differs from the other bioactive biomaterials in that it lacks the initial presence of calcium, phosphorus or other ions known to play a role in the bone-bonding process. Various implantation studies have demonstrated that calcium phosphate crystals can spontaneously precipitate in the copolymers. Radder *et al.* showed, using X-ray diffraction techniques, that the calcification formed during implantation was mainly composed of carbonated apatite [18]. Implantation studies in rat and goat cortical bone using transmission electron microscopy, revealed that bone-bonding, characterized by the presence of an electron dense layer at the bone /biomaterial interface, was observed only related to calcified areas of the copolymers. In non-calcified areas the bone was usually separated from the implant by a fibrous tissue layer. These studies indicated that calcification of the copolymer is a prerequisite for bone-bonding [17, 19–22].

The exact calcification mechanism of PEO/PBT copolymers has not completely been unravelled as yet, but starts with absorption of body fluids due to the hydrogel characteristics of the PEO/PBT copolymers. Studies with PEO- or PTMEG-based poly(ether)-urethanes indicated that the ether segments are able to specifically complex calcium ions from solutions [23, 24]. We assume that a similar complexation of calcium ions can take place in the PEO segment of PEO/PBT copolymers. The phosphate ions present in the body fluids can subsequently precipitate with the complexed calcium ions within the copolymer matrix. The importance of the PEO segment in the calcification mechanism was confirmed by several *in vitro*

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studies with a series of copolymers with increasing PEO/PBT ratio and PEO molecular weight, which resulted in an increase in both water uptake and the amount of calcification [19, 25].

In view of the above, it can be concluded that the post-operative presence of calcium phosphate in PEO/PBT copolymers, and in or on top of bioactive materials in general, is crucial for the bone-bonding process. Although the PEO/PBT copolymers already spontaneously calcify during implantation, we were interested to see if the pre-operative addition of calcium ions or calcium phosphate crystals to PEO/PBT copolymers would further stimulate calcification *in vivo* and as a result the bone-bonding rate. In order to obtain the maximum effect of the different pretreatments we decided to use PEO/PBT 55/45 copolymers that showed relatively slow calcification and bone-bonding characteristics in previous implantation experiments [19].

We recently described methods either to add calcium ions to PEO/PBT copolymers or to induce calcium phosphate depositions in PEO/PBT copolymers *in vitro*. These "precalcification" methods are based on the water uptake and calcium complexing capacity of the copolymers. Incubation in calcium-containing solutions of increasing concentrations resulted in copolymers with higher amounts of calcium ions. Subsequent incubation in phosphorus - containing solutions resulted in calcium phosphate depositions located either on the surface (surface precalcification) or as a continuous 10 μm thick layer underneath the surface in the copolymer matrix (matrix precalcification). In order to make a first selection of the most promising treatments for stimulation of bone-bonding we compared the behaviour of different pretreated copolymers in a cell culture system. We used the *in vitro* bone-forming system described by Maniatopoulos *et al.* [26], in which mineralized nodules are formed on bioactive substrata showing similar interface structures to those observed in bone.

2. Materials and methods

2.1. Precalcification of PEO/PBT 55/45 copolymers

In all experiments 2 mm thick dense compression moulded plates of Polyactive™ with a 55/45 PEO/PBT proportion and PEO molecular weight of 1000 Dalton were used (HC Implants, Leiden, The Netherlands). Precalcification was performed according to previously described methods [25].

Calcium-enriched Polyactive™. Copolymers were incubated in calcium chloride solutions for two periods of 3 days. We used different concentrations of calcium chloride solutions (0.1 M, 1 M and 3 M $CaCl_2.2H_2O$) in order to increase the amount of calcium in the copolymers.

Precalcified Polyactive™. Copolymers were first incubated in a calcium-containing solution for 3 days followed by incubation in a phosphate containing solution for 3 days. In between and after the two incubation periods all copolymers were briefly rinsed with distilled water and dried at 37 °C. Two combinations of calcium- and phosphate-containing solutions were used. The 3 M $CaCl_2.2H_2O$/6 M $NaH_2PO_4.H_2O$ (pH 4) combination resulted in matrix precalcification and the 1 M $CaCl_2.2H_2O$/1 M $Na_2HPO_4.2H_2O$ (pH 10) combination in surface precalcification.

Control Polyactive™. Copolymers were incubated in distilled water for two periods of 3 days.

2.2. Bone marrow cell culture

Rat bone marrow cells were isolated from the femora of 100–120g male Wistar rats according to the method described by Maniatopoulos *et al.* [26]. The femora were removed and washed three times in α-minimal essential medium (α-MEM-RNA/DNA, Gibco) containing antibiotics in a ten times higher concentration than usual (see below). After removal of the epiphyses the bone marrow was flushed out of the femora using 15 ml α-MEM that contained 15% foetal calfs serum (FCS, Gibco), 0.1 mg/ml penicillin G (Boehringer-Mannheim), 50 μg/ml gentamycin (Gibco), 0.3 μg/ml fungizone (Gibco) and freshly added 50 μg/ml ascorbic acid (Gibco), 10 mM Na-β-glycerophosphate (Sigma) and 10^{-8} M dexamethasone (Sigma). The marrow cells of both diaphyses were collected in 50 ml plastic tubes (Costar) and the cell suspension was resuspended by careful aspiration in a 25 ml syringe with a 20 G needle.

Droplets of 200 μl of the cell suspension were placed on the different samples and incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, to allow cell attachment to the substrate. After 4 h, 1 ml of culture medium was added to each well and the samples were placed in an incubator. The cells were refed after 2 days with freshly prepared medium and then refed every other day. At day 14 and 21 cells were washed in PBS, fixed in 1.5% glutaraldehyde in 0.14 M Na-cacodylate buffer pH 7.4 for 1 h at room temperature.

2.3. Transmission electron microscopy

After fixation the cells were rinsed three times in phosphate - buffered saline (PBS) and subsequently post-fixed in a freshly prepared aqueous solution of 1.5% potassium ferrocyanide and 1% OsO_4 for 16 h at 4 °C. The samples were dehydrated through a graded series of alcohol and routinely embedded in Epon. Semi-thin ($\pm$ 2 μm) sections were prepared and stained with toluidine blue in order to study nodule formation in the cell layers on the sample. Calcium-specific Alizarin red staining was used to detect mineralization in the cell cultures. Next, ultra-thin sections were processed on a LKB ultramicrotome using a diamond knife. The sections were routinely stained with uranyl acetate and lead hydroxide and examined in a Philips EM 201 transmission electron microscope working at an accelerating voltage of 80 kV.

2.4. Calcium concentration in culture medium

In order to measure the calcium release from the different samples we incubated supplemented α-MEM

in the absence of cells on the samples and followed the culture conditions as described above. Every other day the medium was collected in a 2.5 ml tube and stored at − 20 °C. The calcium concentration in the medium was determined using atomic absorption spectrometry (Varian SpectrAA 300/400). The experiments were performed in triplicate and the average of the samples was calculated. The calcium concentration in the culture medium was plotted accumulatively over 21 days. All data were normalized to the calcium concentration present in standard supplemented α-MEM culture medium exposed to similar culture conditions of tissue culture polystyrene (TCPS).

3. Results

3.1. Calcium release in culture medium

The calcium release from the PEO/PBT copolymers (Polyactive™) was analysed using atomic absorption spectrometry (AAS). Fig. 1 shows the accumulative calcium release in culture media of the control, calcium-enriched and precalcified PEO/PBT copolymers normalized to the calcium concentration in standard supplemented α-MEM medium. Only minimal fluctuations in the calcium concentration in the culture medium (approximately 12 ppm) in the presence of control copolymers were observed (Fig. 1a and 1b).

Copolymers incubated in solutions with a low calcium chloride concentration released only 14 ppm of calcium during the first culture day. Copolymers incubated in the other two more concentrated calcium chloride solutions initially released high amounts of calcium (220 and 1050 ppm, respectively), although the calcium concentration in the culture medium remained at a constant level after the third day (Fig. 1a).

The two precalcification methods of the copolymers had different effects on the calcium concentrations of the culture media. Matrix precalcified copolymers released a total amount of 50 ppm calcium during the 3 weeks of the experiment. Surface precalcified copolymers, however, induced a decrease of approximately 100 ppm in the calcium concentration of the culture media during the first 7 days. This decrease slowly continued during the rest of the experiment with a total amount of 135 ppm calcium (Fig. 1b).

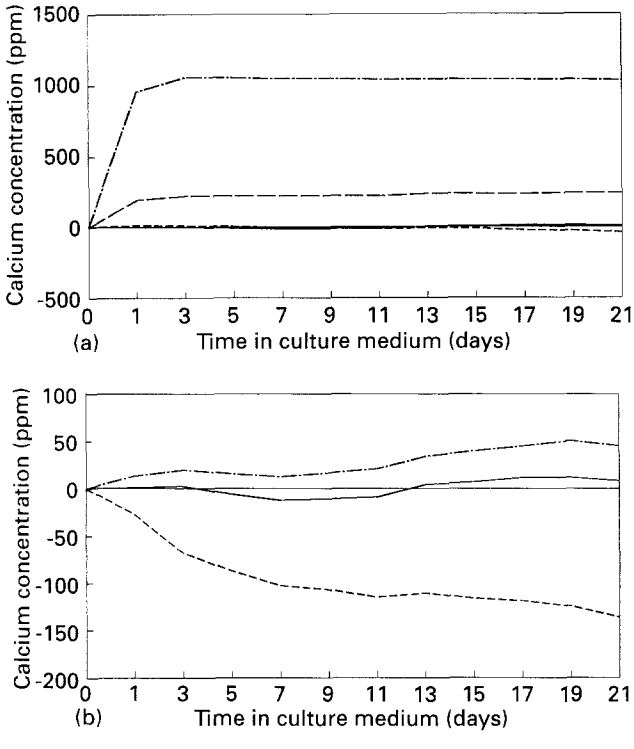


Figure 1 Atomic absorption data showing the accumulative calcium release from (a) control and calcium-containing PEO/PBT copolymers and (b) control and both precalcified PEO/PBT copolymers over 21 days. The released calcium is normalized to the calcium concentration in standard supplemented culture medium (a) (—— control; - - - low; — — medium; —·— high). (b) (—— control; - - - low; — — surface; —·— matrix).

3.2. Morphology of control bone marrow cell cultures

Light microscopy on control bone marrow cell cultures on tissue culture polystyrene (TCPS) without the presence of a biomaterial showed nodule formation at 2 weeks. Mineralization of these nodules was first observed in the 3-week culture period, using calcium-specific Alizarin red staining (Fig. 2). Analysis of the culture medium with AAS showed a decrease in the calcium concentration of the culture medium after 15 days, indicating precipitation of calcium ions during the mineralization process (Fig. 3). Transmission electron microscopy (TEM) demonstrated that the nodules were composed of cells surrounded by an extracellular matrix (ECM) that contained collagen fibres characterized by a banding pattern. The cells in the unmineralized part of the ECM were morphologically similar to osteoblasts, since they showed a well-developed cytoplasm, rich in endoplasmatic reticulum, Golgi complexes, mitochondria, intracellular glycogen and fat inclusions. These osteoblast-like cells were often separated from the mineralized ECM by an unmineralized, osteoid-like layer composed of collagen. The crystals in the mineralized ECM were more electron dense at the osteoid interface than closer to the substratum (Fig. 4).

3.3. Evaluation of materials

The results of the bone marrow cell cultures on the different Polyactive™ samples were compared using light microscopical and TEM techniques and are summarized in Fig. 2. One to three continuous cell layers were formed on all copolymers, except on the materials that were incubated in the calcium chloride solution having the highest concentration. TEM showed that the cells present on this material did not adhere to the substratum, the cell membrane was disrupted, intercellular structures could not be distinguished and calcium phosphate precipitates were observed.

Non-treated, control PEO/PBT copolymers and copolymers incubated in low (0.1 M $CaCl_2$) calcium chloride solutions both showed nodule formation (Fig. 2), although no mineralization of the extracellular matrix was observed during the 3-week culture periods (Fig. 5).

Bone marrow cell cultures on PEO/PBT copolymers incubated in intermediate (1 M $CaCl_2$) calcium

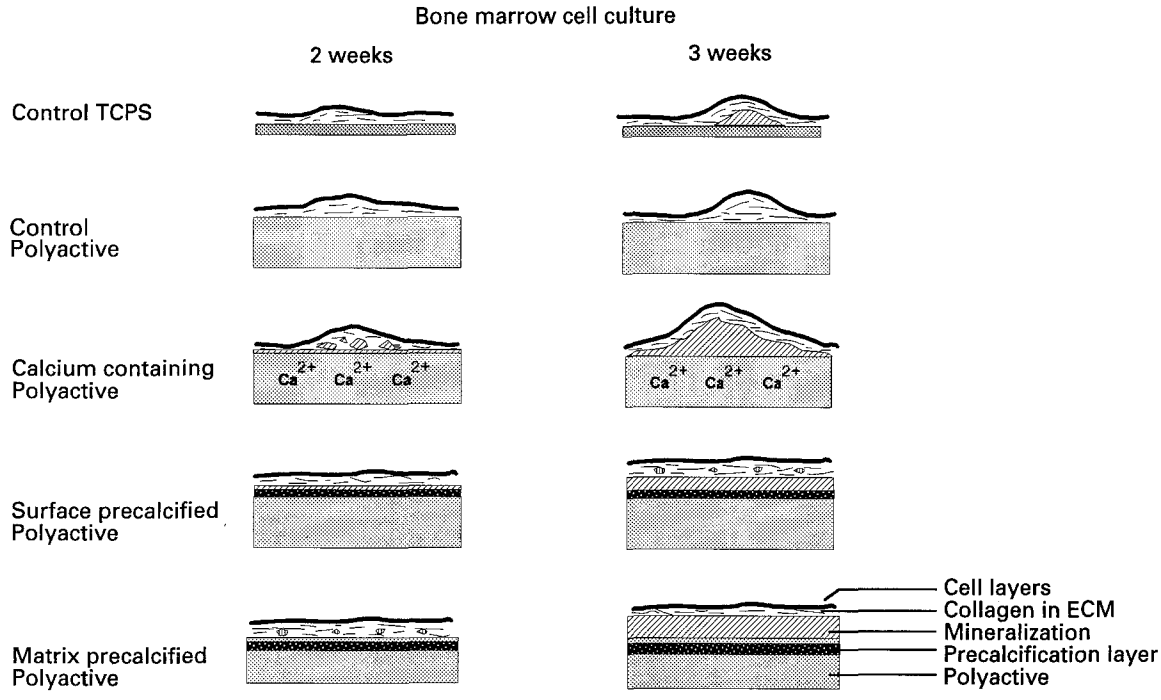


Figure 2 Overview of light microscopical and transmission electron microscopical results of 2 and 3 weeks bone marrow cell culture on PEO/PBT copolymers. Control cultures on TCPS and non-treated Polyactive are compared with Polyactive incubated in 1 M $CaCl_2$, surface precalcified and matrix precalcified Polyactive.

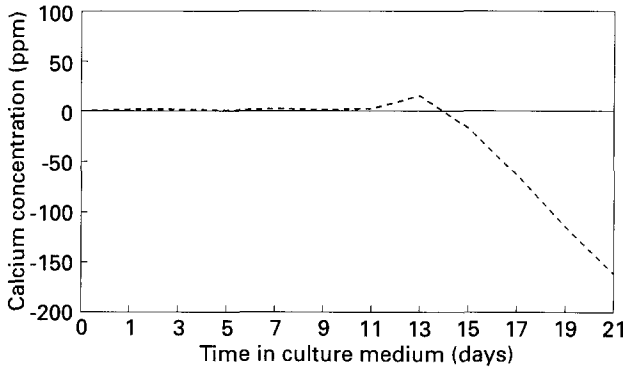


Figure 3 Atomic absorption data showing the accumulative calcium concentration in culture medium of control bone marrow cell cultures (– – –) on TCPS over 21 days. The calcium concentration is normalized to the calcium concentration in standard supplemented culture medium (——).

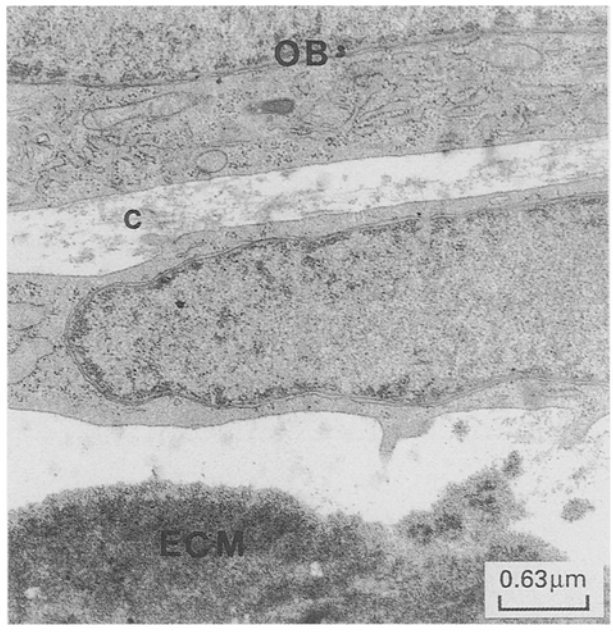


Figure 4 Transmission electron micrograph showing mineralized extracellular matrix (ECM) in control bone marrow cell cultures on tissue culture polystyrene for 3 weeks. Osteoblasts (OB) characterized by a well-developed cytoplasm rich in endoplasmatic reticulum, golgi complexes and mitochondria are present in the unmineralized, collagen- (c) containing ECM.

chloride solutions showed nodule formation from the 2-weeks culture period on. TEM demonstrated that mineralization foci were formed in the ECM. In addition, a continuous mineralized layer was present in close contact with the surface of the copolymer (Fig. 6a). Sometimes an electron dense layer, indicative for bone-bonding, was observed at the interface of the mineralized layer and the copolymer (Fig. 6b). The 3-weeks bone marrow cell culture on these materials resulted in more mineralization of the ECM compared to the 2-weeks evaluation period (Fig. 2).

No nodules were observed in bone marrow cell cultures after 2 and 3 weeks on both precalcified copolymers using light microscopical techniques (Fig. 2). After 2 weeks the initially present calcification layer on surface-precalcified materials seemed to be thicker than prior to the culture experiment. Unmineralized collagen fibres were in close contact with this calcified layer. After 3 weeks, the first signs of mineralization of collagen fibres in the ECM further away from the interface were observed (Fig. 7). The thickness of the calcified layer at the interface further increased and collagen fibres from the ECM were in close contact with this layer.

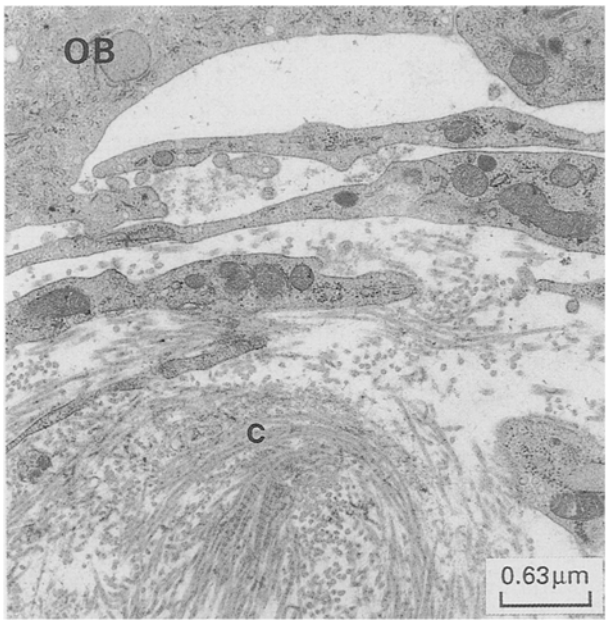
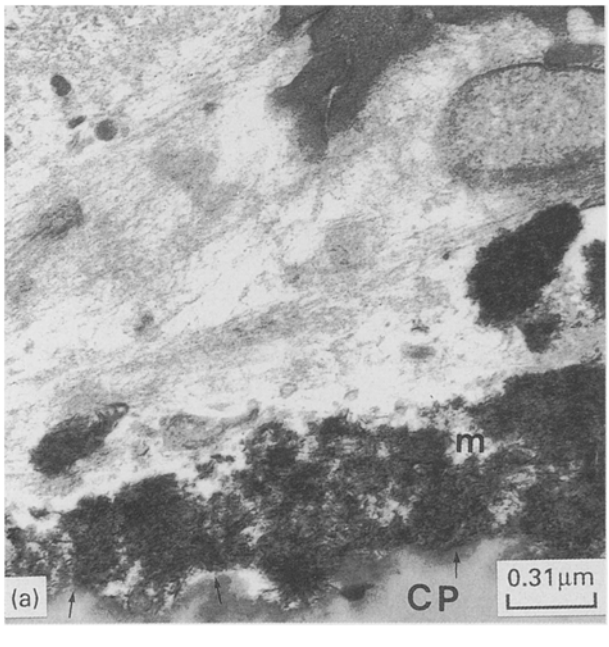


Figure 5 Transmission electron micrograph of bone nodule formed in a 3-week culture of osteoblasts (OB) on control PEO/PBT copolymers. Note the absence of mineralization in the collagen fibres (c) in the ECM.



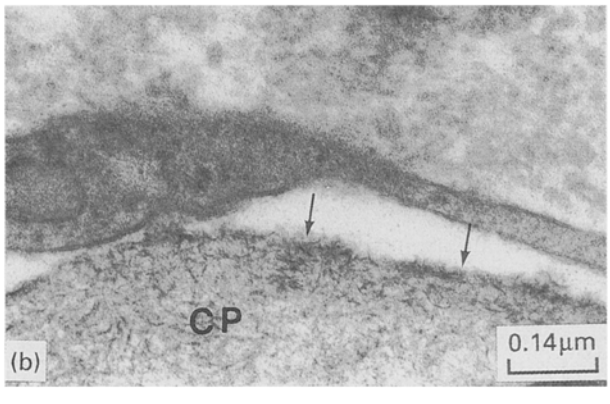


Figure 6 Transmission electron micrograph of cultured osteoblasts (OB) on calcium-containing PEO/PBT copolymers (1 M $CaCl_2$). (a) Small arrows indicate the continuous mineralized layer (m) in close contact with the copolymer (CP) surface. (b) Arrows indicate the electron dense layer at the interface with the copolymer (CP).

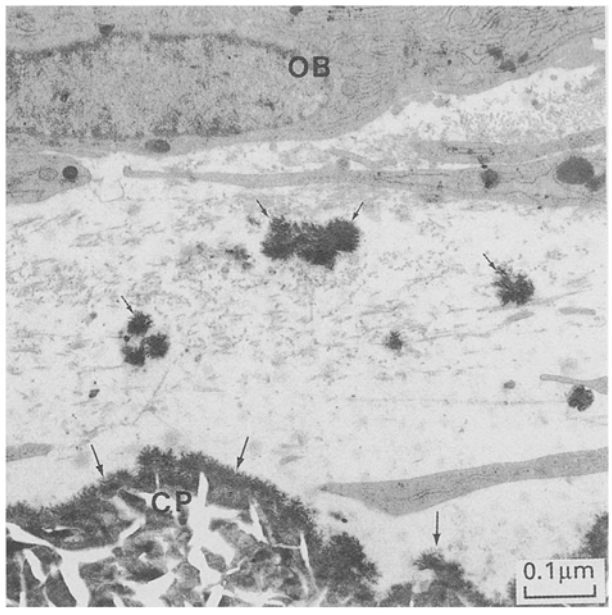


Figure 7 Transmission electron micrograph of cultured osteoblasts (OB) on surface precalcified PEO/PBT copolymers (CP). Note the mineralized layer in close contact with the surface of the copolymer (arrows). Small mineralization foci (small arrows) are formed in the ECM.

In matrix precalcified materials the precalcification layer was present at approximately 10 μm underneath the surface (Fig. 2). TEM demonstrated that the first mineralization foci were formed in the ECM at 2 weeks and that mineralization of the ECM increased with longer culture periods (Fig. 8a). Although the mineralized ECM was in close contact with the surface of the copolymer (Fig. 8b) after 3 weeks, no contact was observed with the precalcification layer in the copolymer matrix.

4. Discussion

The hydrogel properties of PEO/PBT copolymers allow absorption of solutions [19]. It was demonstrated that incubation in calcium chloride solutions of varying concentrations resulted in uptake of different amounts of calcium by the copolymers. This initially present calcium chloride can be released into surrounding fluids. Drying of these calcium-enriched copolymers and subsequently incubating in phosphate-containing solutions resulted in calcium phosphate precipitation. It was previously shown that, depending on the pH of the phosphate-containing solution, calcium phosphate crystals were precipitated either on the surface, the so-called "surface precalcification", or underneath the surface, the "matrix precalcification" of the copolymers [25].

We compared the effect of the different pretreatments of the copolymers on the bone-bonding properties in an *in vitro* bone-forming system described by Maniatopoulos *et al.* [26]. Previous studies with bioactive materials in this cell culture system showed similar interface reactions as in bone *in vivo* [27–30]. Mineralization of control bone marrow cell cultures started after 15 days, indicated by a decrease in calcium concentration of the culture

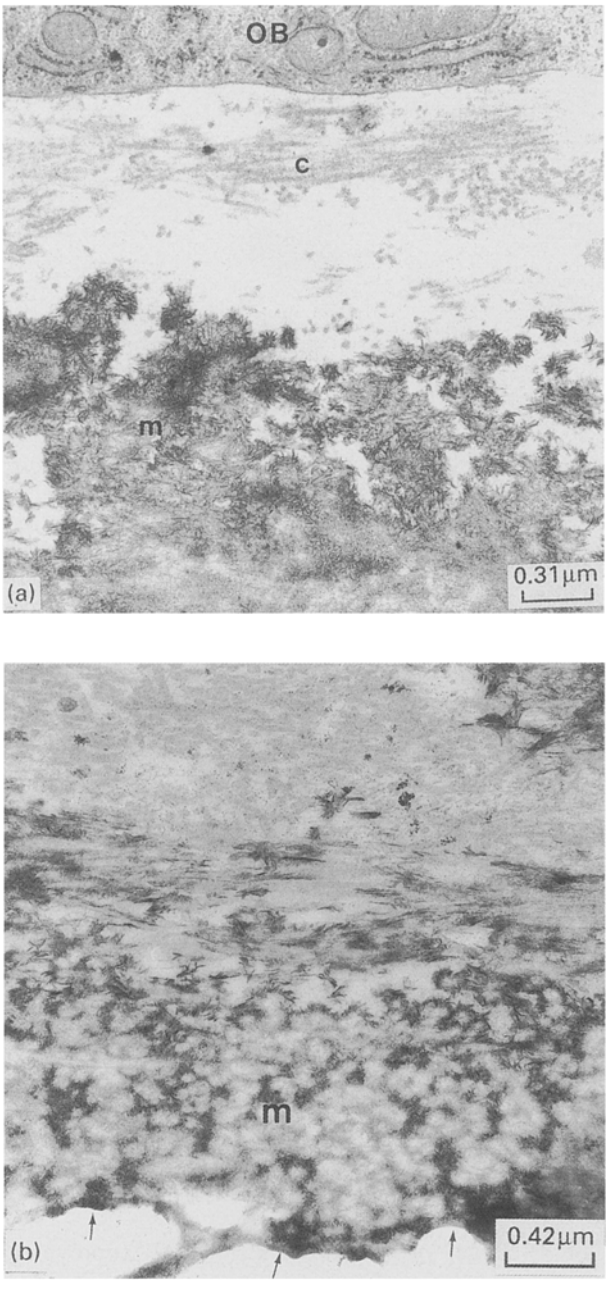


Figure 8 Transmission electron micrograph of cultured osteoblasts (OB) on matrix precalcified PEO/PBT copolymers. (a) Shows non-mineralized, collagen (c) containing ECM and abundant mineralization (m) of the ECM closer to the interface. (b) Arrows indicate close contact between the mineralized ECM (m) and the copolymer surface.

medium. However, the first visible signs of mineralization in the ECM became obvious using TEM after a 3-week culture period.

Bone marrow cell cultures on control PEO/PBT copolymers did not show mineralization of the ECM even after 3 weeks. A possible explanation for this phenomenon is that the hydrophillic surface characteristics of the PEO/PBT copolymers delay initial cell attachment [31, 32]. This will in turn affect the general proliferation of the bone marrow cells and mineralization of the ECM will probably start later.

PEO/PBT copolymers containing calcium, but no phosphate ions, release calcium into the culture medium during the first 3–5 days of the bone marrow cell culture. Only a small calcium release was observed from copolymers incubated in the lowest calcium-containing solution and therefore nodule formation and mineralization of the ECM was not affected. Copolymers with an intermediate amount of calcium induced earlier mineralized nodule formation in the cultures in comparison with control copolymers, which can be explained by results from a previous report, that calcium-containing biomaterials enhance cell adhesion and spreading [33]. Copolymers with the highest amount of calcium resulted in a calcium concentration of the culture medium which was toxic for the bone marrow cells.

Although no nodules were formed, both precalcified PEO/PBT copolymers had a different effect on mineralization in the bone marrow cell cultures. The thickness of the initial calcium phosphate layer on surface precalcified copolymers increased during the experiment, possibly due to spontaneous calcium phosphate precipitation. This hypothesis is supported by the finding that the calcium concentration in the culture medium was decreased. Due to the relatively low calcium concentration of the culture medium, we assume that mineralization of the ECM will be less in comparison with control cultures. On matrix precalcified copolymers a different phenomenon was observed. AAS results indicated an increase in the calcium concentration of the culture medium, which probably led to fast mineralization of the ECM, even before nodules were formed.

Based on the results of this study we conclude that the pre-operative addition of calcium or calcium phosphate to PEO/PBT copolymers (Polyactive™) can stimulate mineralization in bone marrow cell cultures. Calcium release from the calcium-containing and matrix precalcified copolymers seemed to stimulate mineralization of the ECM, although the amount of calcium release can be critical for the survival of the bone marrow cells. We expect that an initial release of calcium ions from the implants can *in vivo* stimulate the formation of an apatite layer on the surface of the implant and thus promote bone apposition.

Surface precalcified copolymers, characterized by a decrease in calcium concentration of the culture medium, stimulated calcium phosphate precipitation directly on the surface of the copolymers, which probably resulted in less mineralization of the ECM. *In vivo* we expect that the surface precalcification layer can also lead to further calcium phosphate precipitation and thus acts in a manner similar to the apatite layer formed on other bioactive materials. Bone apposition can directly start on this layer and would thus lead to faster bone-bonding.

It became obvious form this study that the pre-operative addition of calcium ions alone, or calcium phosphate crystals to Polyactive™ copolymers stimulated mineralization *in vitro*. Further research needs to be performed on the effect of these pretreatments on stimulating the bone-bonding characteristics of PEO/PBT copolymers *in vivo*.

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