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# Growth of human osteoblast-like cells on beta-tricalciumphosphate (TCP) membranes with different structures

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**Abstract** Bioresorb®, a bioactive, bioresorbable bone replacement material, consisting of pure beta-tricalciumphosphate (TCP) ceramic, was evaluated in cell culture with human osteoblast-like cells. The aim of our study was to investigate the influence of TCP on the growth behavior of human osteoblast-like cells. Different granule sizes and plate sizes were used in this study: Granule sizes 500–1000 $\mu$m, 1000–2000 $\mu$m and 2000–5000 $\mu$m; plate sizes 1.7 mm, 2.0 mm and 2.2 mm. Under scanning electron microscopic (SEM) observations the cell colonization on the surface of the biomaterial and the tissue compatibility were studied. Thin sections were used to study the growth of human osteoblast-like cells inside the biomaterial. It could be clearly shown that all investigated biomaterials are tissue compatible and that the size and structure (granule or plate) of the biomaterial effects the colonization rate. Bioresorb® plates enhance the colonization comparable to granule. The results obtained by SEM and thin sections were confirmed immunhistochemically by the nonradioactive assay EZ4U – EASY FOR YOU.

In conclusion, all investigated sizes and structures of Bioresorb® are tissue compatible but the cell growth is much better on plates than on granule small in size. The results suggest that the plates may be favourable useful as scaffold for regrowth of bone.

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## Introduction

Tricalciumphosphate (TCP) membranes, porous ceramic, are candidate materials for use in reconstructive surgery [1]. They serve as bone substitute materials in the orthopedic and cranofacial field to augment bone defects caused by resorption, trauma or surgery. These membranes are non-reactive, biocompatible, resorbable and non cytotoxic. TCP acts as a scaffolding for bone ingrowth, undergoing progressive degradation and replacement by bone. TCP membranes also allows the ingrowth of vessels [2]. Such membranes serve as alternative to autogenous or allogenic bone transplants [3]. Additionally, TCP devices as carriers for bone morphogenetic proteins [4, 5]. Reported studies with TCP membranes describe the biological response of calvarial bone towards TCP, the replacement of TCP-ceramic implanted into an artificial bone defect by newly formed bone as well as effects of TCP on cellular proliferation, cytokine gene expression and protein secretion [1, 6, 7]. Krajewski et al. [8] examined the mineralization and calcium fixation within a ceramic mixture of hydroxyapatite and beta-TCP [8]. Also animal studies exist with TCP to compare this material with autogenous bone, to study the degradation of TCP and the bone regeneration [9–12]. In humans the bone formation in tricalcium phosphate-filled lesions was studied after grafting [13]. The experiments showed that TCP has an osteogenic potential and is degradable but it was also observed that these processes are of long duration.

However, the growth of human osteoblast-like cells on such tricalciumphosphate membranes and especially the ingrowth of cells into TCP membranes were rarely studied. Rice *et al.* [14] studied the cell adhesion and cell response of human osteoblasts and monocytes on biphasic calcium phosphate granule with different percentage of TCP within the biphasic calcium phosphate granule. The higher content

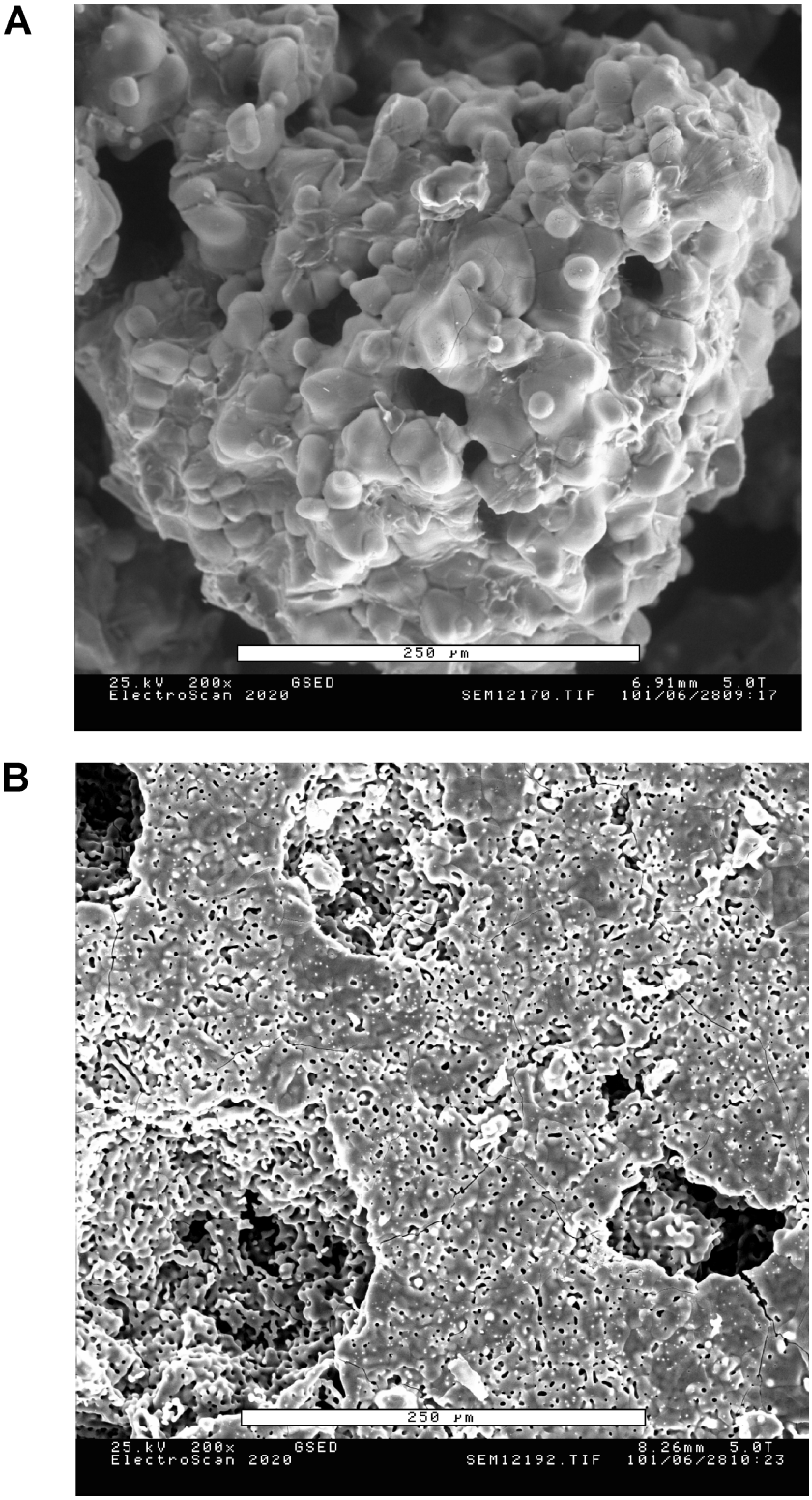



**Fig. 1** Scanning electron microscopy study of beta-TCP as granule **(A)** or plate **(B)** without human osteoblast-like cells

of TCP showed a detrimentel effect in cell adhesion. No study exists about the colonization of the cells on the surface as compared inside TCP scaffolds different sizes and structures. The question wether the size and structure of TCP membranes might influence the growth of human osteoblast-like cells on such biomaterials was analyzed in this study. For this purpose we used six different sizes and structures (granule and plates) of Bioresorb® to examine the proliferation and colonization of human osteoblast-like cells on these scaffolds. Such biomaterial/cell constructs could have the

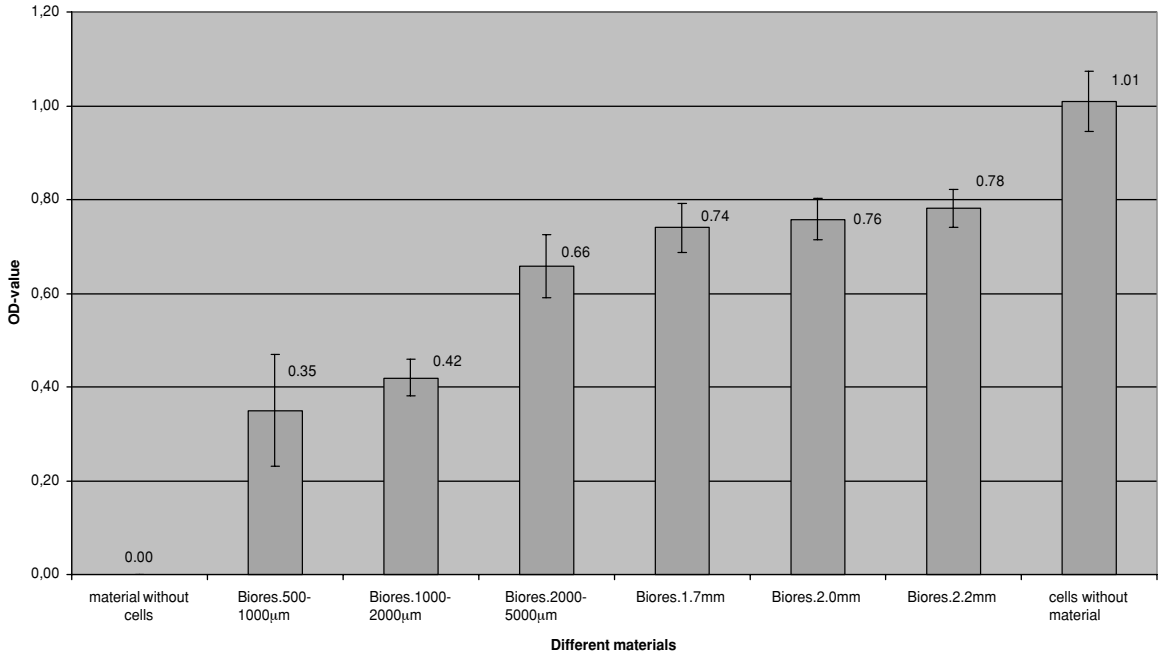



**Fig. 2** Cell proliferation analysis of human osteoblast-like cells seeded onto beta-TCP as granules or plates with different sizes. The standard deviations are indicated as error bars

advantage especially in treating long or large bone defects. The prerequisite is an optimal environment for the normal function of osteoblasts and the capability that osteoblasts can not only proliferate on the surface but also inside the biomaterials. The culture conditions for the cultivation of human osteoblast-like cells isolated from bone on various biomaterials were evaluated recently [15].

In future, the combination of such materials together with cellular components such as osteoblasts and vascular endothelial cells combined with e.g. growth factors could be a very effective approach in the field of tissue engineering. Such constructs could be vascularized very fast and the healing process of critical bone defects could be accelerated and much more effective.

## Materials and methods

### Biomaterials

BioResorb® (Oraltronics, Bremen, Germany), a bioactive, bioresorbable bone replacement material, was used in this study. It consists of pure beta-tricalciumphosphate ceramic, tertiary calcium salt of orthophosphoric acid. Six various structures of this biomaterial were investigated for the cultivation of human osteoblast-like cells:

1. Granule size 500–1000 $\mu$m
2. Granule size 1000–2000 $\mu$m
3. Granule size 2000–5000 $\mu$m
4. Plate size 1.7 mm
5. Plate size 2.0 mm
6. Plate size 2.2 mm

### Isolation and cultivation of human osteoblast-like cells

The cells, isolated from cortico-lamellar bone of the maxilla during biopsies from five patients, were seeded on culture flasks (25 $cm^3$, Greiner, Frickenhausen, Germany) using Opti-minimal essential medium (Opti-MEM, Gibco Laboratories Life Technologies, Inc, Grand Island, NY, USA) pH 7.2 with 10% foetal calf serum (FCS) and kept in a humidified atmosphere of 5% $CO_2$ at 37°C (Heraeus, Hanau, Germany).

The osteoblast-like cells which migrated onto the floor of the culture dish form a confluent layer after 4-5 weeks (primary culture) and the first passage was used for the growth experiments on BioResorb®.

### Preparation of the cell culture plates

Before cell seeding onto the biomaterials the 24 well culture plates (Costar, NY, USA) were humidified with culture medium.

### Seeding the cells onto BioResorb®

The confluent osteoblast cultures (primary culture) were detached from the culture flask by incubation with 0.5%

trypsin (Gibco, Paisley, Scotland) in phosphate buffered saline (PBS) for 8 min at 37°C. The bone cell solution was filtered through a 100 $\mu$m cell-strainer (Falcon, Heidelberg, Germany) in a 50 ml tube (Falcon, Heidelberg, Germany), centrifuged (Biofuge Stratos, Heraeus, Hanau, Germany, 1120 × g, 12 min, 30°C) and resuspended in 1 ml culture medium. The cells were transfered in a 75 $cm^3$ culture flask (Greiner, Frickenhausen, Germany), filled up with 25 ml

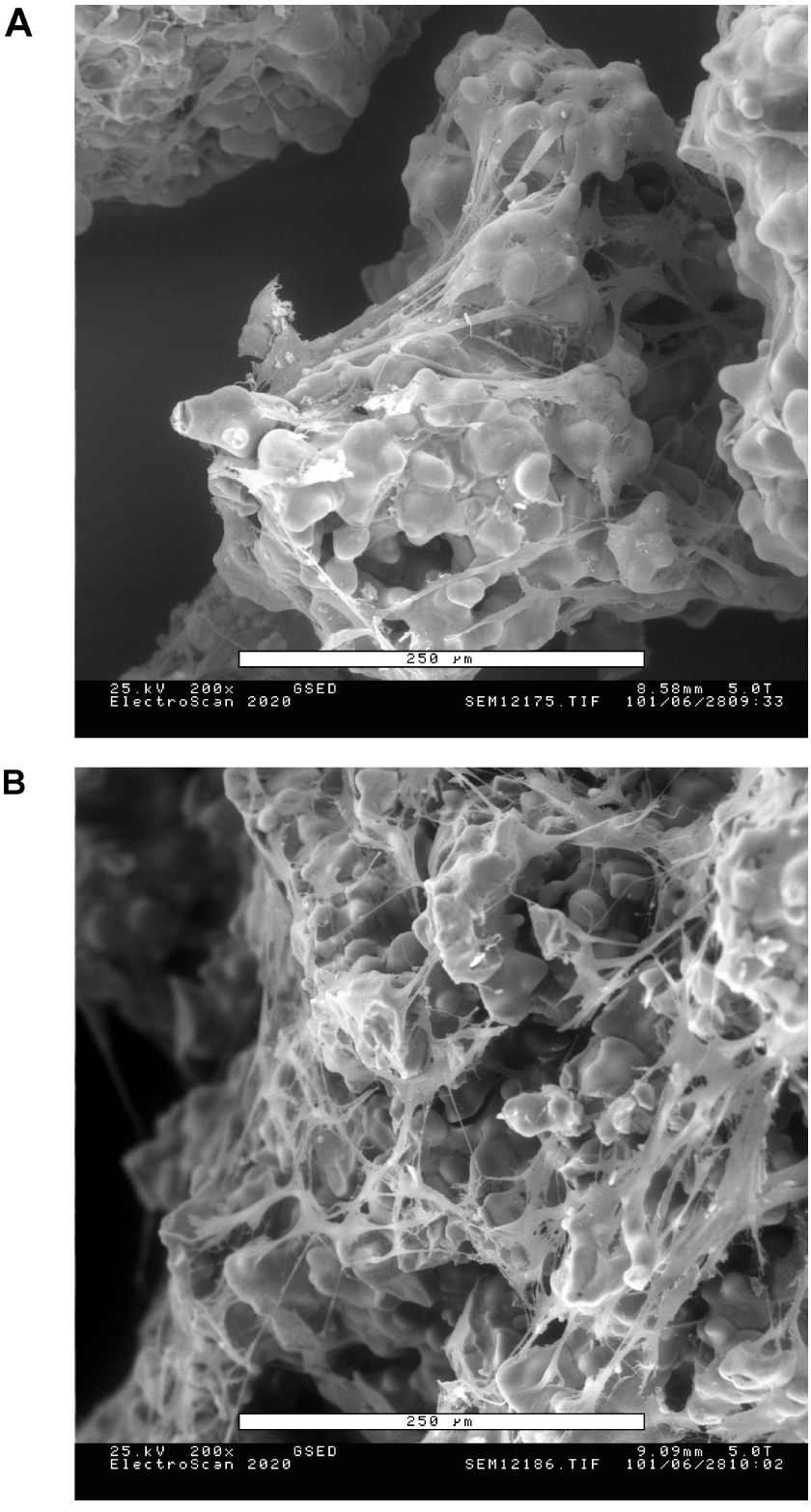



**Fig. 3** Scanning electron microscopy study of beta-TCP granules different sizes cultivated with human osteoblast-like cells: 500–1000 $\mu$m **(A),** 1000–2000 $\mu$m **(B)** and 2000–5000 $\mu$m **(C)**

**Fig. 3** (*Continued*)

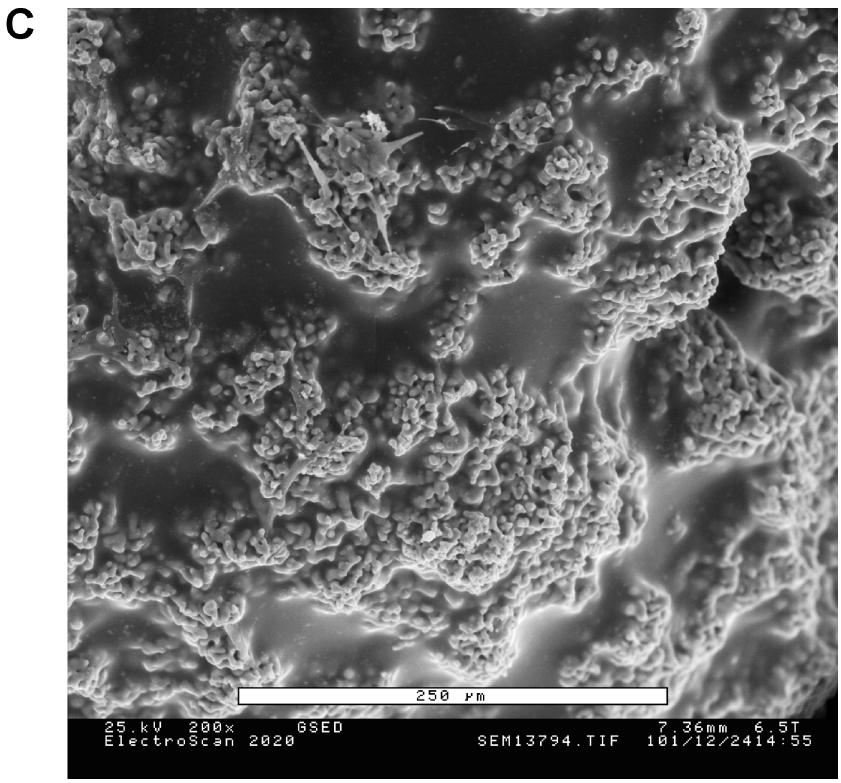
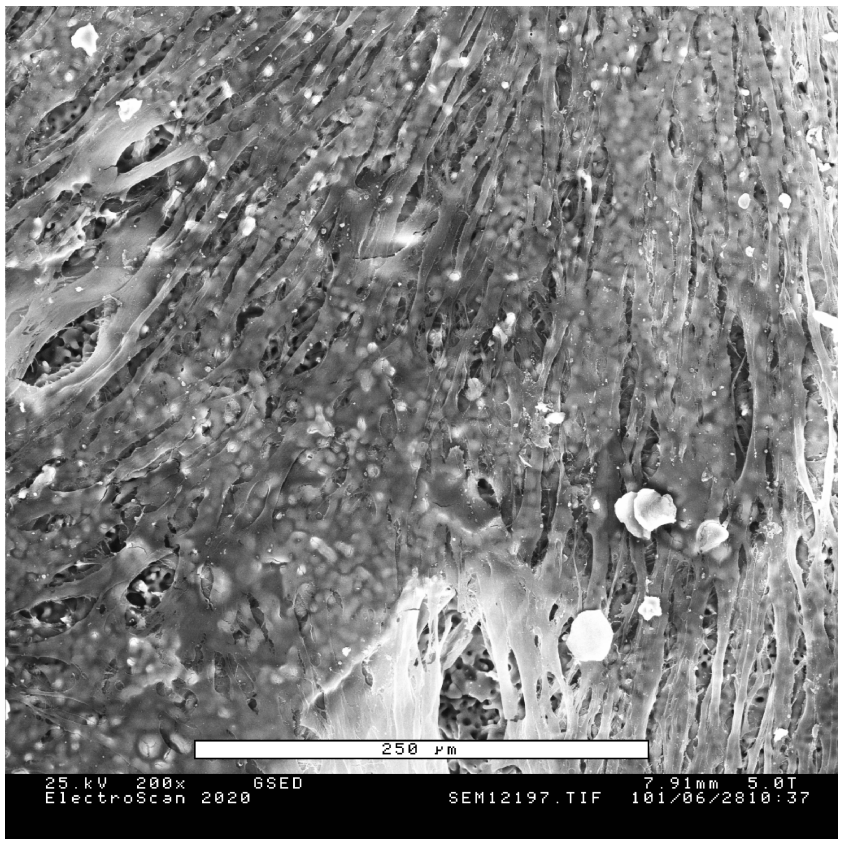



**Fig. 4** Scanning electron microscopy study of beta-TCP plate (2.0 mm) cultivated with human osteoblast-like cells



culture medium. After 14 days the cells of the first passage were detached again from the culture flask with 0.5% trypsin, centrifugated and resuspended in 1 ml culture medium. After staining with trypanblue (1:1;v/v) the cells still alive were counted in a chamber by light microscopy (Zeiss Axiovert 135, Jena, Germany). Then the cells ($1 \times 10^5$ cells/ml) were seeded onto BioResorb® with different structures by a sterile syringae. The experiments were done at least five times. The cells were incubated for 2 h at 37°C in 5% $CO_2$ atmosphere before culture medium to a total volume of 1 ml was added. After three days of incubation in total, scanning electron microscopic studies, thin sections and proliferation assays followed.

Cell proliferation analysis

For cell proliferation analysis the nonradioactive assay EZ4U—EASY FOR YOU (Biozol diagnostica GmbH; Eching, Germany) was used. This method is based on the finding that living cells are capable of reducing slightly yellow coloured tetrazolium salts to intense red coloured formazan derivates by an intracellular reduction system, mostly located in the mitochondria [16]. These formazan derivates are excreted into the culture medium and the absorbance can be measured with a microplate reader. The amount of coloured formazan derivates correlates with the amount of living cells in the sample. The proliferation assay was carried out according to the manufacturers instructions.

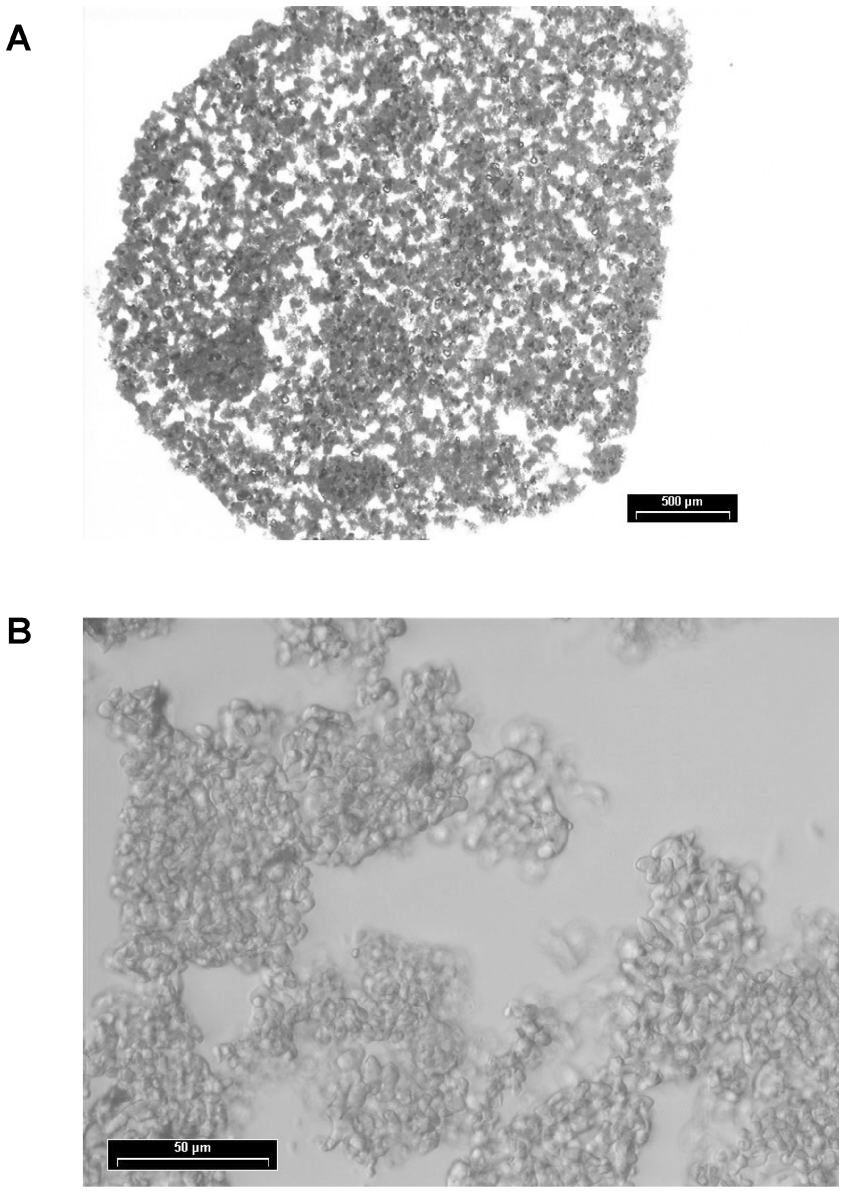
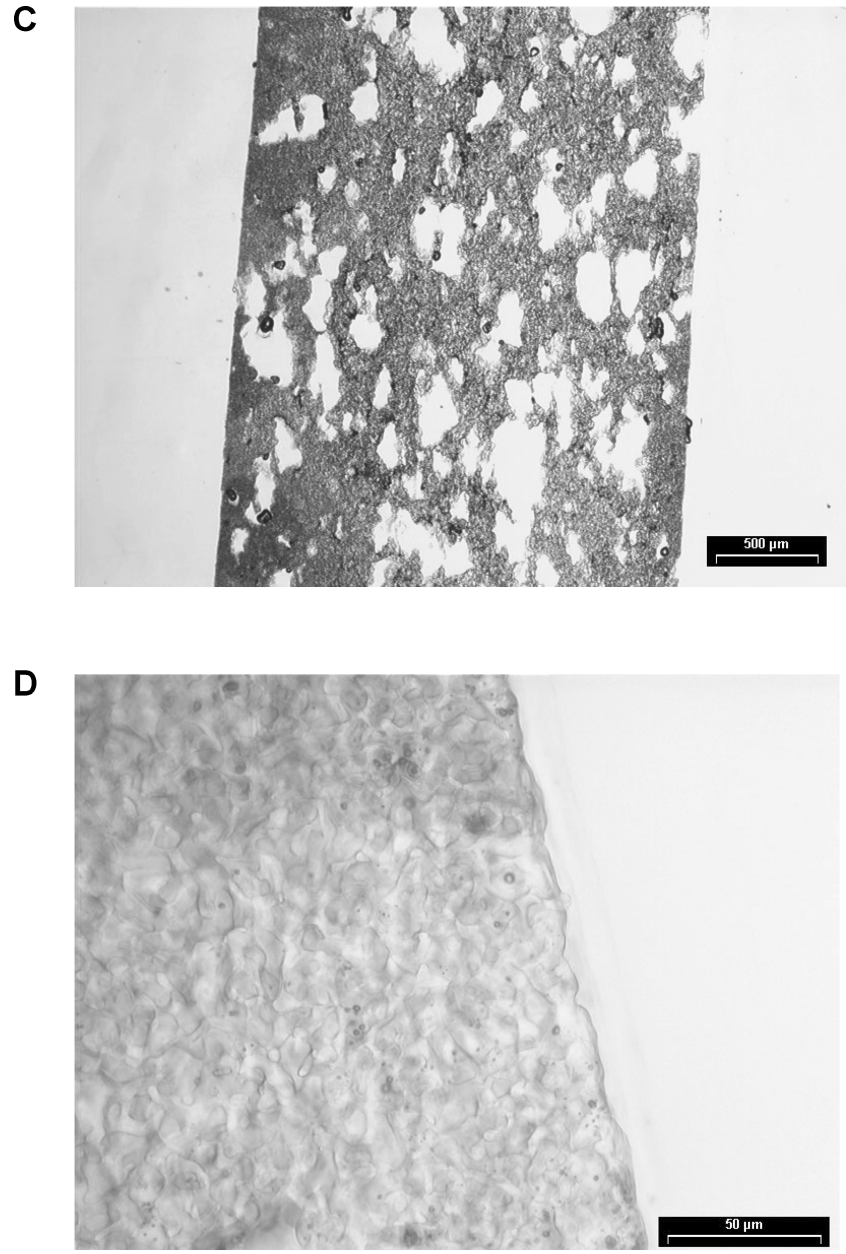



**Fig. 5** Thin sections of beta-TCP granules (**A and B**) and beta-TCP plates (**C and D**) each without osteoblasts

**Fig. 5** (*Continued*)




Cell colonization analysis: Scanning electron microscopy and thin sections

The cell colonization analysis was assessed by scanning electron microscopy and thin sections. For scanning electron microscopy the samples were fixed in 4% paraformaldehyde for 2 h at room temperature and incubated in 8% formaldehyde for 2 days at 4°C. The samples were dehydrated in graded alcohol (30%, 50%, 70%, 80%, 90%, each one time and two times in 99.8%). After critical point drying (CPD 030 Baltec, Wallruf, Germany), according to standard procedure using liquid carbon dioxide, the samples were sputtered with goldpalladium (Plano, Wetzlar, Germany) in the SCD 040 (Balzers Union, Wallruf, Germany). The probes were examined via Zeiss Leo 32 scanning electron microscope (Zeiss, Kochern, Germany) at 10 KV. Images were digitized. Thin sections were performed according to Donath and Breuner [17]. Over an amounting alcohol gradient the segments were dehydrated and infiltrated with glycolmethacrylate (Technovit 7200 VLC®, Kulzers GmbH, Friedrichsdorf, Germany). The blocks were embedded and polymerized by light with a wavelength of 200–400 nm in a light polymerization apparatus (Kulzer-Exact®, Friedrichsdorf, Germany). Afterwards the blocks were cutted in 200–300 $\mu$m sections with a diamond bandsaw under permanent cooling. With the exact grinding unit they were ground to a final thickness of about 50 $\mu$m and stained with toluidine blue. The sections were examined by light microscope (Axioskop,

Zeiss, Oberkochen, Germany) with 25× and 400× magnification.

## Immunohistochemical stainings

Bone cells were characterized to a set of parameters, including alkaline phosphatase (ALP) activity, synthesis of collagen type I and production of the bone-specific non-collagenous protein osteocalcin [18, 19].

The **ALP activity** of osteoblasts was evaluated by an alkaline phosphatase assay kit (Sigma, Deisenhofen, Germany). In brief, the culture dishes were air dried, fixed in a citrate-aceton-formaldehyde solution for 30 sec and rinsed gently with Distilled water. Incubation with alkaline phosphatase staining solution for 15 min protected from direct light and a washing step with distilled water followed. The citrate-aceton-formaldehyde solution as well as the alkaline phosphatase staining solution was prepared according to the manufacturer's instructions. The culture dishes were counterstained with neutralred for 5 min, rinsed with Distilled water and dried mounted with cover slips. Positive staining for alkaline phosphatase (red-violet) was identified by lightmicroscopy and evaluated by morphometry using

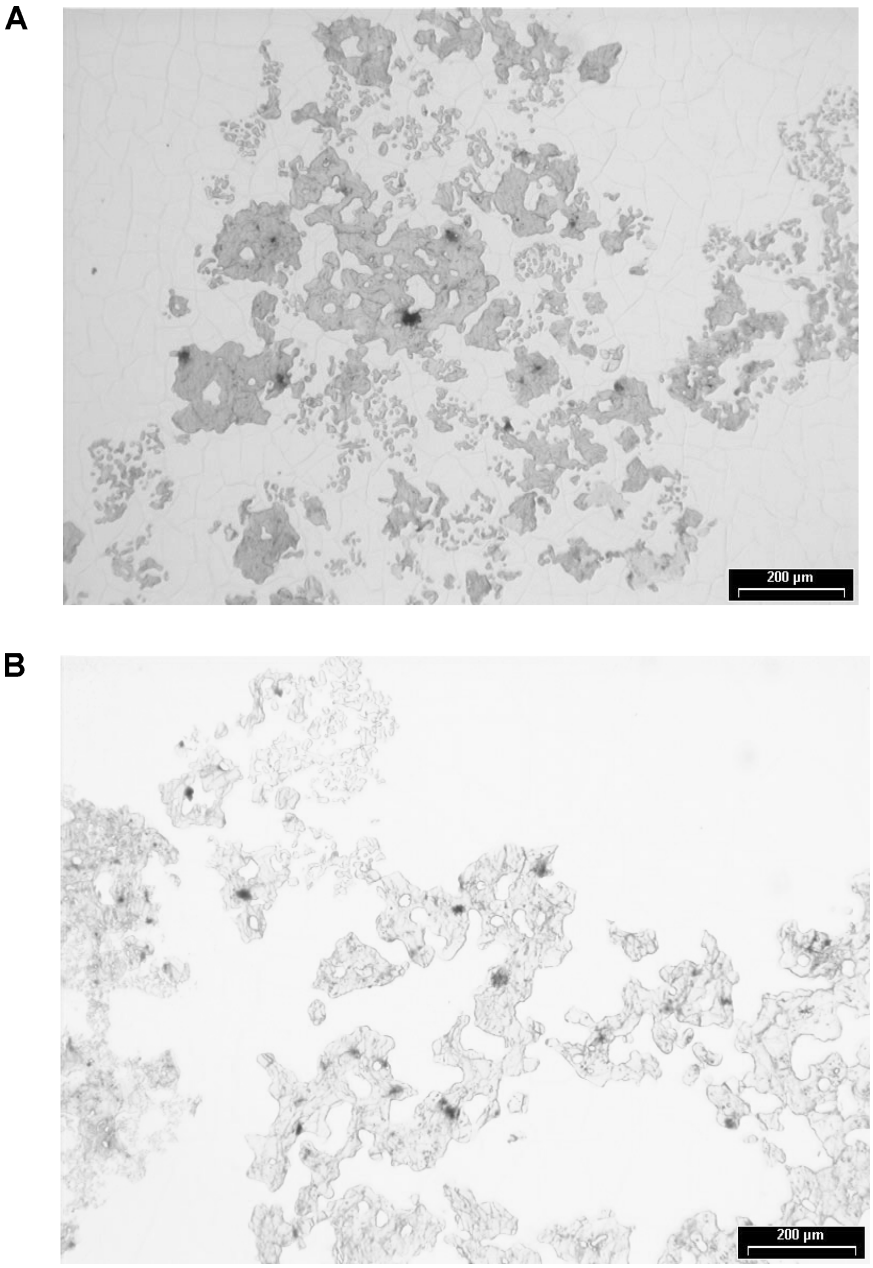
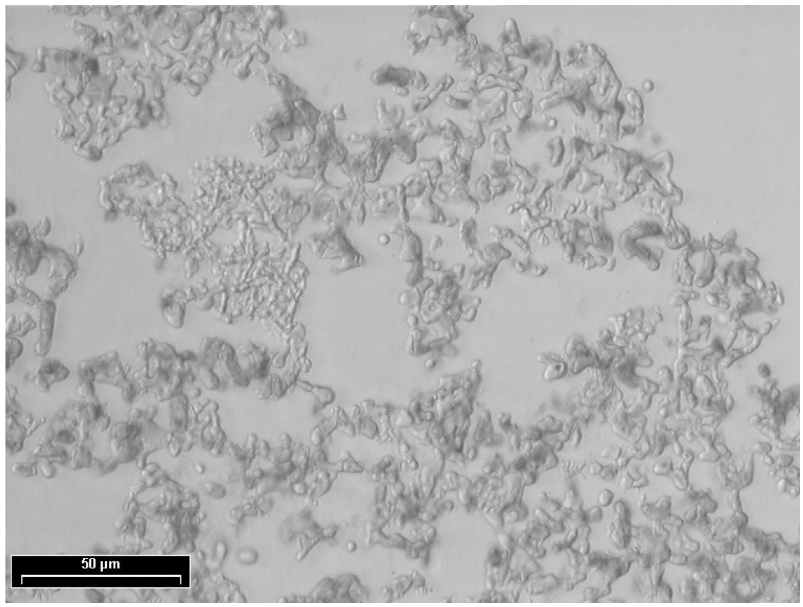



**Fig. 6** Thin sections of beta-TCP granules. **A**: 500–1000 $\mu$m, **B**: 1000–2000 $\mu$m and **C**: 2000–5000 $\mu$m in size cultivated with human osteoblast-like cells (blue stained)

**Fig. 6** (*Continued*)




the computer program Analysis 3.1 (Soft Imaging System, Münster, Germany).

For the **quantification of type I collagen** the cells were washed in phosphate buffered saline (PBS) for 5 min, fixed with 70% ethanol for 1 h, washed in PBS for 5 min, allowed to air dry and washed again in PBS for 5 min. After an incubation of 0.3% $H_2O_2$ in methanol for 30 min, unspecific immune reactions were blocked with 1% bovine serum albumin for 10 min, before the anti-collagen I antibody (Sigma, Deisenhofen, Germany) was administered for 1 h. An incubation of the biotin-conjugated secondary antibody (Vectastain Elite Kit, Vector Laboratories, Burlingame USA) for 45 min and an incubation of avidin mixed with biotin-conjugated peroxidase (Vectastain Elite Kit, Vector Laboratories, Burlingame USA) for 30 min followed. Sections were rinsed between each incubation step three times with PBS for 5 min. The immunreaction was developed by diaminobenzidine-solution (0.05 mg/l DAB/0.05 M Tris-HCl pH 7.3/ 0.01% $H_2O_2$) at room temperature. The sections were counterstained with hematoxylin (Merck, Darmstadt, Germany) for 10 sec and mounted in 40% glycerin (Merck, Darmstadt, Germany) in PBS. The evaluation was done by lightmicroscopy and the computer program Analysis 3.1 (Soft Imaging System, Münster, Germany).

For the **quantification of osteocalcin** in the cell culture supernatant of human maxillar osteoblast-like cells the osteocalcin ELISA (DAKO, Glostrup, Denmark) was performed according to the manufacturer's instructions. In brief, the standards, the curve control and the cell culture supernatants were premixed with biotinylated osteocalcin, incubated in microwells precoated with anti-osteocalcin for 1 hour, washed and incubated with peroxidase-conjugated streptavidin for 15 min, which binds strongly to the biotinylated osteocalcin. After a further washing step the chromogenic substrate was added and incubated for 30 min. The reaction was stopped by 2 M $H_2SO_4$ and the absorbance at 450 nm was measured. Osteocalcin is exclusively synthesized by osteoblasts and is believed to prevent premature mineralization of newly formed, but yet disorganized bone matrix [20].

## Results

In this study Bioresorb®, a bioactive, bioresorbable bone replacement material, consisting of pure beta-tricalciumphosphate ceramic, was evaluated in cell culture with human osteoblast-like cells. Six different sizes of Bioresorb® was used: Granule with a size of 500–1000 $\mu$m, 1000–2000 $\mu$m, 2000–5000 $\mu$m and plates with a size of 1.7 mm, size 2.0 mm and 2.2 mm. Figure 1 shows a scanning electron microscopy study of Bioresorb® as granule (Fig. 1A) or plate (Fig. 1B) without human osteoblast-like cells.

The vitality and the proliferation capacity of the osteoblast-like cells seeded onto the six different sizes of the biomaterial was studied by the EZ4U – EASY FOR YOU test. The average of the measured absorbance is shown in Fig. 2. The values of the standard deviation are indicated as error bars. Osteoblast-like cells seeded on plates showed in each test significantly more vital cells than on granule. Nearly the same proliferation rates could be measured using the different plate sizes (Fig. 2). Whereas differences were measured using the different granule sizes: The lowest proliferation rate was measured with the granule size of 500–1000 $\mu$m and the highest with the granule size of 2000–5000 $\mu$m (Fig. 2).

Under scanning electron microscopy observations the cell colonization, cell morphology and tissue invasion were studied. Thin sections were used to examine the growth of human osteoblast-like cells inside the biomaterial. It could be clearly shown that the size and structure (granule or plates) influence the colonization on the surface of the biomaterial.

Figure 3 shows the growth of the cells on Bioresorb® granule and Fig. 4 the cell growth on Bioresorb® plates analyzed by scanning electron microscopy. It could be observed that the cell colonization on the plates with different sizes were increased compared to the colonization on granule. Scanning electron microscopic observations showed on all granule with different sizes much more not intact osteoblast-like cells, round cells as well as incomplete cell sheets (Fig. 3). If the three granule different in size are compared with each other the granule with 2000-5000 $\mu$m in size shows a more dense cell monolayer than the other two investigated granule. In contrast, the surface of the Bioresorb® plates were completely covered with osteoblast-like cells. The cells were intact, no round-shaped cells were visible and a confluent monolayer of cells had adhered to the biomaterial (Fig. 4). No differences were observed in dependence of the plate size (data not shown).

To study the growth of human osteoblast-like cells inside the biomaterial thin sections were performed. Figure 5A and

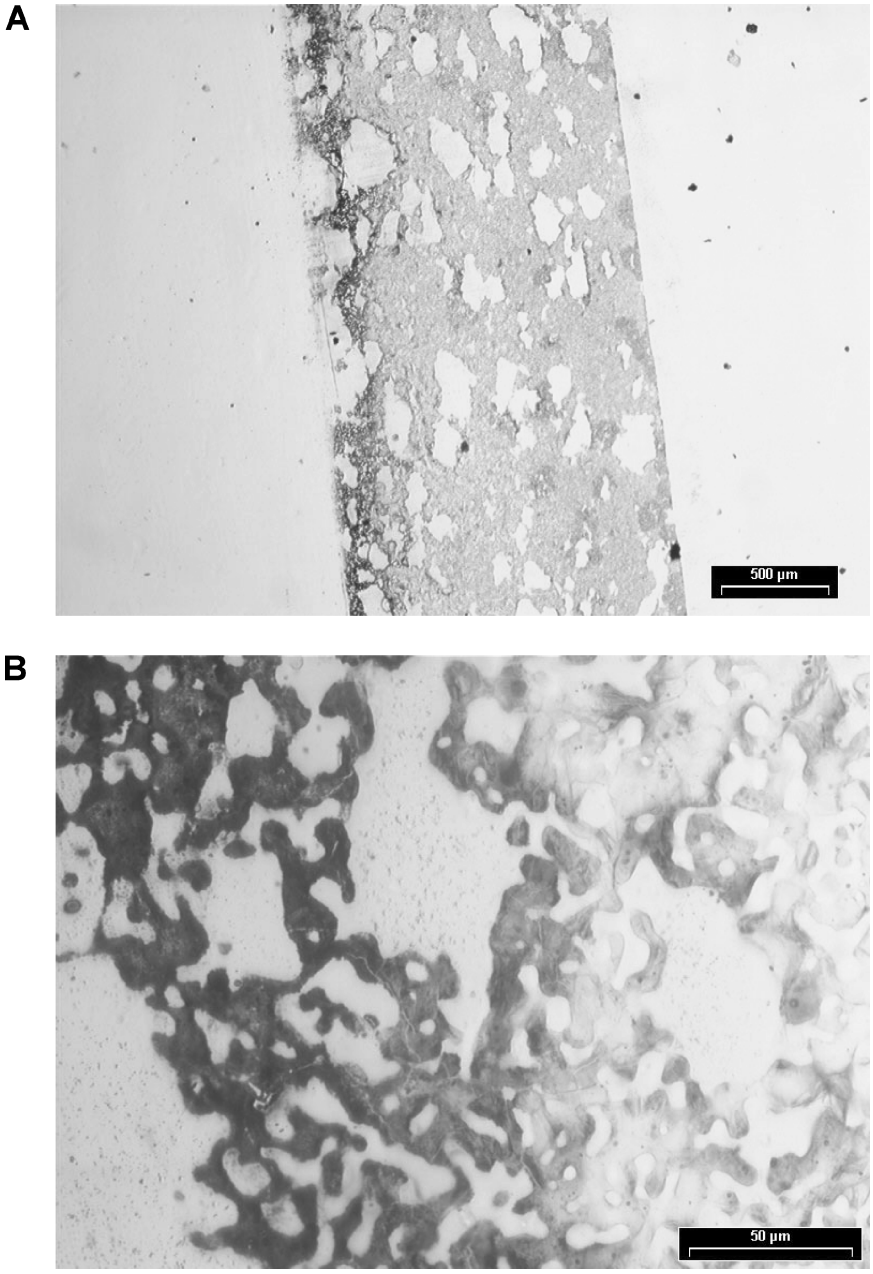



**Fig. 7** Thin sections of Bioresorb® plates different sizes. 1.7 mm (**A and B**), 2.0 mm **(C)** and 2.2 mm **(D)** cultivated with human osteoblast-like cells (blue stained)

**Fig. 7** (*Continued*)

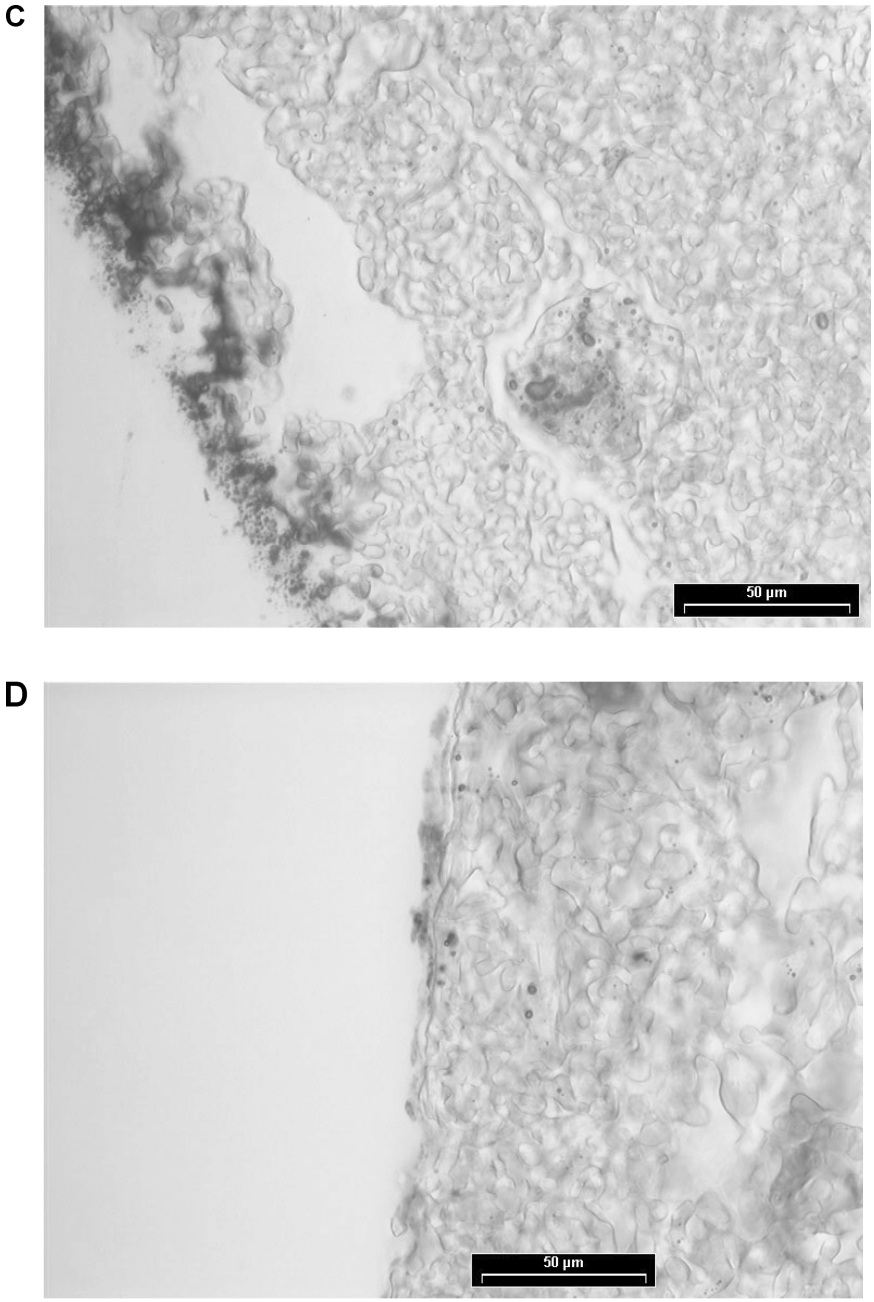



Fig. 5B shows thin sections of Bioresorb® granules whereas Fig.5C and Fig. 5D shows Bioresorb® plates each without osteoblasts. Figures 6 and 7 show thin sections of Bioresorb® granules and plates cultivated with human osteoblast-like cells (blue stained). Figure 6 A–C show the Bioresorb® granule different in size. The osteoblasts grow well inside the biomaterial, the cells are able to infiltrate the porous structure of the TCP scaffold. In Fig. 7 A–D thin sections of Bioresorb® plates of different sizes are shown. The osteoblasts are well attached to the surface of the biomaterial and grow into the micropores of the scaffold. The cells proliferate in a multilayer fashion. The peripheral region but not the whole plate of the biomaterial is packed with osteoblasts. No differences were observed using different plate size.

The use of osteoblasts from five different patients showed no differences in the proliferation assay, scanning electron microscopy or thin section observations.

Markers of osteoblastic differentiation, the alkaline phosphatase (ALP) activity, collagen type I and the osteocalcin production, were measured. The osteoblasts seeded onto the TCP scaffolds produced all bone markers tested. The amount of osteocalcin was 5.7 $\mu$g/l. The alkaline staining of these cells typically resulted intensively positive (about 55%) whereas the control, the human gingival keratinocytes,

did not. Immunocytochemistry of the fixed cells showed the presence of type I collagen in about 63% of the cells.

## Discussion

Biodegradable calcium phosphate bioceramics have been applied as bone substitutes in the field of reconstructive surgery for several decades. The use of such resorbable bone substitutes, e.g. for alveolar ridge augmentation, has the advantage that no second-site surgery for autograft harvesting is necessary [21]. These bone substitutes are mechanically suited for bone function and support osteoinduction of the cells to form new bone. The Ca/P ratio of the calcium phosphate ceramics is similar to those of natural bone. Such bone substitutes provoke little if any inflammatory response and have further advantages that complications associated with bone grafts for example the transfer of diseases from the graft or the demand of the surgical removal are avoided [22, 23]. Tricalciumphosphates (TCPs) also devices as carriers for bone morphogenetic proteins. It is well known that the nature and degree of tissue response depend on characteristics of the material such as its chemical composition, surface texture, porosity, density, shape and size [24–26]. This study was performed to examine the influence of the structure and size of Bioresorb® , consisting of pure beta-TCP, to the human osteoblast growth.

In this study human osteoblast-like cells used for the seeding procedure could be characterized as osteoblasts by the determination of osteocalcin, type I collagen as well as by the enzymatic activity of alkaline phosphatase. ALP and collagen type I belong to early marker of osteoblastic differentiation whereas osteocalcin belongs to the late marker [5]. Osteocalcin, an extracellular non collagenous matrix protein, produced exclusively by osteoblasts, was found in all of our seeded cells in an amount of 5.7 $\mu$g/l. The positive alkaline phosphatase staining (55%) was reported in many studies as an indicator for osteoblasts, for the formation of new bone and as a marker for cell differentiation in cultures [27, 28]. Type I collagen was expressed in approximately 63% of the cells. The levels of type I collagen may depend on the duration of the cultivation and the supplements [28].

Pioletti et al. [29] described in their study that small TCP particles are less biocompatible than large ones. Additionally, they observed that the viability and proliferation of rat osteoblasts were influenced by the particles used. These results confirm the results of our study carried out with human osteoblast like cells. The lowest proliferation rate of the cells was measured on granule with 500-1000 $\mu$m in size (Fig. 2). Recent studies supposed that particles small in size induces the phagocytosis process of osteoblasts [29, 30, 31]. It was supposed that the phagocytosis could lead to a decrease of osteoblast adhesion and consequently to a disorder in the osteoblast function. This disorder could disturb the formation of new bone. Furthermore, Pioletti *et al*. [29] decribed the possibility that the phagocytosis of calcium phosphate particles leads to an accumulation of calcium in mitochondria. This accumulation could lead to the lysis of mitochondria and consequently to the cell death.

Ginebra *et al*. [32] reported about the effect of the particle size on the micro and nanostructural features of calcium phosphate cement. They describe that protein adsorption and cell adhesion strongly depends on the nano-sized roughness of the interface. They could show that the higher specific surface, produced by the reduction of the particle size, accelerates the hydrolysis of TCP into calcium-deficient hydroxyapatite.

The thin sections of our study showed that the cells were grown well inside the biomaterial and that they were able to infiltrate the porous structure of the TCP scaffold. These results correspond with the study of Fredericks *et al*. [33]. They used a porous tricalciumphoshate bone void filler as an alternative to autograft in bone grafting and reported that the tricalcium phosphate pores exhibited bony infiltration.

In conclusion, the tested tricalcium phosphate scaffolds, Bioresorb® plates and Bioresorb® granule, are both well tissue compatible but Bioresorb® plates enhance the colonization comparable to granule. The results suggest that the plates may be favourable useful as scaffold for regrowth of bone and that they are a good alternative to autograft in bone repair and regeneration. They are a good tool for cell-based tissue engineering.

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