



Volumetric analysis of osteoclastic bioresorption of calcium phosphate ceramics with different solubilities

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ABSTRACT

Commonly, to determine osteoclastic resorption of biomaterials only the resorbed area is measured. The depth of the resorption pit, however, may also be important for the performance of a material. To generate such data we used two calcium phosphate ceramics (Ca_{10} and Ca_2). The solubility of the materials was determined according to DIN EN ISO 10993-14. They were scanned three-dimensionally using infinite focus microscopy and subsequently cultivated for 4 weeks in simulated body fluid without (control) or with human osteoclasts. After this cultivation period osteoclasts number was determined and surface changes were evaluated two- and three-dimensionally. Ca_{10} and Ca_2 showed solubilities of 11.0 ± 0.5 and $23.0 \pm 2.2 \text{ mg g}^{-1}$, respectively. Both materials induced a significant increase in osteoclast number. While Ca_{10} did not show osteoclastic resorption, Ca_2 showed an increased pit area and pit volume due to osteoclastic action. This was caused by an increased average pit depth and an increased number of pits, while the average area of single pits did not change significantly. The deduced volumetric osteoclastic resorption rate (vORR) of Ca_2 ($0.01\text{--}0.02 \mu\text{m}^3 \mu\text{m}^{-2} \text{day}^{-1}$) was lower than the remodelling speed observed in vivo ($0.08 \mu\text{m}^3 \mu\text{m}^{-2} \text{day}^{-1}$), which is in line with the observation that implanted resorbable materials remain in the body longer than originally expected. Determination of volumetric indices of osteoclastic resorption might be valuable in obtaining additional information about cellular resorption of bone substitute materials. This may help facilitate the development of novel materials for bone substitution.

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1. Introduction

Biomaterials are increasingly used to repair and regenerate diseased and damaged organs [1]. For improved healing of skeletal defects, autologous bone transplants have already been used for more than 100 years [2,3]. The gold standard in this organ system is autografting trabecular and cortical bone, typically taken from the iliac crest. These transplants have the advantage of being osteoinductive and osteoconductive, which means they stimulate bone formation and act as a scaffold for this new bone. Due to a high bending strength of approximately 160 MPa [4], cortical bone grafts can also be used in load-bearing regions of the skeleton. However, there are also disadvantages to the use of autologous transplants: a longer operating time with an associated higher risk of complications, pain at the site of explantation and limited availability. The nearly unlimitedly available allografts and xenografts, on the other hand, carry a certain risk of immunoreaction or transmission of diseases. Due to these problems, the development of

artificial biomaterials for use as bone substitutes started in the middle of the last century [5]. Two important properties of an ideal bioactive bone substitution biomaterial would be to have the same biomechanical competence as autologous bone and to be remodelled in the same fashion [6,7]. This requires osteoclasts to resorb the material at the same speed as osteoblasts lay down new bone on its surface, until the material is completely replaced by new, living bone. Using such a biomaterial would have many advantages. It would prevent stress shielding and material fatigue and make a second operation for implant removal unnecessary.

Resorbability of materials is often equated with solubility and, therefore, the materials are commonly tested according to DIN EN ISO 10993-14. However, materials that qualify as “resorbable” under these testing conditions have been found to be incompletely resorbed even years after implantation [8,9]. Resorption of materials in vivo is not only dependant on the fluid components of the body, but also on cellular activity, mainly of osteoclasts. There have been a number of studies that have addressed this topic and shown that some materials are resorbed by cells (calcium phosphate [10,11], sintered hydroxyapatite [12,13], nanocrystalline hydroxyapatite [14], tricalcium phosphate [11–13],

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carbonated apatite [15], calcite [12,16], aragonite [16] and aluminum calcium phosphorous oxide [13]), while other materials are not (poly(methyl methacrylate) [10], deproteinated bovine bone, coated silica glass and coated methacrylates [17]). Most of these studies, however, only reported the resorbed area. The remodelling speed of bone has been known for more than 40 years ($0.04\% \text{ day}^{-1}$ [18]). The resorption of biomaterials, however, has only been quantified relatively so far. A knowledge of volumetric resorption speed might help in the development of materials for which resorption and replacement by new bone match more closely.

The purpose of this study was to analyse two bone substitute materials which qualify as “resorbable” in the ISO test, to compare their dissolution in simulated body fluid (SBF) with cellular resorption by human osteoclasts and to quantify this resorption two- and three-dimensionally.

2. Materials and methods

2.1. Biomaterials

Two calcium alkali orthophosphate ceramics with different crystalline phases were chosen for the experiments: a $\text{Ca}_2\text{KNa}(\text{PO}_4)_2$ -containing material (ICDD PDF-Nos. 00-051-0579 and 00-047-0178), which has already been investigated in previous studies [19,20], referred to as “ Ca_2 ”; a material similar to tricalcium phosphate, with an alkali substitution, referred to as “ Ca_{10} ” (Table 1 and Fig. 1). This second material contains mixtures of the crystalline phases of $\text{Ca}_{10}\text{Na}(\text{PO}_4)_7$ (ICDD: 00-045-0339 [sodium containing]) and $\text{Ca}_{10}\text{K}(\text{PO}_4)_7$ (PDF-No. 00-045-0138 [potassium containing]). Both materials were manufactured by melting stoichiometric amounts of carbonates and phosphates in a platinum crucible at 1600°C and casting [21]. Powders were prepared by milling and after which discs of 1 mm thickness were formed by compression. For cell culture investigations $5 \times 4 \times 1$ mm cubes were cut from the discs. The solubilities of the materials were determined from the powders ($315\text{--}400 \mu\text{m}$) according to DIN EN ISO 10993-14. The degradation products of the ceramics resulting from storage in Tris-HCl buffer over a period of 120 h can be identified and quantified by this method. Dentin was used as an internal control to determine whether osteoclasts were functional and able to degrade mineralized tissue (Fig. 2), kindly provided by the German Federal Agency for Nature Conservation.

2.2. Cell culture of human osteoclasts

Peripheral blood mononuclear cells (PBMC) were isolated from the buffy coats of healthy volunteers and purified as described previously [10]. Density gradient centrifugation was used to separate mononuclear precursor cells from other formed elements in the

Table 1
Material properties of Ca_{10} and Ca_2 .

Code	Ca_{10}	Ca_2
Main crystalline phases	$\text{Ca}_{10}\text{Na}(\text{PO}_4)_7$ $\text{Ca}_{10}\text{K}(\text{PO}_4)_7$	$\text{Ca}_2\text{KNa}(\text{PO}_4)_2$
Mass (%)		
CaO	44.20	30.67
MgO	0.80	2.45
P_2O_5	46.00	43.14
Na_2O	4.30	9.42
K_2O	2.70	14.32
SiO_2	2.00	–
Density (g/cm^3)	2.938 ± 0.002	2.994 ± 0.002
R_a (μm)	1.9 ± 0.1	3.4 ± 0.9
Solubility (mg/g)	11.0 ± 0.5	23.0 ± 2.2

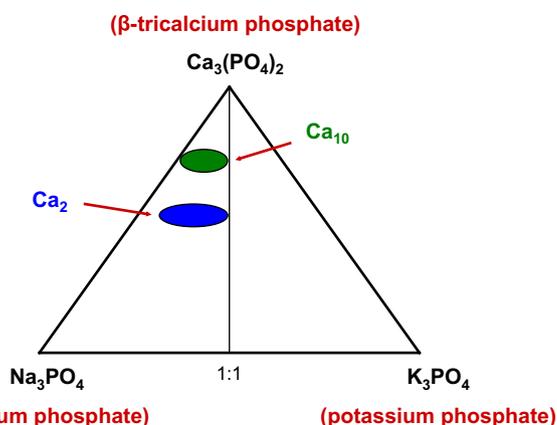


Fig. 1. Schematic representation of the chemical compositions of Ca_2 and Ca_{10} in the pseudo-ternary phase diagram of $\text{Ca}_3(\text{PO}_4)_2$ - CaNaPO_4 - CaKPO_4 .

buffy coats. Phosphate-buffered saline (PBS) and Ficoll Paque Plus (Amersham Biosciences, Uppsala, Sweden) were heated to 37°C . An aliquot of 50 ml of buffy coat cell solution was diluted with PBS to 200 ml. The cell solution diluted with PBS was carefully layered on top of 25 ml of Ficoll Paque Plus per tube in 8×50 ml tubes. The tubes were centrifuged with $350g$ at 20°C for 30 min. After centrifugation mononuclear haematopoietic precursors had accumulated at the interface between the PBS and Ficoll Paque Plus. These cells were transferred to fresh tubes, giving four tubes containing 30–40 ml of mononuclear cells. These tubes were made up to 50 ml with PBS and centrifuged at $350g$ at 20°C for 10 min. The pellets were washed with 50 ml of PBS, then resuspended in 1 ml of PBS. Cells were counted and cultivated on microplates at a density of 2×10^6 cells ml^{-1} with or without ceramic for 28 days ($n = 12$). Simulated body fluid (SBF) consisting of αMEM (PAA, Pasching, Austria) containing 0.22% sodium bicarbonate, 10% foetal bovine serum (Invitrogen GmbH, Karlsruhe, Germany), 1% penicillin/streptomycin (PAA, Pasching, Austria) was used as the cell culture medium. Osteoclast differentiation was achieved using 20 ng ml^{-1} M-CSF and 40 ng ml^{-1} RANKL (Cell Concepts, Umkirch, Germany). Half of the medium was changed every other day. As controls and to determine dissolution of the materials in SBF they were also exposed to SBF alone, without osteoclasts for 28 days ($n = 5$).

2.3. Tartrate-resistant acid phosphatase (TRAP) staining and evaluation of osteoclast numbers on each surface

After cultivation the cells were fixed with 4% buffered formaldehyde and stained for TRAP as described [10]. Cells staining positive for TRAP and with three or more nuclei were counted as osteoclasts.

2.4. Immunofluorescence and confocal laser microscopy

For actin staining cells on coverslips were dehydrated in -20°C acetone for 5 min and air dried on parafilm. They were then incubated in rhodamine-phalloidin (Invitrogen GmbH), diluted in PBS 1:40 for 20 min in the dark. After 3×5 min washes the cells were mounted with Fluosave and stored overnight at 4°C . Analysis was carried out with a Zeiss confocal laser microscope at a wavelength of 568 nm.

2.5. Scanning electron microscopy

After cultivation the materials with or without cells were critical point dried and sputtered with gold (Sputter Coater 108 auto

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