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In vivo behaviour of three calcium phosphate cements and a magnesium phosphate cement

F. C. M. DRIESSENS*, M. G. BOLTONG*, M. I. ZAPATERO‡, R. M. H. VERBEECK§, W. BONFIELD#, O. BERMÚDEZ#,**, E. FERNÁNDEZ*, M. P. GINEBRA*, J. A. PLANELL*

* *Dept de Ciencia dels Materials i Enginyeria Metal.lúrgica, Universitat Politècnica de Catalunya, Avda. Diagonal 647, 08028 Barcelona, Spain*

‡ *Centro de Investigación y Desarrollo Aplicado, Centro Industrial Santiga, C/Argenters 6, 08130-Santa Perpètua de Mogoda (Barcelona), Spain*

§ *Laboratory for Analytical Chemistry, University of Ghent, Krijgslaan 281-S12, B-9000 Gent, Belgium*

Interdisciplinary Research Centre in Biomedical Materials, Queen Mary and Westfield College, Mile End Road, London E1 4NS, UK

Three types of calcium phosphate cements and one magnesium phosphate cement were implanted subcutaneously in rats under exclusion of direct cellular contact. Retrieval times were either 1, 2, 4 or 8 weeks. Before and after retrieval the compressive strength, the diametral tensile strength, the quantitative chemical composition, the qualitative phase composition, the FTIR spectrum and the microstructure were determined. The three calcium phosphate cements maintained their strength during implantation. The phase DCPD was completely transformed into a Na- and CO_3-containing apatite, the phases DCP and CDHA only partially. It could not be ruled out that OCP is also transformed into a bone-mineral–like apatite to a certain extent. That this latter process occurs much faster during the turn-over of living bone, is probably due to the very small crystal size of the OCP particles in bone.

1. Introduction

Since the first preparation of calcium phosphate cements [1] their biological properties have been studied, as the main area of their application is thought to be that of a biomaterial. In the following, abbreviations are used according to Table I.

Gruninger *et al.* [2] tested a PHA cement obtained from a mixture of TTCP + DCP both *in vitro* and *in vivo*. *In vitro* testing included human red blood cell hemolysis, acute mouse oral toxicity, mouse fibroblast cloning efficiency, and the Ames test for mutagenicity. *In vivo* testing included subcutaneous guinea pig and rat tibia implantations of compressed, preformed cement implants. Implanted rats were sacrificed weekly through 7 weeks, and tibial bone was removed for evaluation by radiograph and standard histology. The cement appeared to be neither toxic nor mutagenic. Preformed implants were well tolerated by the animals and showed no adverse tissue reaction. After 7 weeks the tibial implants were not resorbed but were tightly situated within the holes.

The biocompatibility of this same cement was tested by Sugawara *et al.* [3] by subcutaneous implantation in Donryu rats. They found very slight inflammatory reactions around the cement implants as well as around HA ceramic implants used as controls.

As far as bioresorbability of calcium phosphates and their cements are concerned, two types may be distinguished: a material might be bioresorbable because it is not stable in the body fluids, this being without consideration of cellular activity. This type of bioresorbability might be called passive in comparison with the active type mediated by cellular activity. A typical example of a material having passive bioresorbability is plaster of Paris or gypsum. According to its solubility in near-neutral solutions [4] a DCPD cement is also expected to have passive bioresorbability. However, calcium phosphate cements of the types OCP or CDHA are expected to show only active bioresorbability: the activity of the osteoclasts is necessary and sufficient to make them resorb. It is questionable whether PHA cements are bioresorbable, because it is not evident [5–7] that osteoclast activity is sufficient to make them resorb. Compare also the fact that SHA ceramic granules implants are integrated but not resorbed into a bone structure [8–13].

β-TCP implants are as osteoconductive as SHA ceramic implants are, but simultaneously they are

** Present address: Dept de Física, Universidad del Cauca, Popayán, Colombia.

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TABLE I Names, abbreviations and formulae of the calcium phosphates occurring in this study

Name	Abbreviation	Formula
Monocalcium phosphate monohydrate	MCPM	$Ca(H_2PO_4)_2 \cdot H_2O$
Dicalcium phosphate	DCP	$CaHPO_4$
Dicalcium phosphate dihydrate	DCPD	$CaHPO_4 \cdot 2H_2O$
Octocalcium phosphate	OCP	$Ca_8(HPO_4)_2(PO_4)_4 \cdot 5H_2O$
Beta-tertiary calcium phosphate	β-TCP	Ca_3PO_4
Alpha-tertiary calcium phosphate	α-TCP	Ca_3PO_4
Calcium deficient hydroxyapatite	CDHA	$Ca_9(HPO_4)(PO_4)_5OH$
Precipitated hydroxyapatite	PHA	$Ca_{10}(PO_4)_6(OH)_2$
Sintered hydroxyapatite	SHA	$Ca_{10}(PO_4)_6(OH)_{2-2x}O_x$

replaced by new bone tissue after some time [8–13]. This type of biodegradability is certainly not passive [14–18]. Hence, β-TCP combines the property of active biodegradability with that of osteoconductivity, in such a sense that the process taking care of its biodegradation is exactly that also inducing the new formation of bone. For this reason one might call this unique combination of biological properties osteotransductivity. On the basis of their natural involvement in bone mineral turnover [19–21] we expect the same osteotransductivity for calcium phosphate cements of the type OCP or CDHA.

Sugawara *et al.* [3] implanted a PHA cement made of TTCP + DCP in surgically formed pockets in the lower jaws of dogs. They found no inflammatory reactions in tissue areas adjacent to the implants. The cement mass was covered with new bone and periosteum whereas some cement particles were replaced by bone. They did not mention the retrieval time of the implants.

Xie and Monroe [22] placed a PHA cement made of TTCP + DCPD in the jaws of rats. Retrieval times were 4 and 12 weeks. When the cement had been mixed with a $Ca(OH)_2$ solution for setting, there was good osteoconductivity. However, when a H_3PO_4 solution was used connective tissue was found between the implant and the bone.

Constantz *et al.* [23] implanted a DCPD cement in the femora of rabbits. They confirmed that DCPD cements have a passive bioresorbability. However, simultaneously some transformation into bone apatite occurs apparently due to the fact that the dissolution of DCPD is so fast that the bone extracellular fluid becomes supersaturated with bone mineral apatite. After about 8 weeks a Ca/P ratio of 1.61 is found in the remnants of the implant.

Gunasekaran *et al.* [24] implanted a PHA cement and an aggregate of PHA cement with 50 vol % $CaCO_3$ in the femora of rabbits. It is known that $CaCO_3$ is passively resorbable. However, surprisingly its presence in the aggregate seemed to activate the active biodegradation of the PHA part of the aggregate as compared to the PHA cement control. The effect may be similar to that of macroporosity in calcium phosphate ceramics [25].

Hong *et al.* [26] used a PHA cement made of TTCP + DCP for endodontic treatment in the teeth of monkeys. After 1 month they observed minimal adverse tissue reactions. However, adjacent to the cement new bone formation was observed.

Constantino *et al.* [27] evaluated the histologic response of a PHA cement by implanting discs within the heads of nine cats. Three sets of 12 PHA cement discs were made containing either 0, or 10 or 20% macropores by volume. The discs were implanted subcutaneously, intramuscularly, above the periosteum of the skull or directly on to the surface of the calvarium. Animals were killed up to 9 months postoperatively. There were no toxic reactions, implants extruded or wound infections. Histologic examination of the implant–soft tissue interfaces revealed a transient inflammatory response without foreign body reaction. The discs were resorbed during implantation proportionally to their macropores content in all groups except for those discs placed directly on to the surface of the calvarium below the periosteum. In this group, foci of bone formed at the skull–implant interface, with variable replacement of the deep surface of these implants by bone. Implant replacement by bone might occur according to these authors [27] through a combination of implant resorption coupled with osteoconduction.

From these data it is clear that even "PHA" cements are osteotransductive: in older papers Chow *et al.* [28, 29] held that their PHA cement would not be resorbable, but lately Chow explained [30] that it appeared to be "the first calcium-based cement that sets to resorbable apatite and is replaced by bone in an approximate one to one relationship". In other words Chow [30] explained that these cements are osteotransductive. This may also be apprehensible when these cements may be considered as CDHA cements rather than PHA cements, as was argued already by Brown *et al.* [31–34].

Further proof for the osteotransductivity of calcium phosphate cements was found by Munting *et al.* [35]. They filled bone defects made metaphysially in the long bones of adult mongrel dogs with a cement consisting of a dispersion of β-TCP particles in a three-dimensional network of DCPD clusters. Microradiography, histology and scanning electron microscopy was used to evaluate the bone structures obtained after 4 or 7 months retrieval times. These investigations demonstrated the slow resorption of the cement and the simultaneous ingrowth of bone into the defect, in such a way that the original structural

TABLE II Ingredients used for the composition of the powders for preparation of the cements

Cement	Active ingredients	Intended Ca/P	Nucleator added	Reference
A	MCPM + $Ca(OH)_2$	1.13	—	39
F	DCP + α-TCP	1.36	PHA 2%	37
H	α-TCP	1.50	PHA 2%	39
V	DCP + MgO + $MgHPO_4 \cdot 2H_2O$	0.50	—	38

pattern of the bone tended to be restored 7 months after implantation.

The purpose of the present study was to determine the effect of extracellular fluid *in vivo* on the strength and the composition of certain cements. We chose four types according to those mentioned in the literature [36]:

Type A consisting mainly of DCPD
Type F consisting supposedly of OCP
Type H consisting mainly of CDHA
Type V consisting mainly of $Mg_3(PO_4)_2 \cdot 8H_2O$ in which a calcium phosphate was dispersed, in this case DCP.

2. Materials and methods

In Table II the components used to make the four cements are listed. Cylinders were prepared with a height of 12 mm and a diameter of 6 mm. In order to complete the setting reaction they were soaked in Ringer's solution at 37 °C for at least 2 days. They were covered each with a stainless steel gauze and implanted subcutaneously in male Wistar rats weighing between 150 and 200 g. Of each cement, 40 cylinders were implanted, two in each rat after sterilization of the gauzes containing the cement cylinder with γ rays. Diet and water were provided *ad libitum*. Retrieval times were either 1, 2, 4 or 8 weeks after anesthetization with sodium pentobarbital 1% (5 ml/kg i.p.).

After retrieval it appeared that the gauzes had been effective in excluding direct cellular contact from the cement cylinders. The cylinders were inspected visually for any changes of form or size (nonius). Then half of them ($n = 5$ for each cement per retrieval time) was used for determination of the compressive strength, the other half for that of the diametral tensile strength with an Instron Universal Machine Type 4507.

Some of the remaining pieces of cement were ground in a mortar, dried at 80 °C in a stove and used for chemical analysis. The calcium content of the samples was determined by a complexometric titration with ethylenediaminetetraacetic acid and the phosphorus content spectrophotometrically as orthophosphate. The magnesium and sodium content was determined by atomic absorption spectrophotometry and the carbonate content with a gas chromatographic procedure. The relative uncertainties on the amount of Ca, P, Na, Mg and CO_3 analysed are 0.2, 0.2, 2, 2 and 5%, respectively [37].

Other parts of the powdered cements were subjected to X-ray diffraction and to Fourier transform infrared spectroscopy (FTIR), the latter in order to determine whether the carbonate incorporated was of the A or B type. Scanning electron microscope (SEM) pictures were also taken both before and after implantation in order to observe the microstructure.

3. Results

From visual inspection it was derived that none of the cement cylinders had changed their form or size. Data for the compressive strength as a function of the retrieval time are given in Table III, those for the diametral tensile strength in Table IV. Only cement V diminished in strength with time of implantation, the other cements maintained their strength.

The chemical composition of the set and soaked cements before implantation and after 8 weeks of implantation is given in Table V. Cements A, F and H had an increase in their Na and CO_3 content and FTIR showed that this CO_3 was of the B type substituting for phosphate groups. As an example part of the FTIR spectrum of cement F before and after implantation is given in Fig. 1. Cement V had a loss of Mg and of CO_3.

TABLE III Compressive strength (MPa) of some calcium phosphate cements as a function of the time of subcutaneous implantation in rats ($n = 5$)

Implantation time (weeks)	Cements			
	A	F	H	V
0	4.8 (0.3)[a]	28 (4)	36 (4)	11 (2)
1	4.6 (0.7)	28 (3)	33 (10)	12 (2)
2	4.5 (1.0)	29 (5)	36 (7)	6 (2)
4	4.4 (0.7)	27 (5)	32 (7)	5 (2)
8	4.4 (1.0)	26 (3)	41 (6)	3 (1)

[a] Standard deviation in parentheses.

TABLE IV Diametral tensile strength (MPa) of some calcium phosphate cements as a function of the time of subcutaneous implantation in rats ($n = 5$)

Implantation time (weeks)	Cements			
	A	F	H	V
0	1.1 (0.3)[a]	4.8 (0.1)	8.0 (0.8)	3.7 (0.7)
1	1.0 (0.2)	4.6 (0.4)	7.8 (0.9)	3.3 (0.8)
2	1.0 (0.2)	3.9 (0.8)	7.1 (1.2)	2.5 (0.4)
4	1.0 (0.1)	4.1 (1.0)	8.3 (0.6)	1.7 (0.4)
8	1.2 (0.3)	5.3 (1.2)	7.7 (0.7)	0.8 (0.2)

[a] Standard deviation in parentheses.

TABLE V Chemical composition of some calcium phosphate cements before implantation and after 8 weeks of subcutaneous implantation in rats

Cement	Implantation time (weeks)	Na (%)	Mg (%)	Ca (%)	P (%)	CO_3 (%)	Ca/P
A	0	0.305	0.248	30.35	20.20	0.295	1.161 (0.003)[a]
	8	0.692	0.248	30.67	16.89	1.28	1.403 (0.004)
F	0	0.209	0.120	34.96	19.74	0	1.368 (0.004)
	8	0.412	0.131	34.63	19.40	0.283	1.379 (0.004)
H	0	0.251	0.098	36.35	18.60	0.060	1.510 (0.005)
	8	0.485	0.111	36.17	18.38	0.458	1.521 (0.005)
V	0	0.375	12.93	11.35	16.85	1.66	0.520 (0.002)
	8	0.392	9.17	13.67	17.29	0.874	0.611 (0.002)

[a] Standard deviation calculated on the basis of the analytical results.

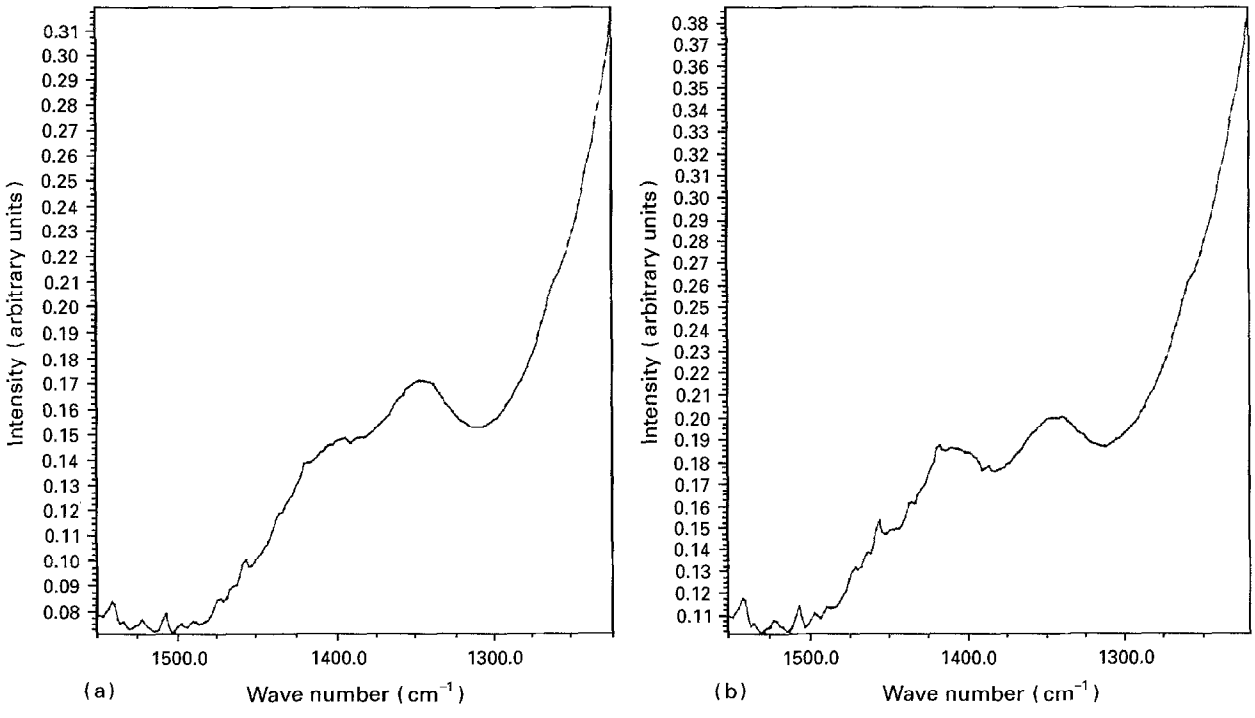

Figure 1 Part of the FTIR spectrum of cement F (a) before and (b) after 8 weeks of implantation. The absorbance at 1412 cm^{-1}, which is typical for β-type CO_3, increases after implantation.

The qualitative phase composition of the cements after complete setting and soaking and after 8 weeks of implantation was derived from their X-ray diffraction pattern. Fig. 2 shows the XRD pattern of cement H as an example. In this cement no changes of the phase composition occurred by implantation. The overall results are given in Table VI. It appears from cement A that DCPD is transformed into an apatite within 8 weeks. This apatite contains some Na and CO_3 as may be concluded from the experimental evidence of the chemical analysis and the FTIR spectra in conjunction with the knowledge that calcium phosphates other than apatite do not incorporate any of these ions. From the data for cements F and V it appears that the transformation of DCP into apatite is much slower. On the other hand, it certainly occurs, because one can calculate from the chemical composition of cement V after 8 weeks of implantation that the Ca/P molar ratio of the mixture of calcium phosphates in that cement is not 1, but 1.12. Furthermore, the apatite phase was clearly observable in its X-ray diffractogram. From the data of cements A and F it is observed that the transformation of OCP into apatite is very slow. However, that it occurs to a limited extent, cannot be ruled out. Transformation of the CDHA of cement H into a bone-mineral–like Na and CO_3 containing apatite occurs, but at a very limited rate.

In Figs 3 and 4 representative SEM pictures of the microstructure of cement H and F, respectively, are given for two different magnifications. No changes were observable. For cement V it was not possible to relate the decrease in strength with any changes in the microstructure. This may be due to the fact that it is not feasible to derive the strength of crystal entanglement simply from pictures of the microstructure.

4. Discussion

The transformations of DCPD as well as DCP into Na and CO_3 containing apatite occur as expected on

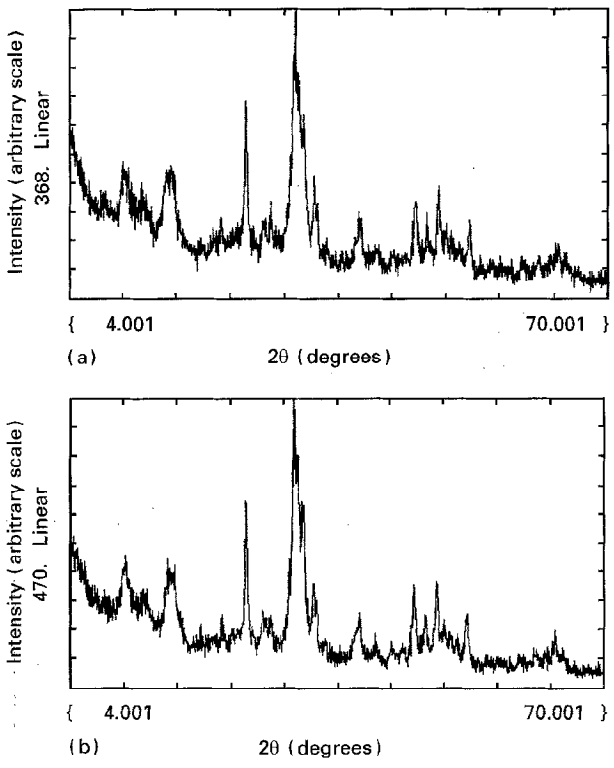

Figure 2 XRD pattern of cement H (a) before and (b) after 8 weeks of implantation. No change in the phase composition is observed.

TABLE VI Qualitative phase composition of some calcium phosphate cements before implantation and after 8 weeks of subcutaneous implantation in rats according to X-ray diffraction

Cement	Implantation time (weeks)	Phase composition
A	0	DCPD + OCP
	8	OCP + apatite
F	0	DCP + OCP + apatite
	8	DCP + OCP + apatite
H	0	apatite
	8	apatite
V	0	DCP + $Mg(OH)_2$ + $Mg_3(PO_4)_2 \cdot 8H_2O$
	8	DCP + $Mg_3(PO_4)_2 \cdot 8H_2O$ + apatite

the basis of their known instability in comparison with apatite in the physiological range [6]. The same holds for the transformation of CDHA into a bone-mineral–like apatite. That the latter transformation is so limited may be due to the transformation of only the surface layer of the CDHA particles up to the point that the extracellular fluid touches only that transformed surface layer.

More unexpected is the fact that the transformation of OCP in cements A and F is so much rate limited. During the continuous remodelling of bone the precursor mineral in the newly formed bone is OCP and this is transformed into a Na and CO_3 containing apatite at such a rate that the half-life of OCP in bone is estimated to be of the order of only 1 month. Thereby, it is thought that this transformation is not influenced by cellular activity but is a purely passive process [38, 39]. This difference in transformation rate between the cements and the bone mineral is probably due to the effect of crystal size. From our SEM observations in Fig. 4 we know that the size of the OCP

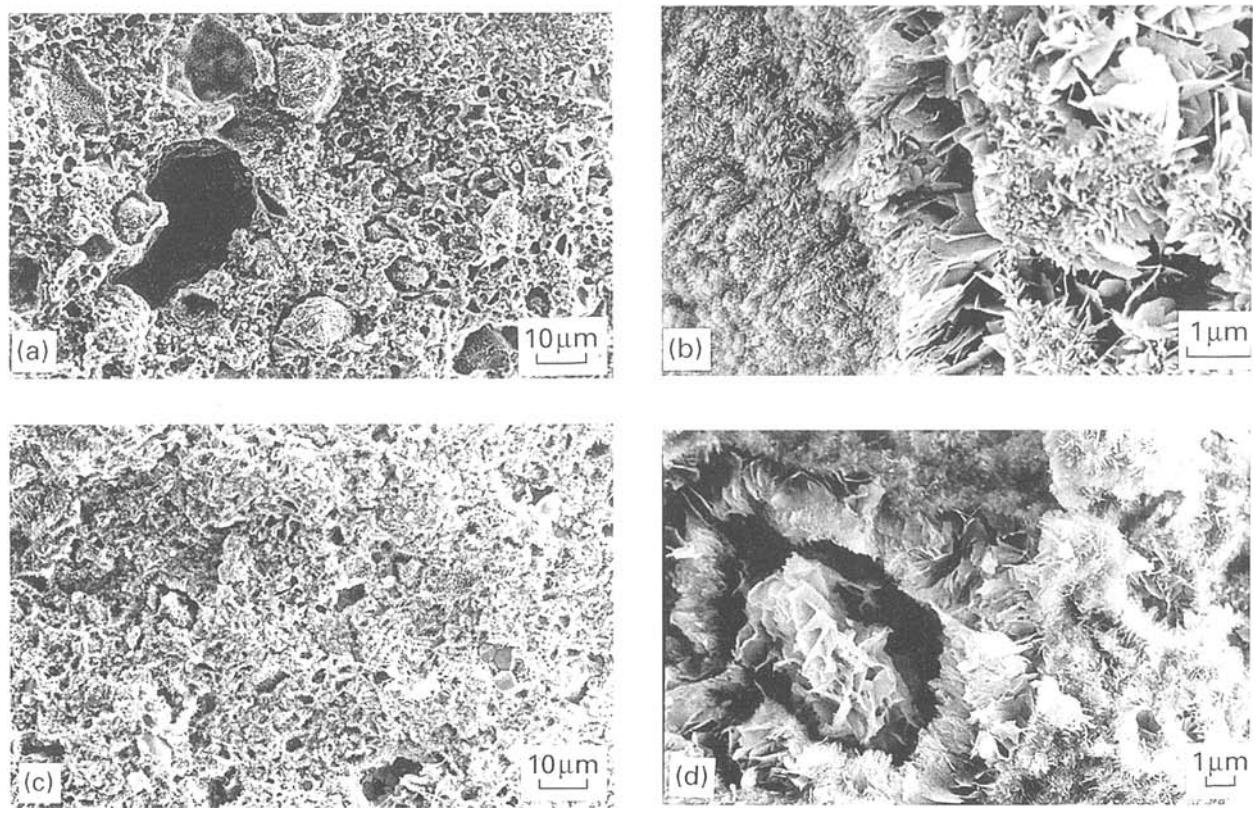

Figure 3 Representative SEM pictures of the microstructure of cement H at two different magnifications before implantation (3a and 3b) and after 8 weeks of implantation (3c and 3d).

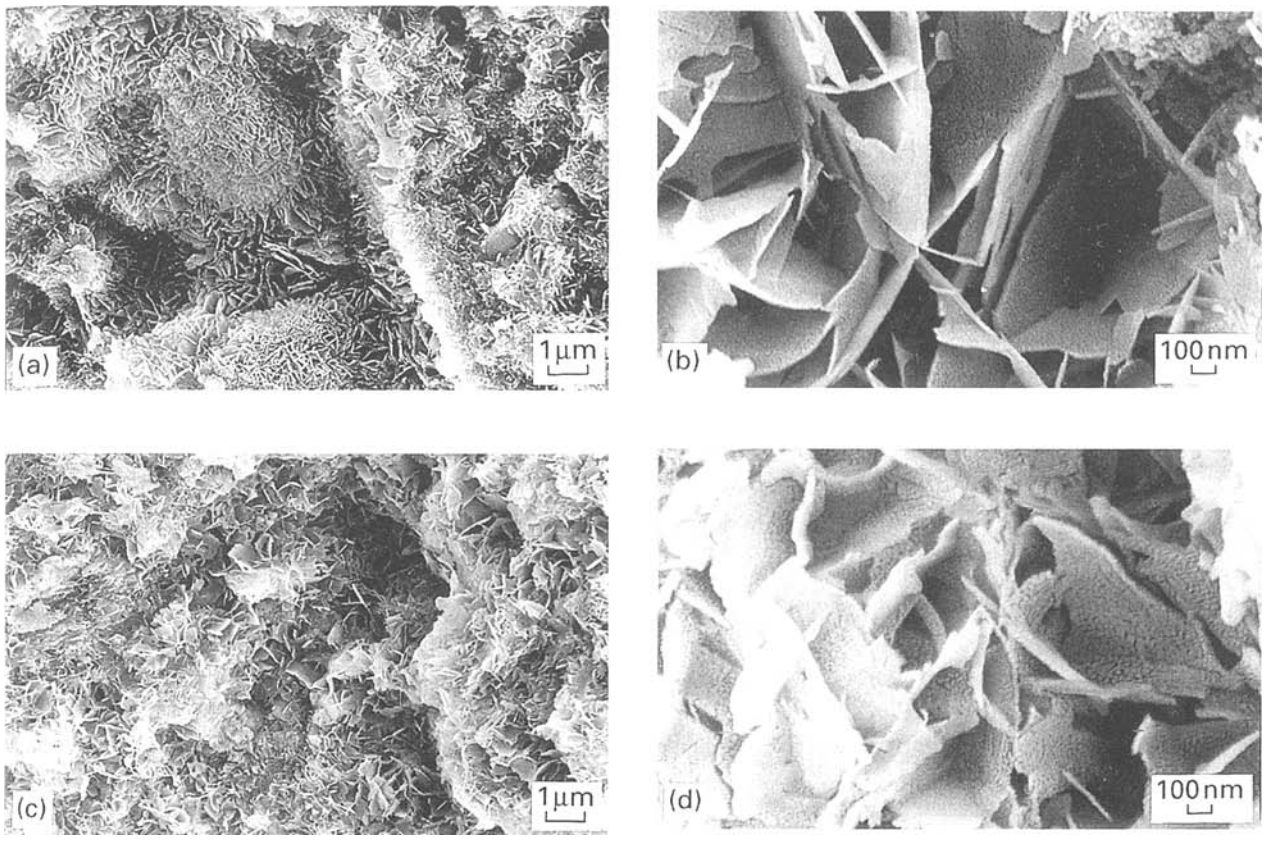

Figure 4 Representative SEM pictures of the microstructure of cement F at two different magnifications before implantation (4a and 4b) and after 8 weeks of implantation (4c and 4d).

crystals in our cements is of the order of 1 μm [40, 41]. However, the apatite crystals of bone mineral have a length of only about 60 nm, a width of 30 nm and a thickness of no more than 5 nm and the apatite crystals may be even somewhat larger than the OCP precursor crystals, from which they are formed [42, 43].

Only cement V, which is in fact not a real calcium phosphate cement but a magnesium phosphate cement, decreased in strength with time of implantation. That the phase $Mg(OH)_2$ was completely dissolved, is probably not the cause of this effect. More likely is that the clusters of $Mg_3(PO_4)_2 \cdot 8H_2O$ crystals dissolve slowly. However, whether this has occurred cannot be derived from the present chemical data which are expressed in weight per cent. For that purpose data of amounts of $Mg_3(PO_4)_2 \cdot 8H_2O$ phase per unit volume would have been necessary.

Follow-up studies are going on to observe the osteotransductivity of some of our calcium phosphate cements upon implantation in bone.

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