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PRELIMINARY *IN VIVO* STUDIES OF A NEW INJECTABLE BONE SUBSTITUTE

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Abstract

Calcium-phosphate materials have been increasingly employed in orthopedic and dental applications in recent years and are now being developed for use in noninvasive surgery or as carriers for drug delivery systems. We developed an injectable bone substitute (IBS) for percutaneous orthopedic surgery which uses a biphasic calcium-phosphate (BCP) mixture composed of hydroxyapatite (60%) and β-tricalcium phosphate (40%), together with a polymer (hydroxy-propyl-methyl-cellulose, HPMC) as a carrier. The best BCP/polymer ratio was determined to achieve the highest mineral phase in the composite and provide the rheological properties required for injectable material.

The *in vivo* biocompatibility and biofunctionality of IBS were tested in rabbits using implants in subcutaneous, intramuscular and cartilage sites and defects created in trabecular bone of the femur epiphysis. The defects were filled with IBS, and histological studies were performed after 1, 2, 4 and 12 weeks on decalcified and non-decalcified sections. Image analysis and backscattered electron analyses were performed by scanning electron microscopy.

This study demonstrated that HPMC is a non-toxic material which can be associated with calcium-phosphates to produce an IBS and create a matrix for deep cell colonization conducive to bone ingrowth.

Key Words: Injectable bone substitute, biphasic calcium phosphate, biomaterial.

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Introduction

In recent years, self-setting calcium-phosphate cements have proved of considerable interest in orthopedic and dental applications [3, 5, 13, 19, 20, 21, 22, 23, 27, 29, 30]. Calcium-phosphate materials have also been used as components or fillers in polymeric composites [2, 14], in association with polymers such as polysaccharides [1] or elastin or collagen of animal origin [16]. However, these products have not proved efficient for bone osteoconduction and ingrowth [25]. Some ionic calcium-phosphate cements have been developed, including one used in clinical studies [5, 20]. Yet it has been clearly demonstrated that macropores are required for bone ingrowth [7, 12], and these materials are not macroporous [13, 25].

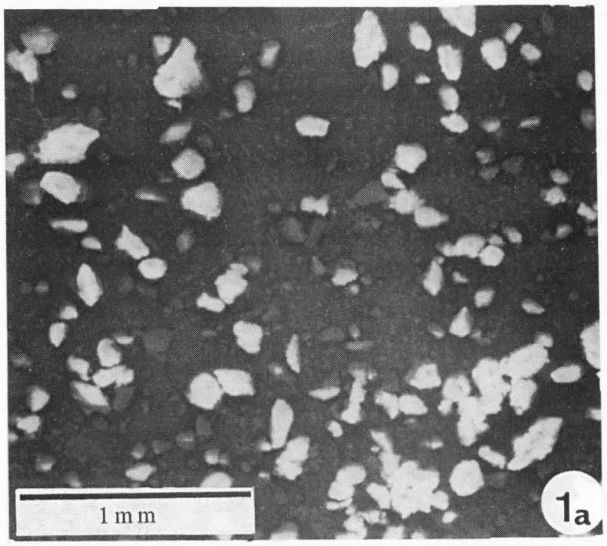
The aim of this study was to develop a ready-to-use injectable bone substitute (IBS) which could be efficiently colonized by osteogenic cells. This material needed to be perfectly biocompatible and potentially resorbable (synthetic bone graft material) and needed to possess an initial plasticity suitable for the filling of bone defects. Good *in situ* cohesion is necessary until the implant is completely replaced by newly-formed bone.

For this purpose, we developed a composite of calcium-phosphate granules and a hydrosoluble polymer [32, 33]. Biphasic calcium phosphate (BCP) was chosen because of its efficiency as a bone substitute and in promoting bone ingrowth [6, 8, 9, 16]. Cellulosic ethers were selected on the basis of their applications in pharmaceutical systems and their biocompatibility *in vitro* [4, 17].

Materials and Methods

Biomaterials

Polymer solution (HPMC) Methylhydroxypropylcellulose (HPMC-MP 824; Aqualon-Herculès, France), with an average molecular weight of 700,000, was prepared in a 2% solution (w/w) by dissolving the powder in bidistilled water under stirring for 48 hours. Solution viscosity was adjusted to 14,000 mPa·s at 25°C by



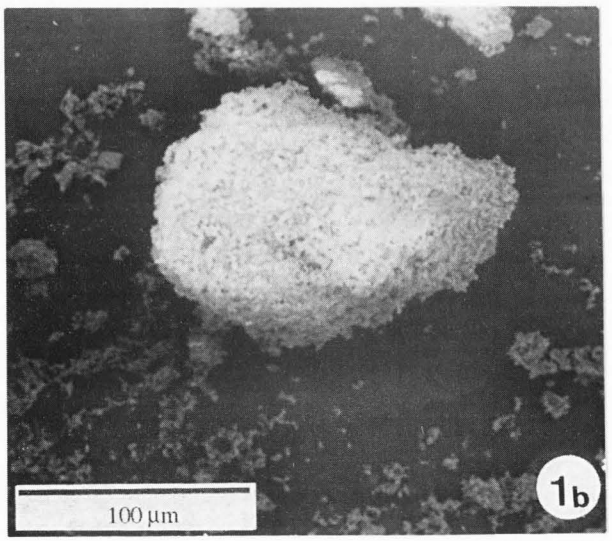


Figure 1. Scanning electron micrographs of BCP grains used for preparation of the composite, showing the wide range of particle size (a) and the aggregates of single crystals at a higher magnification (b).

addition of water or polymer under the control of an LDVI+ Brookfield viscometer (Brookfield, Stoughton, MA).

Mineral Phase BCP granules [60% hydroxyapatite (HA), 40% β-tricalcium phosphate (β-TCP)] with a grain size of 80 to 200 μm were obtained by crushing blocks of Triosite™ (Zimmer, Paris, France) and riddling the powder (Analysette, Fritsch, Germany).

Composite The IBS was prepared before implantation and injected similarly to conventional orthopedic cement {PMMA: poly(methylmethacrylate)}. The w/w ratio was 60% for the mineral phase and 40% for the polymer solution.

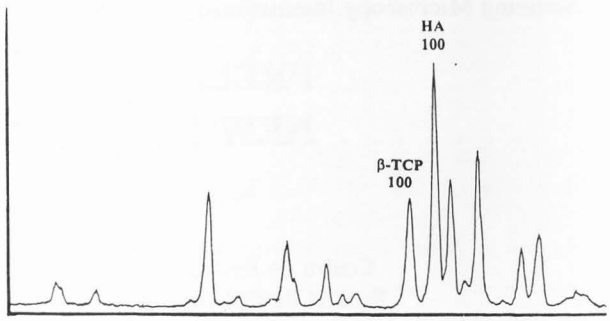


Figure 2. X-ray diffraction pattern (obtained at 40 kV and 30 mA) of the mineral phase used for preparation of the composite.

Sterilization The BCP powder was sterilized by steam at 120°C for 1 hour. The polymer solution was prepared in a clean room (class 100) in sterile conditions. The composite was mixed just before implantation.

Physico-chemical analysis X-ray diffraction (XRD) according to the AFNOR (Association Française de Normalisation) standard (S 94-066) for calcium phosphates was used to check the HA/β-TCP ratio in BCP and in the IBS after air-drying. Powders were examined using a Siemens D5000 Kristalloflex (Siemens, Iselin, NJ) in the range of $20° < 2\theta < 40°$.

Grain size Powder grain size was checked by scanning electron microscopy (SEM; JEOL 6300, Tokyo, Japan) and image analysis (Quantimet 500, Leica, Deerfield, IL).

Animal experiment

In vivo tests were performed according to AFNOR standard NF S90-703 (December, 1989) for surgical implants. The standard recommends the osseous and non-osseous implantation site. Eleven adult, female New Zealand white rabbits (controlled sanitary status), weighing between 2.5 and 3 kg, were selected. Two animals were sacrificed after 1 week, 3 after 2 weeks, 3 after 4 weeks, and 3 after 12 weeks.

Bone defects Bone defects were performed in a trabecular bone site of rabbit femoral and tibial epiphysis. Under sterile conditions, an approach to the knee was made to reach the medial side of the tibia and femur. A bone defect with regular borders (5 mm in diameter and 12 mm in length) was produced by manual excavation parallel to the joint line without breaking the intramedullary canal in the joint space. Femoral bone defects were filled with 0.5 g of IBS, whereas the right tibia defect was filled with BCP blocks, and the left one not filled (control).

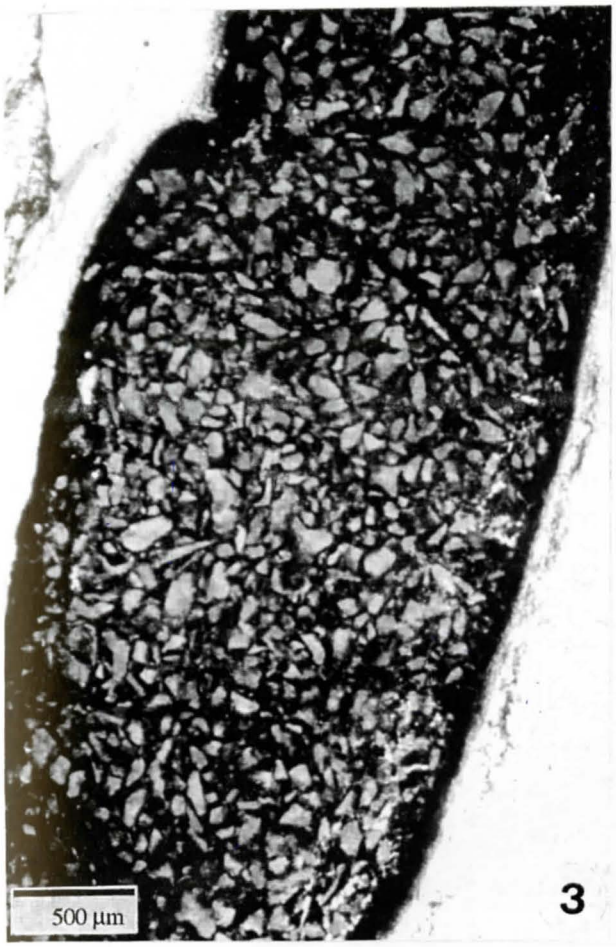


Figure 3. A homogeneous degradation front for BCP grains was observed after 1 week of subcutaneous implantation.

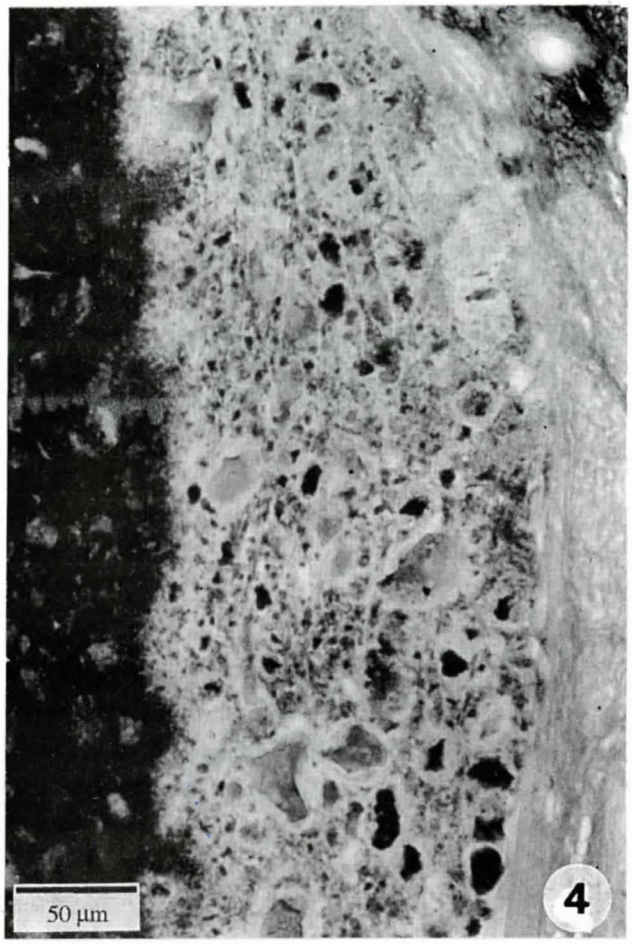


Figure 4. After 2 weeks of implantation, biomaterial appeared less dense from the surface to the core.

Subcutaneous sites Two subcutaneous sites on each side of the ventral face were injected respectively with HPMC alone and IBS.

Muscle sites Four muscle sites in the paraspinal lumbar area were filled in a similar manner (2 with HPMC alone and 2 with IBS).

Cartilage site A new model of cartilage site was developed in the anterior border of rabbit ear. IBS were implanted in the core of the cartilage.

Histological and ultrastructural techniques

Light microscopy Recovered samples were fixed with 10% neutral formaldehyde solution. Half of the samples were decalcified by a 5% HNO_3 solution and embedded in paraffin for histological studies. Sections (7 µm) were stained using the Masson-Goldner trichromic method [26]. The other samples were embedded in methylmethacrylate. All samples were sectioned with a LEITZ 1600 diamond saw (Leitz, Wetzlar, Germany) into 50 to 100 µm thick slices. The slices were stained using Movat's pentachrome and observed in normal and polarized light microscopy. X-ray microradiography was performed at 20 kV on these same sections.

Electron microscopic techniques After diamond saw sectioning, residual blocks were carbon-coated and examined by SEM (JEOL 6300) operating at 15 kV, and using backscattered electron imaging (BSE).

Image analysis was performed on sections using a Quantimet 500 system (Leica).

Results

Before implantation

Viscosimetry HPMC-MP 824 (2%) has a viscosity of between 15,000 and 20,000 mPa·s, without aggregate formation. The non-ionic structure of HPMC was also taken into account in choosing a polymer in order

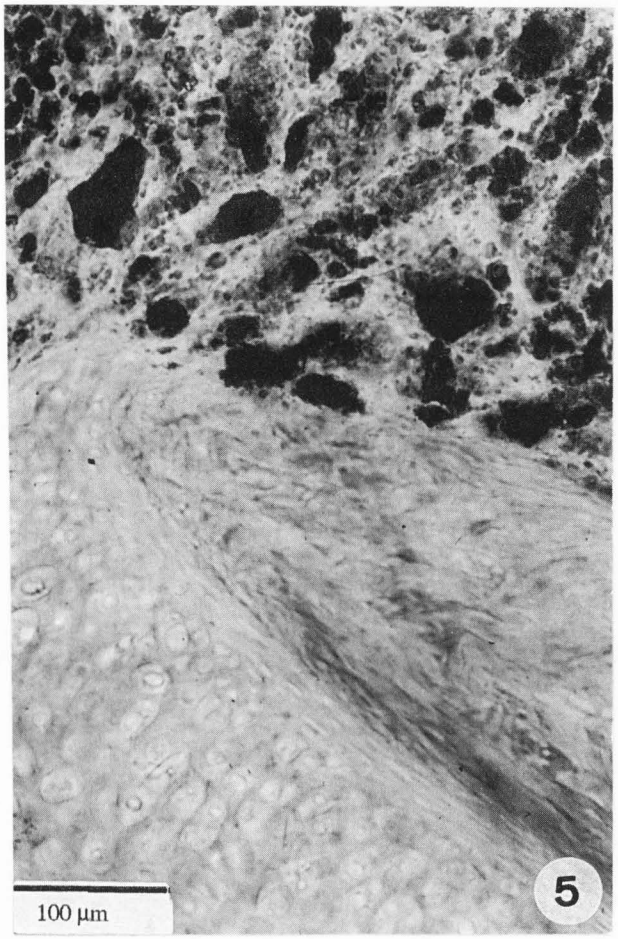


Figure 5. Connective tissue was in direct contact with cartilage after 1 week of implantation in a cartilage site.

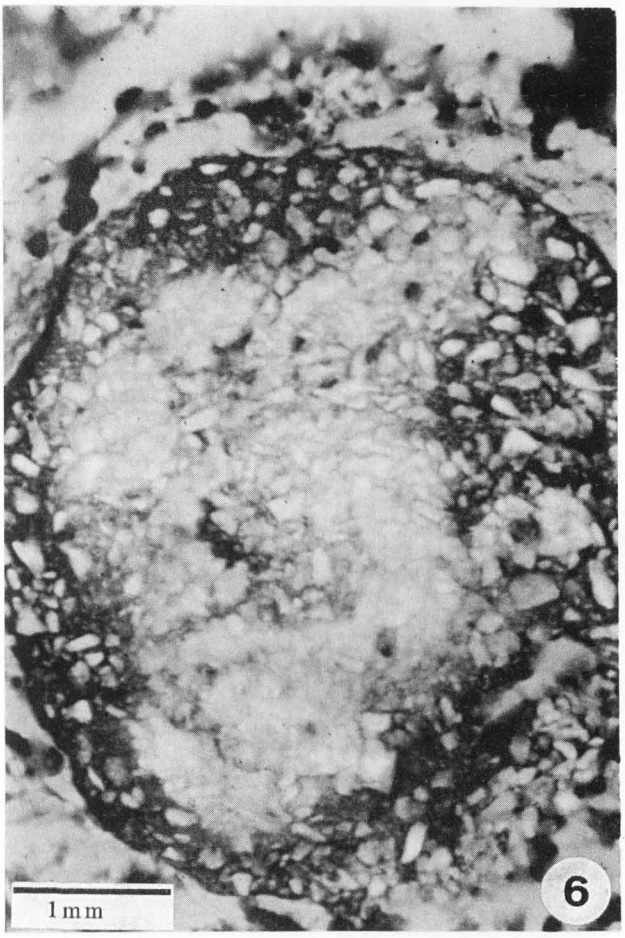


Figure 6. Section of materials in a bone site studied in microradiography after 2 weeks of implantation.

to prevent strong interaction with Ca^{2+}. The optimal ratio for the BCP/polymer solution was 60/40 (w/w), as determined by the inflection of the viscosity curve.

Grain size BCP granules observed in SEM showed a wide range of grain size (Fig. 1a). Measurements indicated that 46% of the volume was composed of particles 60-200 μm, 21.5% was composed of 30-60 μm particles, 26.1% was composed of 10-30 μm particles, and 1.1% was made of less than 10 μm sized particles. SEM revealed that the granules were composed of aggregates of single crystals 1 to 5 μm in diameter which were more or less associated by grain boundaries (Fig. 1b). Microporosity, as determined by image analysis of SEM observations, represented 33% $\pm$ 0.5% of the grain surface area.

Physico-chemical analysis XRD indicated the HA and β-TCP content of the composite (Fig. 2). The HA/β-TCP ratio was determined using the 2θ peaks at 31.81° for HA and at 31.03° for β-TCP, according to the method of LeGeros [24].

Histological results: Light microscopy

(1) **Subcutaneous and intramuscular sites:** After 1 week, no inflammatory reaction or fibrous encapsulation was noted. A homogeneous degradation front was observed between the BCP grains (Fig. 3). After 2 and 4 weeks, the resorption of microparticles was regular, and the biomaterial became less dense from the surface to the core (Fig. 4). No encapsulation membranes were observed with the decalcified technique, and there was an increase in the number of cells in the central zone of the implanted area.

(2) **Cartilage site:** Connective tissue was in direct contact with cartilage after 1 week of implantation. The process appeared to be similar after 2 to 4 weeks of implantation (Fig. 5).

(3) **Bone site:** After 1 and 2 weeks of implantation, a decrease in the density of the biomaterial surface was observed in conjunction with the organization of woven

Table 1. X-rays microanalysis of Ca and P content of the newly formed bone and the BCP grains remaining after implantation expressed in weight percentage (mean/standard error); n = 20; significant differences are < 5%.

Weeks		Ca	P	Ca + P	Ca/P
--	Host bone (n = 60)	27.9/2.0	13.0/0.8	40.9/2.6	2.2/0.1
2	Newly-formed bone	25.7/4.8	11.0/1.9	36.7/6.9	2.3/0.1
2	BCP	32.6/2.5	15.8/1.9	48.3/4.6	2.1/0.1
4	Newly-formed bone	23.3/4.0	10.7/1.9	34.0/5.8	2.2/0.2
4	BCP	34.2/2.2	16.6/1.8	50.8/2.1	2.1/0.2
12	Newly-formed bone	28.8/1.4	10.2/2.0	39.0/2.5	2.8/0.2
12	BCP	39.0/1.3	19.0/2.0	58.0/3.2	2.1/0.2

Table 2. Image analysis performed on the surface area occupied by peripheral bone, newly-formed bone and residual BCP grains (mean/standard error); n = 10; significant differences are < 5%. Op. = operation.

	Host bone	Newly-formed bone	BCP grains
Post op.	-	-	56.3%/5.6
1 week	48.9%/2.9	-	46.3%/5.2
12 weeks	49.3%/3.0	40.9%/2.3	38.1%/2.3

bone matrix all around the larger particles. In microradiography, BCP granules were linked to newly-formed bone (Fig. 6).

After 4 weeks, bridges of woven bone appeared between the granules, but the biomaterial remained very dense in the central zone (Fig. 7).

After 12 weeks, trabecular bone was observed all around and between the larger particles (> 80 μm) (Fig. 8). These residual granules appeared to be completely covered with lamellar bone. Spaces previously occupied by the polymer were totally filled by newly formed bone, and smaller particles were no longer observed.

Scanning electron microscopy SEM revealed bone ingrowth after only 2 weeks of implantation (Fig. 9). After a longer implantation time, lamellar bone was observed in direct contact with the larger BCP grains, without a fibrous interface (Fig. 10). The spaces between the grains surrounded by newly-formed bone were occupied by bone trabecules. Numerous osteocytes with a broad range of sizes were observed.

X-ray microanalysis Table 1 reports the calcium and phosphorus content of newly-formed bone in direct contact with BCP particles after 2, 4 and 12 weeks of implantation of host bone, as well as the number of BCP particles remaining after implantation.

Image analysis Image analysis was performed before implantation and at 12 weeks after implantation. The surface occupied by host bone, newly-formed bone and BCP particles are reported in Table 2.

After 12 weeks, the total surface area occupied by newly-formed bone and residual BCP grain was 79%. The size of these residual grains was 104 $\pm$ 32 μm in diameter.

Discussion

Like Katthagen [19], we chose a model different from other cement-filling models. Defects have generally been created in rabbit femoral epiphysis or in the intramedullary of canine femora [29]. Implantation in trabecular bone in our study was justified to test the efficiency of IBS in filling bone defects without large diffusion into trabecular bone and to determine the resorption and bone replacement which occur to the detriment of such material. To address certain questions raised by surgeons concerning the behavior of BCP granules during injection in non-bone sites, we also tested biocompatibility in subcutaneous, intramuscular and cartilage implantation sites.

Injectable bone substitute cannot be compared to ionic bone cement which is able to undergo a hardening process after mixing of the components [5, 13, 16, 21, 24, 25]. However, bone cells in IBS are able to invade the spaces created by the disappearance of the polymer, so that general bone ingrowth occurs to the detriment of

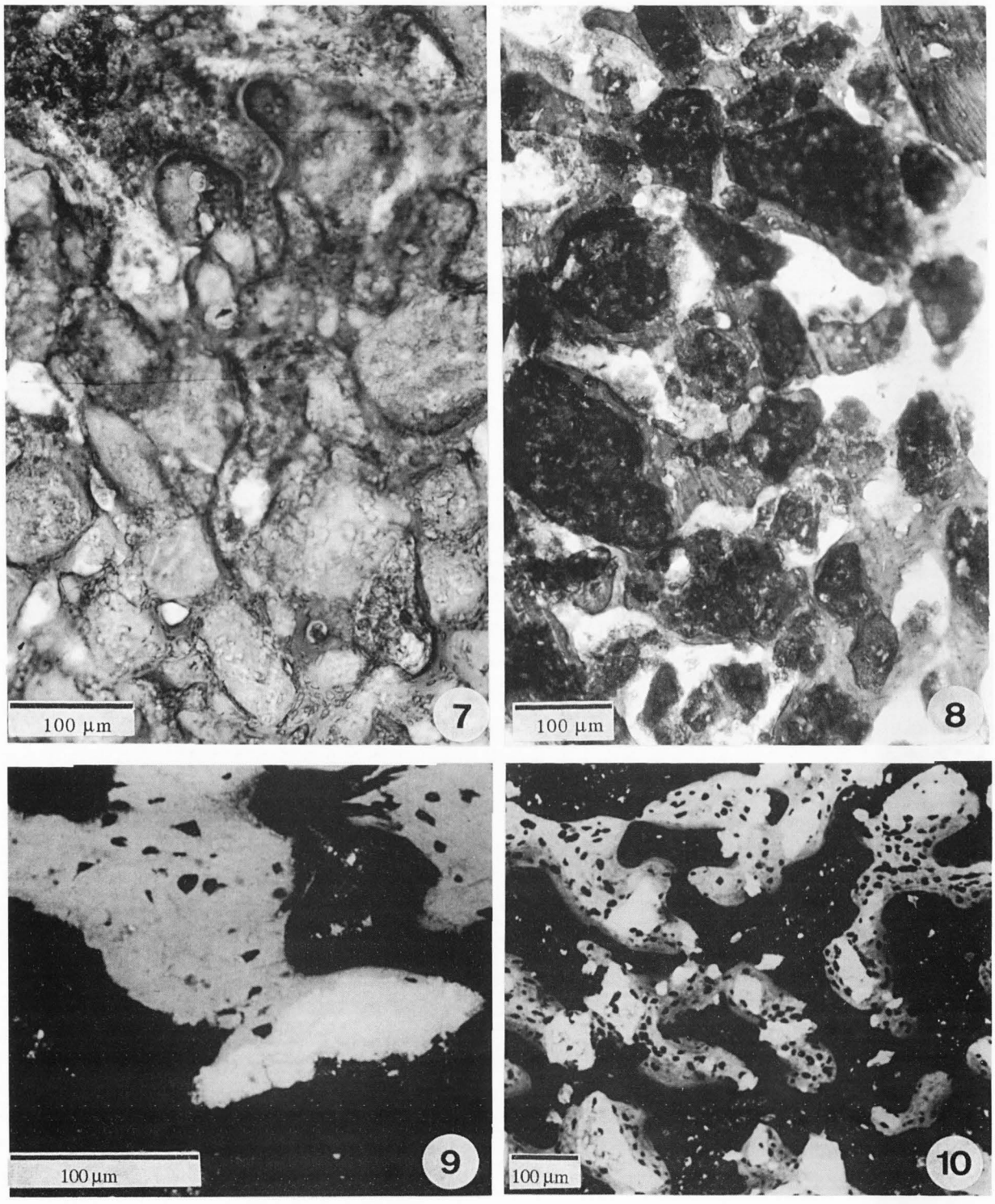


Figure 7. After 4 weeks, bridges of woven bone appeared between the granules.

Figure 8. After 12 weeks, trabecular bone was composed of a network of bone bridges between the larger particles.

Figure 9. Scanning electron microscopy using backscattered electron imaging revealed bone ingrowth after only 2 weeks of implantation in direct contact with BCP grains.

Figure 10. After a longer implantation time (12 weeks), a greater amount of bone was observed in direct contact with the larger residual BCP grain, with no fibrous interface.

BCP grain resorption. The disappearance of the polymer will be due to diffusion, and solubility in extracellular body fluid, or/and cellular activity. In time, a mechanical property can be observed due to the presence of bone [31]. Cellulose and its derivatives have long been used for medical purposes and can be selected to achieve this goal.

The hydrosoluble polymer was mixed with calcium phosphate, a material well-known for its osteoconduction properties [9, 11] and ability to provide a scaffold for cell spreading. The efficiency of BCP is related to its capacity for partial dissolution, the long-term stability of HA, and the enhanced bioactivity of readily-soluble TCP [6, 8, 12]. Methylhydroxypropyl cellulose was chosen for its pseudoplastic properties, as well as its non-ionic structure which prevents strong interaction with Ca^{2+} [17, 32]. The best ratio for the two components, in order to obtain an IBS with a high mineral content and a relatively low polymer concentration, was 60% for the mineral phase and 40% for the polymer, as determined by the viscosity curve [31].

Scanning electron microscopy investigations of reference BCP powder revealed that granules formed agglomerates 80-200 μm in size, made up of 1 to 5 μm individual micrograins. Due to the association with the polymer, a partial dissociation of these clusters released single crystals [15].

Some authors have demonstrated *in vivo* that cellulosic derivative is biocompatible with bone [4, 18, 28]. In a previous study, Grimandi *et al.* [17] tested IBS *in vitro* and demonstrated its biocompatibility. With HPMC alone, no cytotoxicity was noted in short- and long-term assays. Until day 4, cell numbers increased in the same way for BCP, HPMC-MP 824 and composite. However, from day 4 to 21, the curves for BCP and composite showed a similar inhibition of cell proliferation. Fibroblast culture, in the presence of HPMC-MP 824, showed moderate inhibition of cellular growth after 10 days, probably as a result of changes in the ionic homeostasis of media.

In our *in vivo* studies, no extensive inflammatory reaction occurred at any implantation site, whether subcutaneous or intramuscular. Direct contact between the biomaterial and soft tissues was achieved without encapsulation by the membrane. In cartilage sites, the composite was embedded into a well-organized tissue as fibrous cartilage.

The biofunctionality of IBS relates to its two levels of porosity. The microporosity of BCP grains is necessary for fluid circulation and to induce the dissolution/precipitation process for calcium-phosphate crystals [10]. Cellulosic polymer creates spaces between BCP grains which are conducive to angiogenesis and cell colonization (osteogenic and stromal cells). These spaces act like macropores in calcium-phosphate implants. The mechanism of resorption of such biomaterial is centripetal, with a homogenous dissolution front relative to macrophage and/or osteoclast-like cell activities. Macrophages invade the composite from the surface to the core, resorbing smaller BCP particles (less than 20 μm), and releasing the cellulosic carrier. In our study, residual particles were about 100 μm in diameter three months later, which would appear to be a minimal or critical size to be a scaffold for cell adhesion. The mechanism of HPMC disappearance is still unknown. It has never been demonstrated that animal cells can synthesize cellulase. However, phagocytosis, washing out, or oxidative hydrolysis of HPMC may occur.

The calcium/phosphorus ratio of newly-formed bone appeared to be quite constant (around 2.2), similar to that of host bone. The mineral (calcium + phosphorus) content (weight %) of newly-formed bone increased significantly ($< 5\%$) with aging (from 36.7% to 39.0%), while the mineral content of BCP grains increased at the same time (from 48.3 at 2 weeks to 58.0 after 12 weeks, significant difference $< 5\%$). The changes in the mineral content of the implanted area can be accounted for by two processes. First, at the BCP grain level, carbonated apatite (CHA) is precipitated into the micropores. This phenomenon was first described by Daculsi *et al.* [8, 10] and confirmed by clinical studies [6, 11]. These physico-chemical changes in BCP and mineral content increase the mechanical properties of BCP material [31]. Secondly, bone ingrowth completely fills the spaces previously occupied by the polymer. Osteoblasts synthesize an osteoid matrix, forming bone trabeculae between particles. The three-dimensional network of collagen fibers of the extracellular matrix appeared to be well-orientated after the first month, and mineralization progressed regularly. After 4 and essentially 12 weeks, bone remodelling was observed. The physico-chemical process and the cellular events involved bone ingrowth to the detriment of the composite. The mean surface area occupied by BCP grains and newly-formed bone reached 80% of the total area after 12 weeks.

It may be concluded that injectable HPMC-BCP ceramic constitutes a truly new type of bone filler which could provide an alternative to ionic cement for obtaining bone ingrowth within the core of the material. Moreover, this ready-to-use IBS, like macroporous ceramic, can be colonized by osteogenic cells.

Acknowledgments

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Discussion with Reviewers

A.A. Campbell: The authors talk of the micro- and macro-porosity having an effect on bone formation. However, they do not mention the porosity of the BCP particles.

Authors: The microporosity of the BCP grains was 33% according the specification of the initial raw material (TRIOSITE™ Zimmer). About the "macropores" of IBS, we can only make speculation because macropore are defined for bulk materials. For example, the macropore into TRIOSITE™ Zimmer ware in the range of 400 to 600 μm. The spaces in IBS are filled by the polymer and water, the spaces are equivalent or larger to the size of the grains, in our composition, about 100 to 250 μm, and can represent 40% of the total volume.

A.A. Campbell: It is not clear whether the authors are stating that the BCP is undergoing dissolution/reprecipitation, or if the cells are involved in the remodeling of this material.

Authors: Initial studies on BCP have demonstrated that biological fluid involves a dissolution and precipitation process [8, 10, 34, 35, 36]. In addition, cell activity increases the process. These events are largely described in the literature, obtained from both *in vitro* and *in vivo* studies.

R. Todescan: Could the authors provide rationale for using subcutaneous, muscular and cartilage sites for testing this bone substitute material? Would it not be sufficient to use one non-bony site?

Authors: The AFNOR standard recommend osseous and non-osseous implantation site. It is considered that during implantation, particularly for injectable material the material implanted will be in contact with soft tissue.

R. Todescan: Before preparing the IBS, the granulometry of the mineral phase was between 80 and 200 μm, however, granulometry (SEM) before the implantation revealed that 46% of the granules on the IBS were 60-200 μm, and the rest were of smaller size. Is this the result of the composite preparation?

Authors: I agree with your comment the initial granulometry was modified during the incubation of the polymer and the mineral phase. The process will be due to partial dissolution of grain boundary releasing single crystals or smaller grains.

R. Todescan: Information on the polymer degradation (in **Discussion**, referred to as "disappearance") is needed, for example, tensile properties, degradation rates and mechanisms.

Authors: The determination of the process of the disappearance of the polymer still unknown and was difficult to characterize. We can only speculate. It could be due to dissolution in biological fluid, cell activity and others. We are actually trying to determinate the process of dissolution.

R. Todescan: Evidence for the cellular activities (macrophages/osteoclasts): as the authors indicated, cells should "potentially reabsorb" this material. However, there is no evidence showing this phenomena as judged by the micrographs.

Authors: The undecalcified sections used in this study favored data on the mineral phase and mineralized tissue ingrowth. The different cell lines remains difficult to differentiate. We present, however, evidence of multinucleated cells.

K. King: Given that HPMC is water soluble, how do the authors think that the sterilization process affected the stability of the polymer?
Authors: We have tested in previous studies different ways for sterilization. Only steam sterilization does not change the viscosity of the polymer after solubilization. Moreover using FTIR, no change on the structure of the polymer was detected.

K. King: The results of the X-ray microanalysis demonstrated that the Ca/P ratio of the BCP did not vary with time, however, it is assumed that the TCP phase within the cement was being resorbed and that carbonated apatite was being deposited. Would the authors like to comment?
Authors: We said that no significant differences will be detected. The standard error is higher than the difference that we can observed by the disappearance of the TCP content. Moreover, the biological apatite precipitation is constituted of Ca deficient carbonated apatite and not stoichiometric.

K. King: What evidence has lead to the conclusion that 100 μm is a minimum or critical size for cell adhesion?
Authors: This comment is based on the observation. All the osteoconduction process and bone formation observed at the expense of the grains are observed on the larger size particles. We have realized a recent study to demonstrate the critical size of the particle for cell adhesion and/or cell resorption activity, in rats, and we can confirm this observation [37].

Reviewer V: HPMC is not shown in the study to be "non-toxic." Nor is HPMC shown to create a matrix for deep cell colonization leading to osteogenesis. Rather, it shows inhibitory phenomena compared to the undefined control.
Authors: HPMC was demonstrated in the literature to be non-toxic. In the text, we did not indicate that HPMC is a matrix for cell colonization. We said that HPMC involved spaces between the BCP grains in IBS, and the release of the polymer after implantation give spaces for bone ingrowth. However, it is well known by cell biologists that HPMC is a suitable matrix for three-dimensional cell culture, as well as collagen sponge.

Additional References

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