Cells and Materials

Volume 6
Number 1 *Numbers 1-3*

Article 2

1996

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R. Filmon
Université d'Angers

D. Chappard
Université d'Angers

J. P. Monthéard
Université d'Angers

M. F. Baslé
Université d'Angers

Cells and Materials, Vol. 6, No. 1-3, 1996 (pages 11-20) 1051-6794/96$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

A COMPOSITE BIOMATERIAL: POLY 2(HYDROXYETHYL) METHACRYLATE / ALKALINE PHOSPHATASE INITIATES MINERALIZATION *IN VITRO*

R. Filmon[1,2], D. Chappard[1], J.P. Monthéard[3] and M.F. Baslé[1,2*]

[1]Laboratoire d'Histologie-Embryologie, Faculté de Médecine and CHU d'Angers, 49045 Angers Cédex, France
[2]Laboratoire de Chimie Macromoléculaire, Faculté des Sciences et Techniques, 42023 St Etienne Cédex, France
[3]Service Commun de Microscopie Electronique, Faculté de Médecine, 49045 Angers Cédex, France

(Received for publication May 14, 1996 and in revised form November 21, 1996)

Abstract

Bone substitutes are nowadays largely used in orthopedic surgery but they lack osteoinductive properties. Poly (2-hydroxyethyl methacrylate) (pHEMA) has numerous biomedical applications. Alkaline phosphatase (AlkP), an ectoenzyme elaborated by osteoblasts, initiates bone mineralization by hydrolyzing organic phosphates before calcium-phosphorus deposition. We have immobilized AlkP in pHEMA in a copolymerization technic. Histochemical study revealed that AlkP has retained its biological activity. Image analysis of sections using a tessellation method showed a lognormal distribution of the area of tessels around AlkP particles thus confirming an homogeneous distribution of the enzyme in the polymer. Pellets of pHEMA and pHEMA + AlkP were incubated with synthetic body fluids containing either inorganic or organic phosphates (β-glycerophosphate). Mineral deposits with a round shape (calcospherites) were obtained on pHEMA + AlkP pellets incubated in the presence of organic phosphates. No deposits were observed on pHEMA in either incubating conditions or on pHEMA + AlkP incubated with inorganic phosphates. Calcospherites were observed by transmission and scanning electron microscopy. They appeared composed of minute single tablets packed together. X-ray microanalysis showed a Ca/P ratio of 1.42 and X-ray diffraction identified hydroxylapatite. AlkP entrapped in an hydrogel is able to initiate mineralization *in vitro* by a mechanism that closely mimics the cartilage/bone mineralization *in vivo*.

Key words: Alkaline phosphatase, poly (2-hydroxyethyl) methacrylate, methacrylate, mineralization, image analysis, bone biology, biomaterials, bone substitute, polymer.

*Address for correspondence:
Michel Baslé
Laboratoire d' Histologie-Embryologie
Faculté de Médecine, 49045 Angers Cédex - France
Telephone: (33) 241 73 58 64
Fax: (33) 241 73 58 88

Introduction

Polymers based on derivatives of methacrylic acid have been extensively used as biomaterials with medical applications. These synthetic materials are characterized by a high degree of resistance of their backbone against the hydrolytic properties of the body fluids (Kálal, 1984). Poly (2-hydroxyethyl methacrylate) - pHEMA - has been proposed during the last decade for a number of applications ranging from ocular lenses (contact or intra ocular lenses) to contraceptive technics (Chappard *et al.*, 1992; Monthéard *et al.*, 1992, 1996). Considerable interest has been paid to pHEMA in the biomedical literature because the polymer possesses several attracting properties: (a) it is highly biocompatible even in contact with blood and does not activate the complement system (Gobel *et al.*, 1987); (b) it is insoluble in water but can swell and form hydrogels due to the existence of pending primary alcohol groups ($-CH_2-CH_2-OH$) (Wichterle and Lim, 1960). On the other hand, the monomer (HEMA) itself can be dissolved in water. These properties have been used by pharmacologists for the controlled release of drugs (Huglin and Sloan, 1993; Robert *et al.*, 1987), and for immobilization of cells, enzymes and bacteria (Kumakura and Kaetsu, 1983).

When prepared from water-free and bulk-polymerized monomer, pHEMA has an hardness comparable to bone. Several preliminary studies have reported the use of pHEMA as a bone substitute when associated with organic (collagen) or inorganic (β tri-calcium phosphate) compounds (Smetana *et al.*, 1992; Zavrel and Štol, 1993). Today, there is a growing demand for bone grafting materials in orthopedic, neurologic, plastic and dental surgeries. Autograft is by far the best material but suffers from increased surgical time, necessarily limited volumes and possible residual pain at the site of harvesting (Prolo and Rodrigo, 1985). Allografts have been extensively used during the last decade. However, they necessitate the constitution of a bone bank; they are not absolutely safe with respect to bacterial or viral problems, and they use a non-delipidated material (Buck *et al.*, 1989). Xenografts prepared from animal bones

can represent a good alternative when highly purified (Chappard *et al.*, 1993). In the future, synthetic products will supersede natural grafts because it will be possible to obtain homogeneous and unlimited industrial productions of active biomaterials. Immobilization of bioactive factors such as growth factors or bone matrix proteins in a biomaterial may promote and accelerate bone healing (Brink *et al.*, 1996).

Alkaline phosphatase (an orthophosphoric-monoester phosphohydrolase having an alkaline optimum - EC 3.1.3.1.). was found to preserve its mineralizing activity when chemically grafted onto collagen or dentine (Beertsen and van den Bos, 1982; van den Bos and Beertsen, 1994). In the present study, we have physically immobilized alkaline phosphatase in a matrix made of pHEMA. This enzyme is recognized to play a key role in the bone mineralization process (Whyte, 1989). We have tried to determine whether this chemical (organic/synthetic) material can cause mineralization under *in vitro* conditions.

Materials and Methods

The monomer (HEMA)

Commercial 2-hydroxyethyl methacrylate was purchased from Aldrich Chemical (St Quentin Fallavier, France). The monomer is known to contain remnants of methacrylic acid and ethyleneglycol-dimethacrylate which are made during the fabrication process. The polymerization inhibitor 4-methoxyphenol (added by the manufacturer before shipping at a concentration of 200 ppm) also needs to be removed. HEMA was purified and distilled as follows: HEMA was shaken with 3% (w/v) of $NaHCO_3$ in distilled water. $NaHCO_3$ makes water-soluble complexes with methacrylic acid and 4-metoxyphenol. Water was extracted with a large amount of $CHCl_3$ and most of the solvent was removed by rotative evaporation under vacuum. HEMA was made free of chloroform by distillation of the main fraction (bp 75°C at 3 mm Hg pressure). It was collected in screw-capped bottles and kept in the dark at -20°C until use.

Enzyme

The alkaline phosphatase (AlkP) of the intestinal isoform was obtained from Sigma (St. Louis, MO, USA) (catalogue number P 7640) and used without additional purification steps.

Polymer and immobilization process

Thirty milligrams of the enzyme were dissolved in 1 ml of distilled water and the aqueous solution was added to 4 ml of HEMA containing 0.125% (w/v) of benzoyl peroxide, a polymerization accelerator. The enzyme was homogenized by vortexing for 5 minutes. In parallel, tubes containing the same amount of distilled water were used to prepare controls. The polymerization initiator N-N dimethyl paratoluidine was added at a final concentration of 3% and the mixture was vortexed again for 5 minutes. The accelerated and initiated mixture was poured into polyethylene tubes and polymerized at +4°C for 24 hours. The cylinders of pHEMA were collected and sectioned into pellets (1 mm in thickness) on an Isomet saw equipped with a diamond coated blade or into sections (3 μm thick) on a Leica (Rueil Malmaison, France) Polycut S microtome equipped with tungsten carbide knives.

Histochemical controls of enzyme activity

AlkP was histochemically revealed by a simultaneous coupling reaction using Naphtol AS-BI phosphate as the substrate and Fast Blue BB as the diazonium salt. The 3 μm thick sections were incubated for 30 minutes at room temperature, rinsed in distilled water and let dry until mounting in an aqueous medium. Control histochemical reactions were used by adding 0.25 mM of NaF (a competitive AlkP inhibitor) in the staining fluid. The homogeneity of AlkP distribution within the polymer was appreciated by image analysis on histochemically stained sections using a Leica Quantimet Q570 image processor. After image acquisition and interactive thresholding, the image was converted into a binary image (Fig. 1a). The size of each detected particle of AlkP was measured and values were stored in an array and used to provide the histogram distribution frequency. The volume amount of AlkP in pHEMA was determined as the ratio of the surface area of stained particles over the section area. The volume of polymer around each of the particles was determined by measuring their zone of influence. On the analyzer, partitioning of the complementary image of the AlkP particles was automatically provided using the skeleton by influence zones method (skiz) (Serra, 1982). Briefly, the skiz was drawn around each particle so that the background space was sectioned into numerous tessels. All the tessels that were in direct contact with the microscopic frame were not taken into account and were removed from the image (Fig. 1b). The surface area of each tessel was measured and the values were stored in a second array and computed as above.

Incubation of pellets in synthetic body fluids

The pellets were incubated in three kinds of synthetic body fluids.

(a) A standard synthetic medium was prepared which contained only mineral phosphates (Yamada *et al.*, 1994). Its composition (verified on a Technicon (Paris, France) SMA analyzer) was as follows: Na: 142.19 mM; Ca: 2.49 mM; Mg: 1.5 mM; HCO_3: 4.2

mM; Cl: 141.54 mM; HPO_4: 0.9 mM; SO_4: 0.5 mM; K: 4.85 mM.

(b) 10 mM of a phosphate organic molecule (β-glycerophosphate) were added to the previous medium (Tenenbaum *et al.*, 1992).

(c) The minimal essential medium (MEM) cell-culture medium, containing 5% bovine fetal serum (BFS) was used as it closely resembles the *in vivo* conditions.

All media were supplemented with penicillin (100 UI/ml) and streptomycin (500 μg/ml) to avoid mold and bacterial contamination. Pellets were sterilized by UV radiations (360 nm for 3 hours) and distributed in sterile capped vials containing 5 ml of medium and stand from seven to ten days in an humidified oven at +37°C with 5% CO_2. The medium was replaced every two days and the pellets were processed for electron microscopy.

Electron microscopic examinations

Transmission electron microscopic studies were done on a JEOL (Tokyo, Japan) 100CX. Scanning electron microscopy was performed on a JEOL 6301F field emission microscope equipped with a energy dispersive X-ray micro-analysis machine (Link ISIS, Oxford Instruments, Oxford, UK).

X-ray diffraction

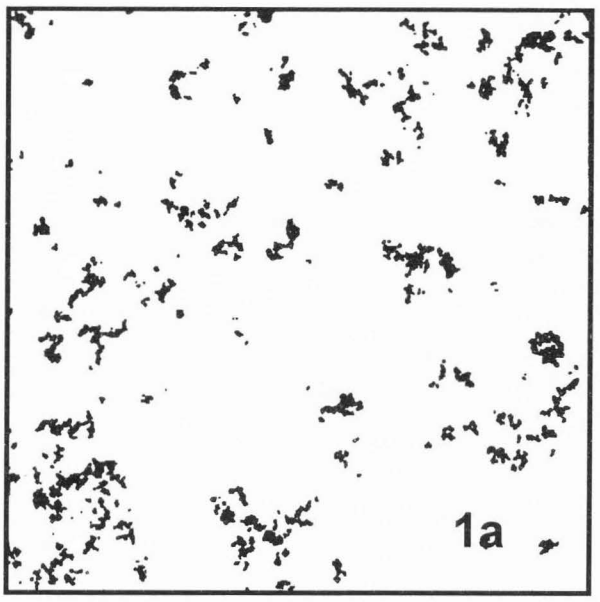
X-ray diffraction was used to characterize mineral deposits. They were transferred onto a metallic stub and examined with a Siemens (Paris, France) D501 X-ray diffractometer with a Cu Kα cathode. The spectrum was obtained after a 25 second period with a 0.02° angular step.

Results

AlkP distribution within the pHEMA.

AlkP was easily identified by the histochemical reaction as blue deposits overimposed on the sites of enzyme activity on the 3 μm sections of pHEMA + AlkP. The enzyme molecules seemed to be homogeneously distributed among the polymer (Fig. 2). On pHEMA alone, the histoenzymatic staining appeared strictly negative (Fig. 3).

Image analysis of AlkP entrapped in the pHEMA gave very reproducible results. The fractional volume of the enzyme particles was 8.0% in the experimental conditions, the particles were rarely isolated, most often grouped into small aggregates. Transmission electron microscopy of ultrathin sections exhibited the same aspects as histochemical sections examined on light microscopy. The size distribution of the AlkP particles was found to follow a lognormal distribution with a mode centered on the 105 μm^2 class. Partitioning of the space around particles by the skiz method provided a set of elementary tessels, the area of which was also found lognormally distributed (Fig. 4) (mode centered on the 1960 μm^2 class), thus confirming the homogeneous entrapping of the enzyme.



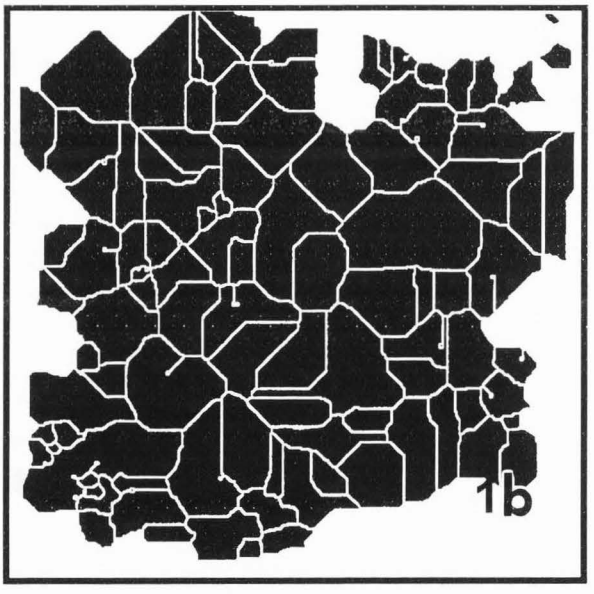


Figure 1. (a) Image analysis of an histochemically stained section of the pHEMA + AlkP. The binary image of the particles is provided by the image analyzer. (b) The skiz around each particles has been automatically drawn and only the tessels which are not in contact with the analysis frame are measured.

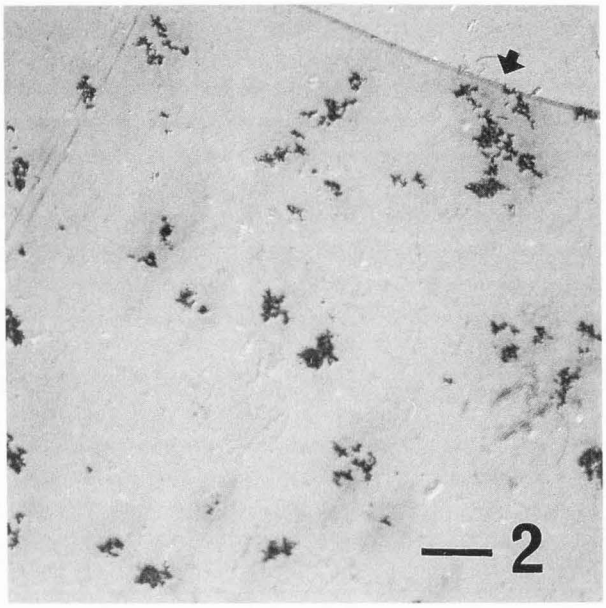


Figure 2. Histochemical identification of AlkP on a 3 μm section of the pHEMA + AlkP; the boundaries of the polymer section are evidenced (→) (Nomarsky interference microscopy). Bar = 100 μm.

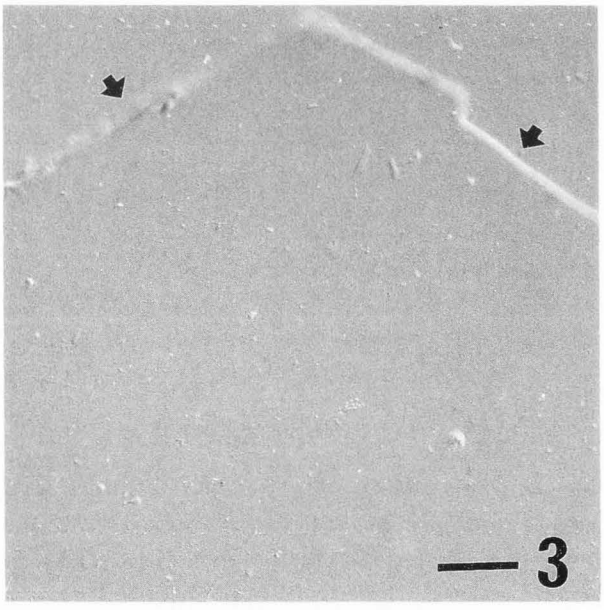


Figure 3. Histochemical identification of AlkP on a 3 μm section of the pHEMA alone, no staining is obtained; the boundaries of the polymer section are evidenced (→) (Nomarsky interference microscopy). Bar = 100 μm.

Incubation of pellets in body-fluids

When incubated in the synthetic body fluid containing only inorganic phosphates, no precipitate could be obtained even after prolonged incubation times. On the other hand, adding 10 mM of β-glycerophosphate was followed by extensive mineral deposition which appeared visually evident from the third day of incubation. The deposits were only present on the pHEMA + AlkP pellets in the form of a thick white layer. Pellets made of pHEMA alone remained translucent. With the MEM culture-medium, similar deposits were observed in the pHEMA + AlkP pellets but not in the pHEMA ones.

Scanning electron microscopic examinations of the pellets incubated in the inorganic phosphate containing medium were unable to find any sign of mineral deposit. On the other hand, in β-glycerophosphate enriched medium or in the MEM-BFS medium, a thick layer of mineral nodules was apparent (Figs. 5a, 5b). These nodules had a rounded shape and appeared made of elementary tablets or plates packed together to form calcospherites. Some pellets were fractured to expose the internal part of the incubated polymer. Mineral nodules were encountered entrapped down to 30 μm in depth. The polymer under this mineralized area appeared homogeneous with a glossy appearance. The surface of pellets made of pHEMA alone appeared totally free of mineral deposit when placed in β-glycerophosphate enriched medium and SEM analysis confirmed the absence of mineral deposition at the surface and depth of the polymer (Fig. 6).

X-ray microanalysis identified calcium, phosphorus and magnesium in the mineralized nodules with a Ca/P ratio of 1.42. (Fig. 7). The X-cartographies of Ca and P were overimposed on the mineralized nodules of the top and superficial zone of the pHEMA + AlkP pellets. On pHEMA pellets, the microanalysis was unable to find traces of Ca and P, the only atoms detectable were C and O, corresponding to the polymer itself.

Transmission electron microscopy of ultrathin sections of pHEMA and pHEMA + AlkP was in total accordance with our previous results. Mineral deposits could only be observed in the pHEMA + AlkP pellets and incubated in β-glycerophosphate enriched synthetic medium or in DMEM-BFS. Round-shape nodules were also recognized and appeared to be composed of thin elementary mineralized tablets which looked like needles when cut transversely (Figs. 8, 9). Nodules appeared to fuse by coalescence and gave the almost continuous superficial layer of mineral.

X-ray diffraction showed that mineral deposits were composed of hydrated octacalcium phosphate $Ca_8H_2(PO_4)_6$, $5H_2O$, hydrated tri-calcium phosphate $Ca_3(PO_4)_2$, xH_2O and hydroxylapatite $Ca_{10}(PO_4)_6(OH)_2$.

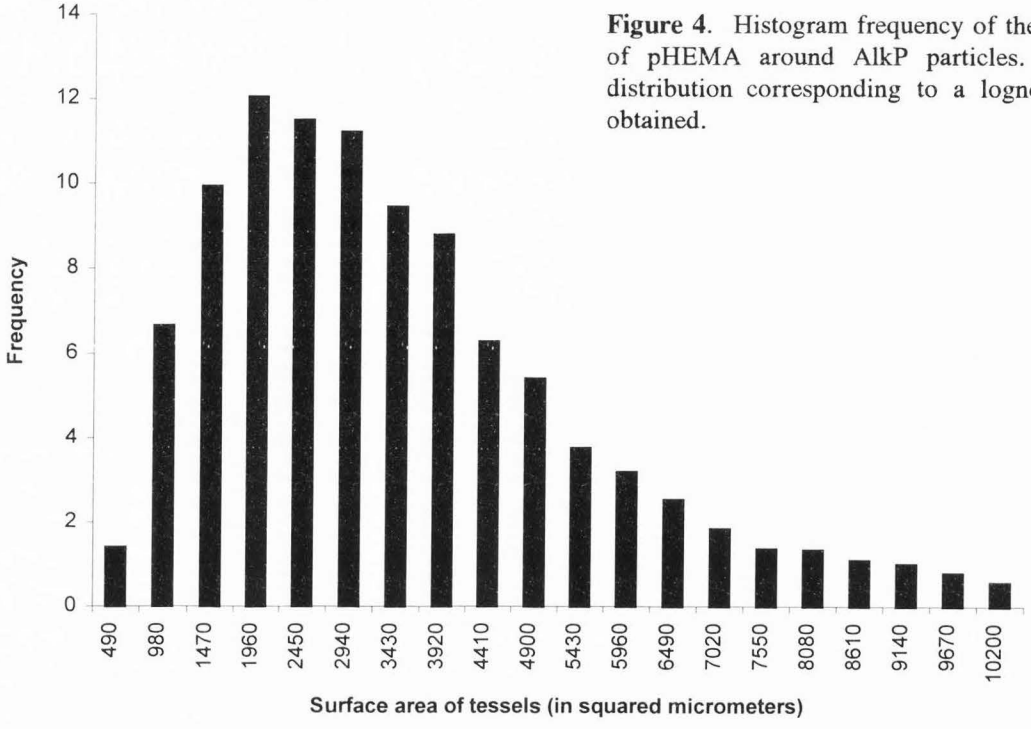


Figure 4. Histogram frequency of the tessels' area of pHEMA around AlkP particles. A skewed distribution corresponding to a lognormal law is obtained.

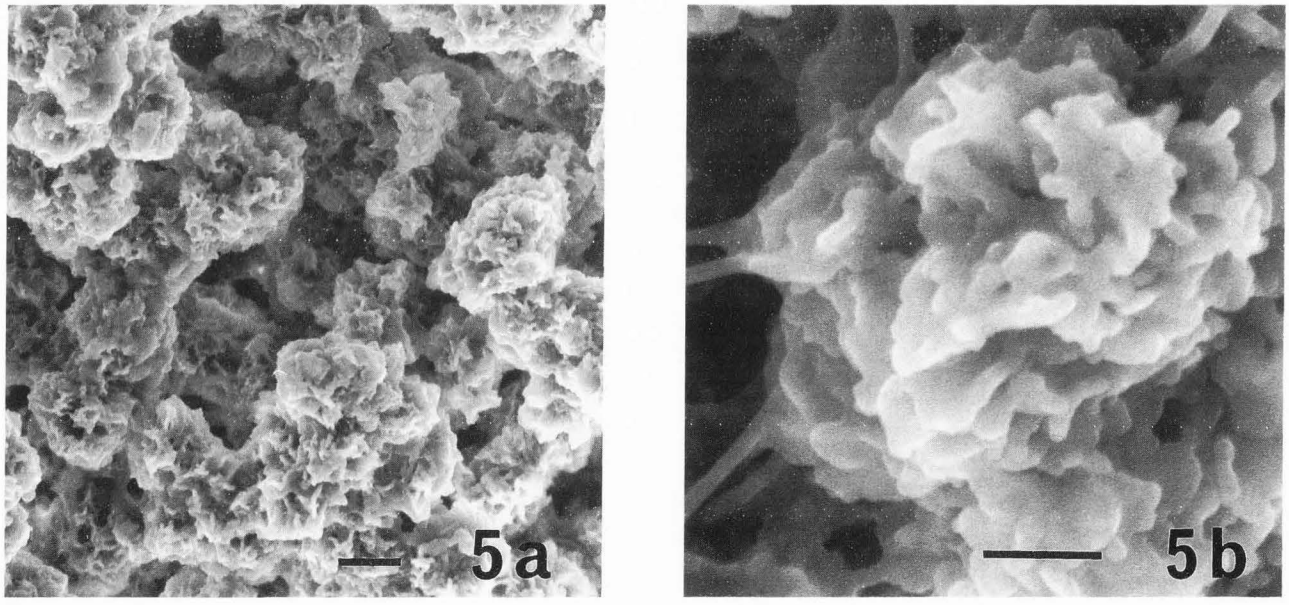


Figure 5. (**a**) Scanning electron microscopy of calcospherites obtained on pHEMA + AlkP incubated for seven days in a synthetic body fluid enriched with β-glycerophosphate. Bar = 1 μm. (**b**) higher magnification (note that calcospherites are composed of small tablets packed together). Bar = 0.5 μm.

Discussion

Immobilization implies the entrapment of a foreign compound (e.g., an organic molecule, a drug, or cells) within a polymeric network whether it is simply confined or grafted onto the polymeric chains. Immobilization of enzymes on solid supports has found a number of biotechnological applications because the enzyme molecules become reusable (see review in Monthéard *et al.*, 1992, 1996). To preserve enzyme activity, radiation-induced polymerization is often reported. Several types of enzymes have been immobilized by grafting onto the

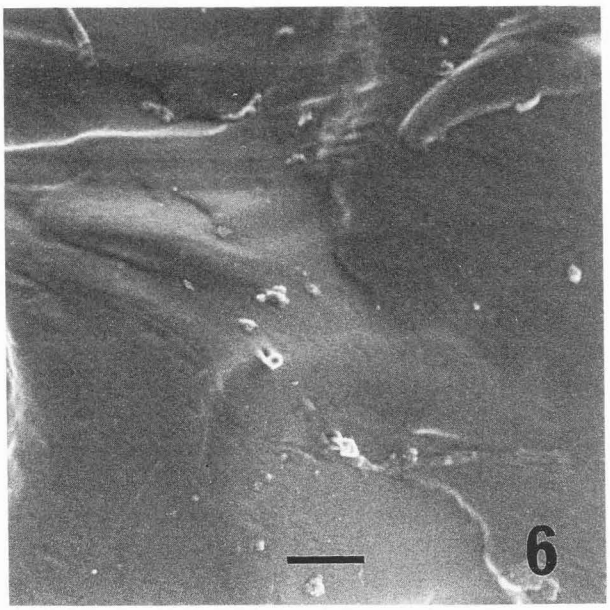


Figure 6. Scanning electron microscopy of the surface of a pHEMA pellet incubated for seven days in a synthetic body fluid enriched with β-glycerophosphate, no calcium/phosphate deposit can be observed on the surface of the pellet. Bar = 10 μm.

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polymer. These include cellulase, trypsin, glucose oxidase and urokinase. Other proteins without enzymatic properties have also been successfully immobilized in pHEMA. α-fetoprotein and collagen have been entrapped within pHEMA by γ-irradiation without molecular modifications. However, radiation polymerization is known to decrease the level of enzyme activity (Kumakura *et al.*, 1984). In the present study, a redox polymerization was used to entrap the AlkP within the polymer chain network and the method is known to preserve enzyme activity (Chappard, 1985). pHEMA embeddings are routinely done for histochemical purposes because enzymes are usually well-preserved. Histochemical demonstration of AlkP could be readily demonstrated in the sections by a classical simultaneous dye coupling. In addition, distribution of the enzyme was found very homogeneous among the polymer as shown by image analysis. In this study, the skeleton by influence zone method was used to study the area (in 2D sections) of the polymer around each cluster of AlkP molecules. The skiz method is a morphological mathematic technic which allows partitioning of a given space by finding the watersheds between particles (Serra, 1982; Russ, 1990). The method produces a compartmentalization of the space into a myriad of tessels in form of a puzzle or a mosaic, each piece of which can be analyzed and measured independently. We found that the distribution of the tessel area was positively skewed in a fashion that can be estimated by a lognormal distribution. Lognormalcy is a general biological law observed in the whole living kingdom and in material science; it indicates that the population under study is homogeneous (Bahr and Mickel, 1987; Russ, 1990). The size distribution of particles such as powder grains is also known to follow a lognormal distribution. In the present study, the AlkP particles and their corresponding tessel areas were found lognormally distributed. This means that the AlkP particles were homogenized in the polymer.

Alkaline phosphatases are ubiquitous dimer glycoproteins associated with zinc. They are anchored in the cell surface membrane by a phosphatidyl inositol glycan ridge (PIG tail) attached to their carboxy terminus (Fishman, 1990; Harris, 1990; Harrison *et al.*, 1995). Four genes have been shown to code for AlkP isoenzymes: (a) the tissue unspecific isoenzyme is usually referred as the bone/liver/kidney AlkP, (b) the intestinal type, c) the placental, and (d) the placental-like isoenzymes. The AlkP genes have been cloned and the amino acid sequences have revealed homologies of about 87% between the different iso-proteins (Harris, 1990). Immunohistochemical technics have shown cross reactions between the various isoenzymes. In fact, the study of carbohydrate moieties have revealed an organ specific pattern of AlkP glycosylation that is responsible for differences in electrophoretic mobility noted between the bone/liver/kidney isoenzymes. Bone and liver AlkP isoenzymes are different due to post translational modifications implying the manner of sialic acid linkage and the attachment of the O-linked sugar moiety (Miura *et al.*, 1994). However, all the AlkP isoenzymes share in common the ability to hydrolysate organic ester compounds but their precise role in mammalian physiology is not fully understood. In the intestine, AlkP is present on the luminal surface of enterocytes and is thought to hydrolyse phosphate esters which are not normally absorbed in the gut (Harris, 1990). A role in the chylomicron absorption and transfer to the liver has been also proposed (Fishman 1990). In bone, the enzyme has been recognized to play a key role in the mineralization process for several decades although its precise mode of action remains elusive. A gross deficiency of the bone isoenzyme leads to hypophosphatasia, a disease associated with defective osteogenesis due to an impaired mineralization process (Caswell *et al.*, 1991). Serum AlkP is now recognized as the most common marker of bone formation in human metabolic bone diseases. The precise role of AlkP in the bone mineralization process seems to be age-dependent:

- during endochondral mineralization, AlkP is present on the surface of matrix vesicles (released by chondro-

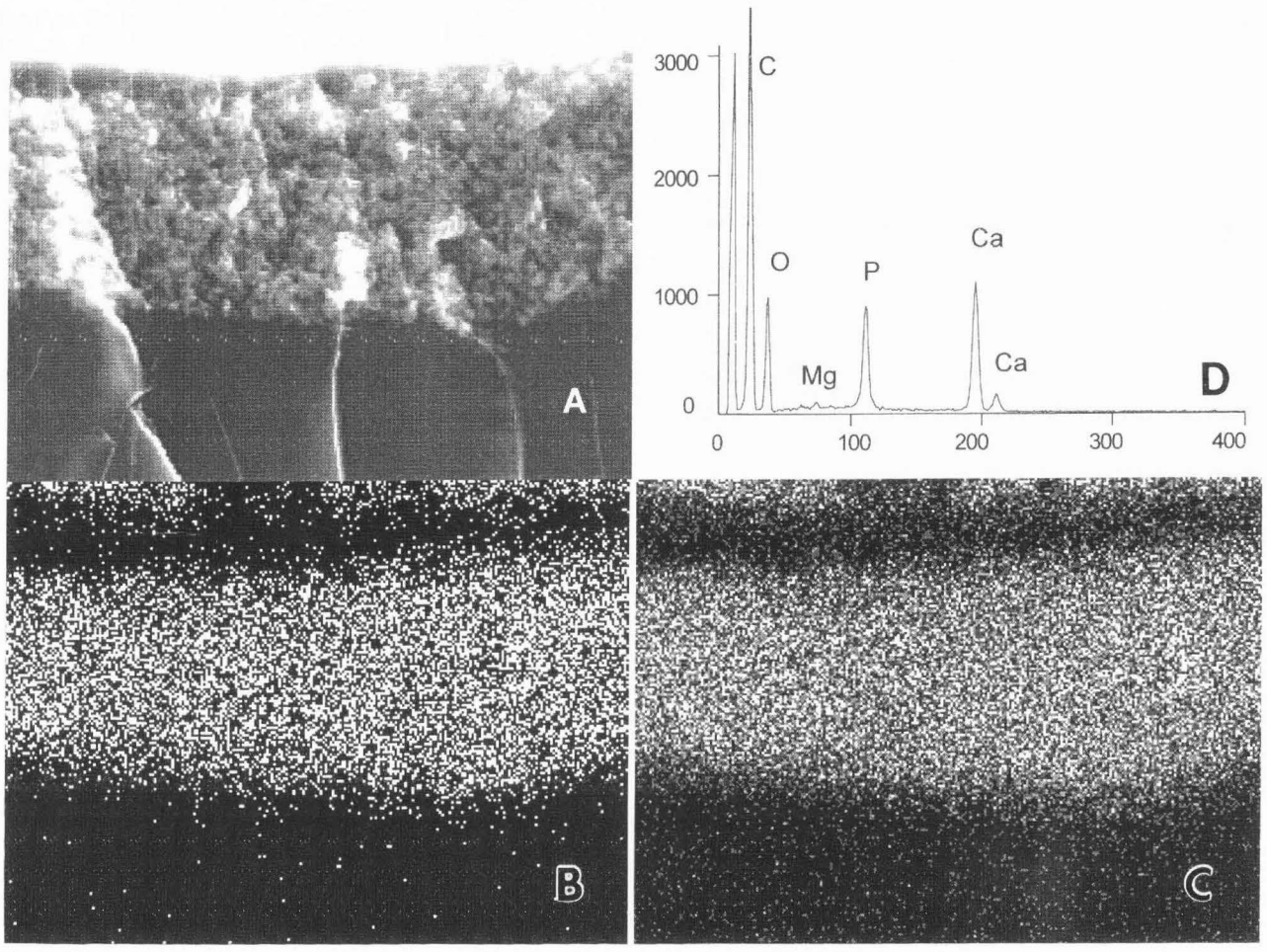

Figure 7. X-ray microanalysis of calcospherites obtained on pHEMA + AlkP incubated in a synthetic body fluid enriched with β-glycerophosphate. (**A**) transverse section of a pellet showing calcified calcospherites on the top, unmineralized polymer at the bottom. (**B**) Calcium cartography of the same picture. (**C**) Phosphorus cartography. (**D**) Spectrum of the deposits exhibiting calcium, phosphorus, oxygen, carbon and traces of magnesium.

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blasts and osteoblasts) and entrapped in the collagenous matrix. The enzyme concentration in the vesicles is 15 up to 20 times the amount in the cells. These vesicles have been recognized as the earliest sites of mineral deposition in the cartilage (Wuthier, 1982, Golub *et al.*, 1992).

- in mature lamellar bone, matrix vesicles have not been identified. AlkP is present at high concentrations in the mineralization fronts of trabecular bone and its distribution is in parallel with the double tetracycline labeling. AlkP and other non- collagenous proteins of the bone matrix may initiate mineral deposition (i.e., nucleation of apatite crystals) and crystal growth is thought to proceed further spontaneously.

In the present study, we have found that the intestinal isoenzyme of AlkP is able to initiate the mineralization process, a finding also observed by others (Beertsen and van den Bos, 1982; van den Bos and Beertsen, 1994). The mineral formed was found analogue of the naturally occurring bony hydroxylapatite. This confirms that AlkP, whatever its origin, possesses the ability to hydrolyze organic phosphate esters and to elevate local concentration of inorganic phosphates (P_i) which can complex Ca^{2+} to give calcium phosphate. In contrast, the enzyme does not appear to act as a P_i transport system because no mineralization is observed when a synthetic body fluid containing only P_i is used. The mineral deposits observed in our medium enriched with β-glycerophosphate have a continuous growth and progressively form calcified nodules on the surface of pHEMA + AlkP. The early calcification steps have been clearly observed as small needle-like structures or tablets of calcium phosphate entrapped within (or at the surface of) the pHEMA. In addition, the elemental distribution

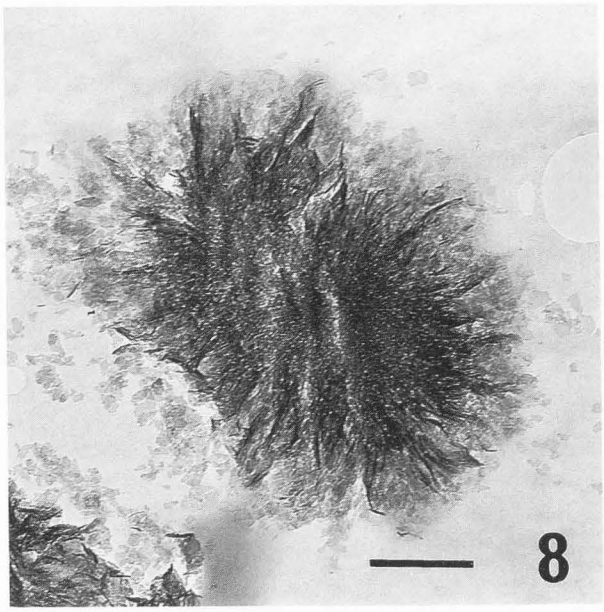

Figure 8. Transmission electron microscopy of calcospherites obtained on pHEMA + AlkP incubated in a synthetic body fluid enriched with β-glycerophosphate. (Note that they are composed of small tablets packed together). Bar = 0.5 μm.

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maps of phosphorus and calcium are strictly superimposed over the microscopic sites of mineral deposition. The Ca/P ratio clearly evidences calcium phosphate and X-ray microanalysis confirmed that hydroxylapatite was obtained. Magnesium is known to stabilize Ca-P deposits and was also evidenced in this study. The morphology of these crystals and nodules are very similar to those described during the physiological mineralization process (Arsenault and Ottensmeyer, 1984; Bonucci, 1987, Harrison *et al.*, 1995). It has been demonstrated that such nodules could only be observed when AlkP is immobilized on a cell membrane via its phosphatidyl inositol tail. In the present study, the enzyme was physically immobilized inside the polymer chains of a pHEMA network, a condition that would have mimicked the PIG-tail anchorage. When prolonged incubations of pHEMA + AlkP pellets are done in synthetic body fluid containing only inorganic phosphates, a slight leaching of the enzyme was observed (data not shown), this was due to the swelling in surface of the polymer down to a 30 μm depth. Several authors have reported that pHEMA was able to interact with calcium (Van de Mark and Lian, 1987) and to mineralize spontaneously when implanted into the body during prolonged periods (Šprincl and Novàk, 1981). In the present study, no calcification of the pHEMA alone could be observed either in synthetic fluids or when an organic phosphate was added. This study points out the leading role of AlkP in the process of inducing mineralization in the presence of organic phosphates. When induced, mineralized nodules appear able to grow spontaneously by nucleation of the mineral.

Acknowledgements

This work was made possible by grants from Pôle Grand Ouest de Génie Biologique et Médical (contract 1995-96) and by TRANSPHYTO SA, Clermont-Ferrand, France. Mrs. N. Gaborit is thanked for her help with the histochemical identification of AlkP.

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Discussion with Reviewers

Reviewer II: Since the media used are not directly comparable with any body fluids, the term "body fluid" should be replaced by "medium."

Authors: The term "body fluid" is now common in the scientific literature and has been reported by numerous authors. We do not agree to substitute it by "medium;" the synthetic fluid used here is the reflection of the ionic composition of human extracellular body fluids.

Reviewer II: The authors added 10 mM β-glycerophosphate to the incubation mixture in order to provide a suitable source of phosphate ions. Is this concentration comparable to the concentration of monophosphate esters in body fluids of living organisms?

Authors: The 10 mM β-glycerophosphate concentration has been shown by numerous authors to be the optimal source of organic phosphates for cultured osteoblastic cells (Anagnostou *et al.*, 1996). This concentration corresponds to the organic phosphate level of the serum

(Ecarot and Glorieux, 1991). In the present study, the Dulbecco's Modified Eagle's Medium (DMEM) culture medium free of β-glycerophosphate but containing organic phosphate compounds was found to provide strictly similar results.

Reviewer II: To what extent has the biomaterial the property to mineralize when installed in the animal body?
Authors: The aims of this study were to present the preparation, characterization and *in vitro* properties of the material. Cultures of osteoblast-like cells have been done on this material and the cells exhibited a preferential adhesion to the crystals. Animal studies are also being conducted in this laboratory. A recent paper (Piatelli *et al.*, 1996) has reported that alkaline phosphatase coated implants produce an increase amount of bone.

P. Li: Is any precipitation in the solutions containing a phosphate organic molecules during incubation with the composite?
Authors: There is some precipitation in the organic phosphate solutions around pellets when incubated with the composite material but this is a known phenomenon (that was not studied in this study) which is due to free enzyme activity.

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