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TRACE ELEMENTS ANALYSES IN A SYNTHETIC ARAGONITE 55% STUDIED AS BONY BIOMATERIAL

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ABSTRACT: Calcium carbonate, in aragonite form, porous at 55% and associated with gentamicin sulphate antibiotic, was synthesised in our laboratory and characterised with physical-chemical methods and then experimented *in vivo* as bony biomaterial The aim of this study was to evaluate its biocompatibility and particularly its mineral composition transformation versus time. Trace elements like phosphorous and strontium and major element like calcium in synthesized aragonite were analysed by PIXE method (Particle Induced X-ray Emission). These elements present a high interest in biomedical field and indicate the bony quality according to the phosphocalcic ratio. Aragonite porous at 55% was inserted in concellous bone of femur diaphysis of nine ovine. Samplings were carried out at one, three, six and twelve months after implantation. For each sample, biological assessments and *in vivo* mineral composition evolution of trace and major elements were studied. Trace atomic elements distribution obtained with PIXE method showed the concentration increasing of phosphorous and strontium and the concentration decreasing of calcium in the aragonite biomaterial. Results obtained have been correlated with histological studies.

Introduction

The development of synthetic biomaterials in orthopaedic surgery is due to the control of their pure mineral composition, their porosity and their physical-chemical properties. Their biocompatibility offers a wide range for applications in restorative and osseous surgery.

Calcium carbonate in the aragonite form was synthesised and associated with gentamicin sulphate antibiotic. Samples of powder, were compacted to obtain the cylindrical form porous at 55%, they were experimented for the *in vivo* studies. Cylinders were inserted in the concellous femur sites of nine ovine. One, three, six and twelve months after implantation, animals were sacrificed and samples were extracted. The goal of this study was to evaluate the morphology and the purity of our synthesised powder and then to evaluate qualitatively and quantitatively the *in vivo* behaviour of $CaCO_3$ compound with gentamicin by analysing trace and major elements (Ca, P, Sr) in pure carbonate calcium, in pure bony matrix and in each sample extracted versus time after implantation.

Various physical-chemical and biological interdisciplinary approaches were required.

Scanning electron microscopy (SEM) and X-ray diffraction (XRD) were used to identify the morphology and the crystalline structure of biomaterial. PIXE method was applied to follow different mineral transformations of implanted biomaterial. It was coupled with histologic studies to correlate the obtained results. Atomic elements studied in this work present a high interest in the biomedical field. The optimal value of phosphocalcic ratio Ca/P (Iyengar et al., 1978; El Solh, Rousselet, 1981; Iyener, Tandon, 1999) is of 1.7 to 1.9 in a mature bone. It varies versus age. The modification of this ratio can be responsible of bony damaging. Strontium element favours the calcification mechanism and develops the enzymes action and the bony metabolism.

Materials and methods

The calcium carbonate in the aragonite form was synthesised by precipitation from boiling aqueous solutions of potassium hydrogenocarbonate $KHCO_3$ and calcium chloride $CaCl_2$ both 0.1 M (Lucas-Girot, 1996). The chemical reaction used for this synthesis is:

$$2\,KHCO_3 + CaCl_2 \uparrow CO_2\text{-} + H_2O + CaCO_3 + 2\,KCl$$

The carbonate precipitate was filtered, washed and dried at 110°C. Calcium carbonate was associated with gentamicin sulphate antibiotic from Micromonospora

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purpurea by Sigma-Aldrich, France used for osteomyelitis treatment according to an original chemical procedure. This original association allows the diffusion of antibiotic since the implantation and avoids bone surgical difficulties.

Powder obtained was compacted with isostatic mode, mixed with porogens to create micro- and macroporosity (55%) and then shaped to obtain samples in cylindrical form with 4 mm in diameter and 12 mm in length. Different porosities were induced depending on the porogens quantities (naphthalene: $C_{10}H_8$) introduced. In this work, we focused on the porosity of 55% with pore sizes of 200 to 300 μm mixed with gentamicin antibiotic.

The *in vivo* experiments were carried out in medicine faculty, at Rennes (France). A general animal anaesthesia was made. The side where the surgical operation would be made was shaved. The bone was uncovered with minimum damage and the biomaterial $CaCO_3$ in the cylindrical form (sterilised before with gamma rays at 25 kGray) was inserted and stabilised. The use of an adaptable device permitted the location of the different biomaterials implanted (Figure 1). Animal experiments were made according to the European norm: EN 10993-22 isonorm. Biopsies have been carried out at 1; 3; 6 and 12 months after implantation.

Extracted samples of 3 mm in thickness and 15 mm in diameter were composed of the biomaterial and the bony matrix. They have been dehydrated, dried and plated by a thin layer of carbon to favour the charge voiding. This study was completed with biological studies carried out on demineralised samples.

To evaluate the kinetic ossification and the consolidation of biomaterial, cartographies of Ca, P and Sr atomic elements at the biomaterial surface, at the bone surface and at the interface (biomaterial – bone), (Figure 3) were achieved with the PIXE method at the CERI – CNRS Laboratory in Orléans, France. This method is based on the interactions between protons beam of about 2.2 MeV in energy and target placed in room analysis device (Figure 4) (Johanson, Campbell, 1988; Choi, 1996; Oudadesse, Guibert et al., 2002). The intensity is about 1 nA and the beam size of about 300 μm. This beam size was chosen to avoid the effect of porosity which is of 55% and pores size of 200 to 300 μm.

PIXE method presents some advantages like its sensitivity around 1 μg/g depending on the matrix and its multielementary character. It is adaptable for trace elements analyses in biological matrix (Oudadesse, Irigaray, 2002). X-rays are detected by semiconductor based detector such as Si(Li), associated to a funny filter (Chu et al., 1981). The collected spectra of X-rays have been analysed by GUPIX software (Maxwell et al., 1995).

Cartographies were made from the centre of biomaterial which toward interface biomaterial — bone and then mature bone. Repairs were the two guides (one thread of gold and two threads of copper).

This physical analysis technique has been coupled to complementary histologic studies to correlate the obtained results.

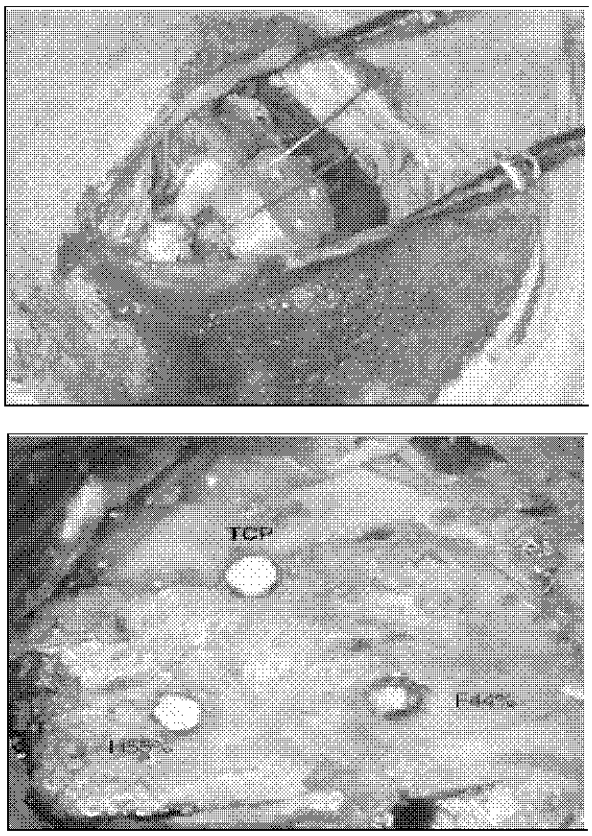

Fig.1. Animal experiments and aragonite $CaCO_3$ (H55%) implantation in concellous bone.

Results and discussion

Synthesised powder, analysed with scanning electron microscopy (SEM) (Lucas-Girot et al., 2001) presents a needle-like grains with an average length of 5 to 10 μm and a wide of 1 to 2 μm (Figure 2).

XRD analysis has been achieved at 291 K with a Siemens D500 diffractometer (Lucas-Girot et al., 1999), with a curved position — sensitive detector (Cu Ka) radiation. Compounds were ground in an agate mortar to obtain a fine powder with diameter less than 0.5 mm. The

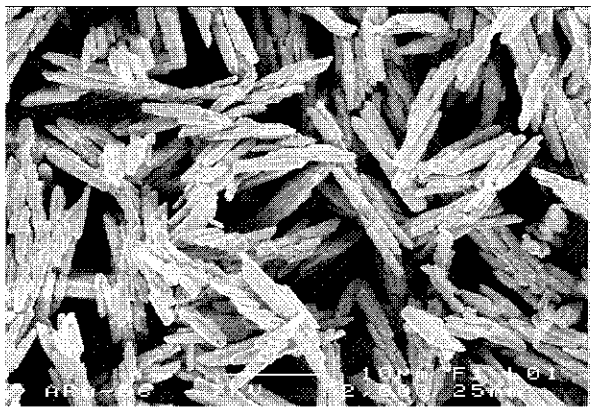

Fig.2. Synthetic aragonite morphology.

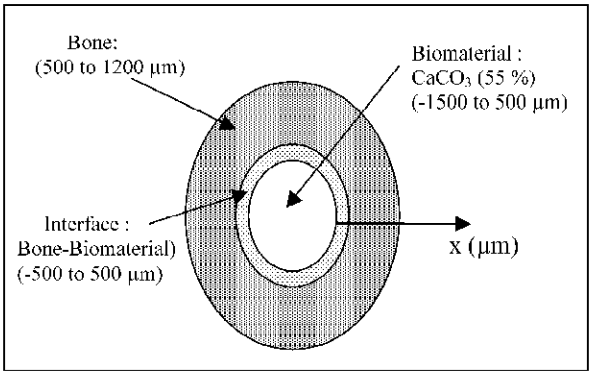

Fig.3. PIXE analyses from biomaterials toward bony matrix.

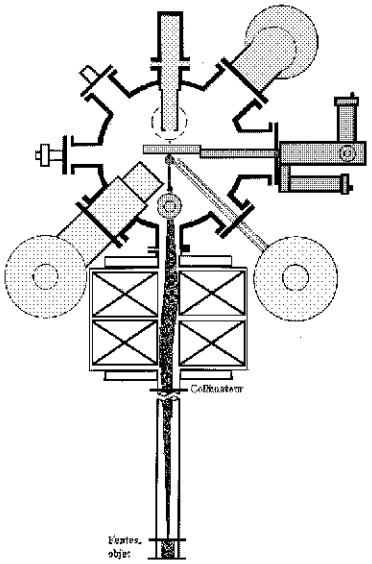

Fig.4. Room analysis device of PIXE method.

identification of phases, the extraction of peak position for indexing and the refinement of cell parameters were carried out with the DIFFRAC+ Software package. Results show that our synthetic calcium carbonate crystallises in an orthorhombic system and presents a pure aragonite without other phases, particularly the calcite, which presents some difficulties to be resorbable.

Cartographies obtained by PIXE method of trace atomic elements Sr and P and of major atomic element Ca, present in our synthetic biomaterial $CaCO_3$ (55%), highlight the concentrations evolution versus time in biomaterial and show the consolidation nature.

Results presented in figures 5, 6 and 7 show the behaviour of these atomic elements.

At one month and at three months after implantation, the surface of synthetic biomaterial (position: -1500 to -500 µm) undergoes some perturbations and the concentrations of calcium (respectively phosphorous) present an irregular decreasing (respectively increasing) and indicate the presence of heterogeneous material.

Six and twelve months after implantation, this heterogeneity persisted and we show some area with neoformed bone and other area with persistent biomaterial.

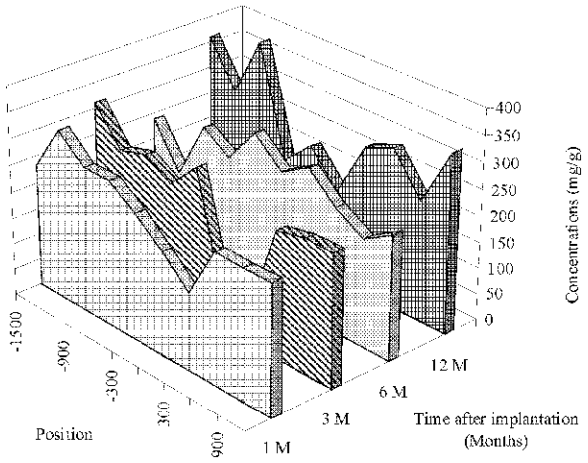

Fig.5. Cartography of Ca in biomaterial, in interface (biomaterial/bone) and in bone.

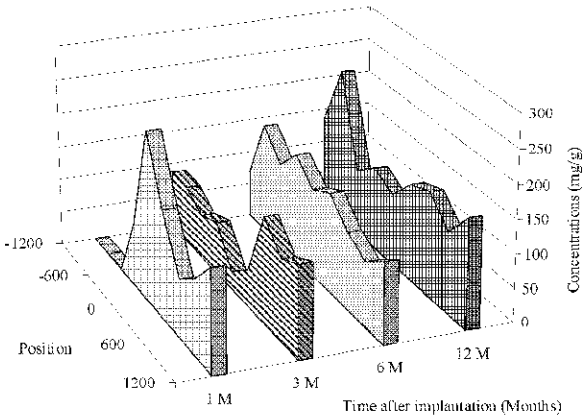

Fig.6. Cartography of P in biomaterial, in interface (biomaterial/bone) and in bone.

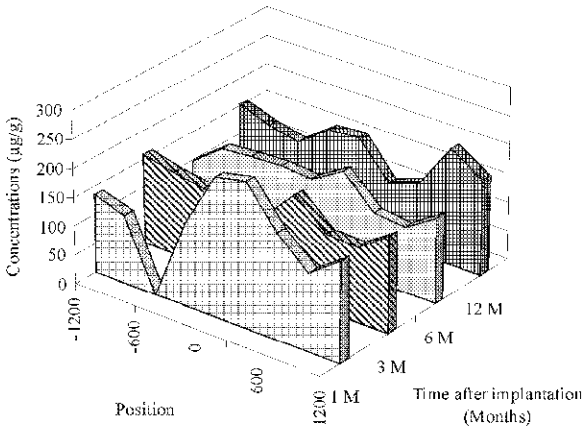

Fig.7. Cartography of Sr in biomaterial, in interface (biomaterial/bone) and in bone.

The strontium was rapidly brought in biomaterial and only one month after implantation, concentrations of Sr are equal to those in the bone. Its distribution in the biomaterial presents a good homogeneity. It is important to indicate that pure carbonate calcium in the aragonite form is metastable and needs the presence of mineral element like Sr to be stable.

Cartographies made in the interface part (position: -500 to +1500 μm) show the same phenomenon (perturbations and heterogeneity) at one and three months after implantation. Six and twelve months after implantation, this area presents an homogeneity with concentrations of about 250 mg/g for Ca, about 130 mg/g for P and about 200 μg/g for Sr. Theses values are equal to those in mature bone.

Cartographies made in bone do not reveal significant variations in the last part (position: 500 to 1500 μm), which corresponds to a bone.

The gentamicin antibiotic associated with this biomaterial and the high porosity are probably responsible of this phenomenon. In our previous works, these perturbations were not observed of calcium porous at 44 % and without any antibiotic association.

The gentamicin antibiotic with acid character can accelerate the solubility of biomaterial and the macroporosity facilitate the vascularisation and the cells and organic matter deposit.

The orthopaedic surgery induces high perturbations in organism, particularly in osseous metabolism. Histologic studies show the formation of light fibrous tissues and inflammatory cells at one month after implantation. At this time, vascularisation and osseous cells like osteoblasts and osteoclasts were observed and show the beginning of cell colonisation (Martin, 2002).

Continuous and homogeneous profiles presented by concentrations of Ca, P and Sr in the interface part (biomaterial – bone) highlight the consolidation between biomaterial in remodelling process and bone and consequently the biointegration of biomaterial at six months after implantation.

Histological studies show an important reduction of fibrous tissues and inflammatory cells at six months after implantation and disappeared completely twelve months after implantation.

The slow-flowing ossification can be attributed to the ovine age (7 to 9 years) responsible of the reduction of cellular activities.

Conclusion

The PIXE method is an original and powerful method to analyse trace, minor and major atomic elements in biological samples.

Atomic element analyses in this work coupled with histological studies permit to have a contribution of the behaviour of pure carbonate calcium in the aragonite form porous at 55 % and associated with gentamicin antibiotic. However, its acid character accelerates the dissolution of biomaterial and induces some perturbations in the resorption mechanism. Atomic elements chosen and analysed in this work characterise an important part of mineral composition in mature bone and show that this composition was reached 12 months after implantation.

The perspectives of this work are to dope our synthetic calcium carbonate with trace atomic elements to improve the neoformed bone qualities and to accelerate relatively its resorption kinetics. We hope to analyse more samples to have significant statistics.

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