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Close to the bone mineral phase, the calcic bioceramics, such as hydroxyapatite (HA) and β-tricalcium phos-phate (β-TCP), are commonly used as substitutes or filling materials in bone surgery. Besides, calcium car-bonate ($CaCO_3$) is also used for their excellent biocompatibility and bioactivity. However, the problem with the animal-origin aragonite demands the new technique to synthesize pure calcite capable of forming 3D bone implant. This study aims to manufacture and evaluate a highly-pure synthetic crystalline calcite with good cytocompatibility regarding to the osteoblasts, comparing to that of HA and β-TCP. After the manufac-ture of macroporous bioceramic scaffolds with the identical internal architecture, their cytocompatibility is studied through MC3T3-E1 osteoblasts with the tests of cell viability, proliferation, vitality, *etc*. The results confirmed that the studied process is able to form a macroporous material with a controlled internal archi-tecture, and this synthesized calcite is non-cytotoxic and facilitate the cell proliferation. Indeed requiring fur-ther improvement, the studied calcite is definitely an interesting alternative not only to coralline aragonite but also to calcium phosphate ceramics, particularly in bone sites with the large bone remodelling.

1. Introduction

Synthetic biomedical ceramics and cements are extensively developed for the treatment of bone defects that arise from trauma, disease or congenital defects. On one hand, the imperatives of orthopaedic patient care and increasing life expectancy multiply the cases of skeletal reconstruction, particularly the use of bone substitutes. On the other hand, the bone auto- or allografts implants of natural origin (coral for example) are no longer well fitted for orthopaedic application, due to their drawbacks such as supply difficulty, biological variability and viral or bacterial contamination risks, *etc*. Nude metallic prosthesis, stainless steel or titanium alloys for instance, are usually and historically the first choice to orthopaedic service. Such biologically inert, biocompatible implants, even optimized through surface coating, can cause bone discontinuities by inducing constraint and wear, due to their incompatible mechanical properties to those of natural bone. The synthetic biomaterial ceramics are so an interesting alternative that they have been widely used currently as bone substitutes [1–3]. Nevertheless, the development and optimization have never been stopped to further improve their biological performance.

Some ceramics, particularly phosphocalcic ones, have a chemical composition similar to the mineral phase of bone. This confers to these bioceramics excellent biocompatibility and osseointegration properties, which make them preferentially used for bone substitutes. The most commonly used among them are hydroxyapatite (HA), often considered as low degradable ceramic, and β-tricalcium phosphate (β-TCP), considered being resorbable. The resorbability, the ability to be assimilated by the living tissue, is indeed important for bone substitutes. It can induce and improve the self-healing capacity of bone, required for the repair of small fractures, by the assimilation of the synthetic bone material with the surrounding living bone tissue. Assuming this is obviously the best solution for the patient (without foreign body reaction, bone reconstruction with continuity and ultimate regeneration of natural bone), it indeed demands specific structural and chemical properties and especially a controlled open macroporous internal architecture for bioceramic implants.

This study aims to manufacture and characterize a calcium carbonate ($CaCO_3$) with regard to the osteoblastic response. Calcium phosphate (CaP) materials have been developed considerably in the past years due to their excellent biocompatibility and bioactivity properties. Three crystalline phases of anhydrous calcium carbonates exist in nature: calcite (rhomboedric, stable), aragonite (orthorombic,

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metastable) and vaterite (hexagonal, unstable). Natural aragonite, mainly issued from coral, is frequently used though seldom studied [4], especially in maxillo-facial surgery, as a cement compound or implant. Although a synthetic aragonite powder does exist [5,6], the great difficulty of producing a volumic scaffold leads to the use of animal-origin aragonite (from coral). Therefore, persistent problems with this source require the solution of synthesizing pure calcite that is able to form a 3D implant, but few studies deal with a high purity synthetic crystalline form. This study aims to manufacture and characterize a calcium carbonate ($CaCO_3$) with regard to the osteoblastic response. As *in vitro* approaches such as the cell proliferation assay are particularly absent from the literature, the studied calcite was compare to hydroxyapatite and β-tricalcium phosphate, whose qualities and properties are well-known from exhaustive studies [7]. To be comparable with regard to a bone reorganization dynamic, the internal architecture of the three bioceramic scaffolds must be identical: the same macroporosity (size and repartition of pores) and the same interconnections between the pores (number and size). In detail, the interconnections must allow the colonization by bone cells and the circulation of biological fluids (to feed the cells and to drain the waste), and they must also ensure bone reorganization and vascularization [8,9]. Therefore, whatever the bioceramic, the samples were manufactured identically following a patented process described in previously study [10].

Since the resorbability of ceramics through primary cultured human osteoclasts was previously studied in our team [11], the osteoblasts MC3T3-E1 were chosen in this study to further characterize the biological response of the bioceramics, by studying the cytocompatibility through the assays of cell viability, cell vitality and cell proliferation.

2. Materials and methods

The manufacturing process of macroporous bioceramic scaffolds is based on the design of an organic construct, impregnated by a ceramic suspension (slurry) filling the interstitial porosity then densified through successive heat treatments to generate a controlled interconnected macroporous material [12]. Dense bioceramics are also directly manufactured by this slip casting method using the slurry. Macroporous and dense ceramics are then used to perform cell cultures.

2.1. Manufacturing and characterization of the bioceramic scaffolds

Polymer balls (the porogen agent) with a fixed size distribution are put in a mould with desired form and chemically stuck together in order to manufacture an organic frame. These chemical welding and chemical forming are ensured by a solvent causing a slow superficial dissolution of the polymer. It allows the realization of bridges at the contact points between balls. This frame is placed in a similarly shaped plaster mould, and an aqueous ceramic slurry is poured into the frame to fill the voids between the polymer particles. After drying and demoulding, a de-binding treatment is carried out at low temperature to allow the porogen agent to be eliminated and to create the macroporosity within the ceramic. The bridgings between the balls generate multiple interconnections between the spherical pores, which are created by the disappearance of those polymer balls. Taking after this process, the dimensions of these interconnections are equivalent to the size of those bridges. After this de-binding stage, the sintering allows consolidating ceramic walls that deliminate the pores. These different steps give rise to a ceramic with controlled macroporous structure. Directly pouring the aqueous ceramic suspension into the mould without the organic frame leads to a dense ceramic.

Polymethylmethacrylate balls (PMMA, diameter 300–400 μm, *Diacron®*, *The Netherlands*), HA, β-TCP and calcite powders used in this study are commercial products. Major physical and chemical characteristics of these commercial ceramic powders are presented in Table 1. The amount of heavy metals in these ceramic powders is measured by inductively coupled plasma element analysis (ICP-AES/OES, ultima 2, Horiba Scientific). These ceramic powders are dissolved in a 0.1 M hydrochloric acid solution at a moderate temperature. The total quantity of trace elements is less than 50 mg/kg (As<3 mg/kg, Ca<5 mg/kg, Hg<5 mg/kg and Pb<30 mg/kg) according to the threshold concentration set by the NF S 94-065 standard, which confirms very high purity of ceramic powders corresponding to the requirements of standards.

Table 1
Physical and chemical characteristics of the used HA, TCP and calcite powders.

Ceramic powder	Supplier	Specific area (m^2/g)	Average diameter (μm)	Ca/P ratio	Theoretical density
HA	Trans-tech USA Bacth n°50192 QC	4.2	1.5	1.66 ± 0.01	3.16
TCP	Siber Hegner DKSH France Batch no. L90508	3.2	1.84	1.50 ± 0.01	3.07
Calcite	SCORA S.A France	2.7	1.5		2.71

For each powder, the aqueous suspension is optimized with regard to concentration in dry matter (70 to 72%) and viscosity (<60 cp). The optimal suspension is ensured through an organic dispersing agent (Darvan C, 0.6 to 1 wt.%, *Vanderbilt Co.*, *USA*) and a planetary milling (180 rpm, 3 h, room temperature). A binder (Duramax B1001, 4 wt.%, *Rhom* & *Haas*, *France*) is then added in order to increase the resistance to the generated constraint from the thermal elimination of PMMA. This step is performed through drying and a moderated thermal treatment of the impregnated shape. The elimination of the organic agent generates the macroporosity. Highly porous and extremely fragile, the ceramics are consolidated by sintering (hydroxyapatite: 1245 °C; β-TCP: 1110 °C; calcite: 890 °C) for 3 h. Calcite sintering is performed in 100% CO_2 atmosphere (0.2 MPa) to avoid decomposing the calcium carbonate into lime [13]. Qualitative

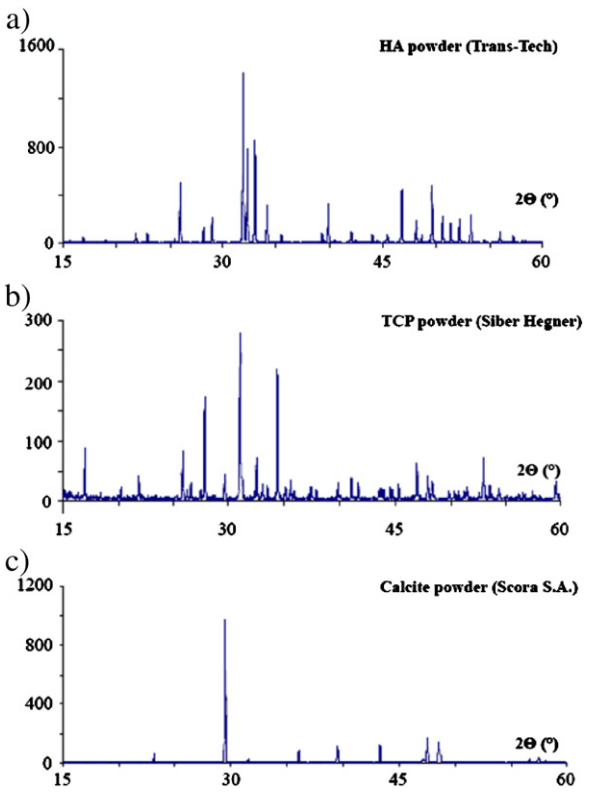

Fig. 1. X-ray patterns of the sintered used ceramics: a/HA, b/TCP and c/calcite.

analysis is performed by Powder X-Ray Diffraction analysis (Rigaku Miniflex). The XRD spectra are collected by employing a step width of 0.02° with counting time fixed to 20 s under 30 kV and 15 mA exciting. X-ray diffraction analyses are performed to assure their high purity after sintering (Fig. 1a–c).

The microstructure of organic frames and finished scaffolds, with and without osteoblasts, is observed through scanning electron microscopy (Hitachi S-3500N, Japan). Dense cylinders are also manufactured (with the same process and the same parameters). Their density is determined through the mercury porosimetry (Pore Sizer 9310, *Micromeritics*, *USA*). The measurement of contact angles is carried out using a goniometer (Kruss, type G1) at 25 °C. Drops of 7 μL are deposited on the surface of dense ceramic samples using a micrometer syringe; ten tests are performed for each solution (water, glycerol, ethylene glycol, diiodomethane, bromonaphtalene, tricresylphosphate, mercury and culture medium supplemented with 10% calf serum). The measurements are taken 1 min after the deposit of the liquid on the samples.

Compression tests are carried out using an instrument (Adamel Lhomargy DY35) equipped with a force cell of 2 kN. The displacement rate of the crosspiece is equal to 5 mm/min. 10 samples per point of measurement are used. The test specimens are all standardized with a height of 20 mm and a diameter of 10 mm.

For the biological tests, 15 mm diameter and 2 mm thick disks are cut (diamond saw) and polished (SiC paper grade 1200). Numerous washings in alcohol and finally in ultra-pure water are performed before drying (2 h at 60 °C) and UV sterilization (1 h for each face).

The concentrations of Ca and P are measured with the Diagnostics kits (587-A and 360–3, Sigma, respectively) using the colorimetric methods to quantify these elements in the different media. Calcium is revealed by the complexion of Ca^{2+} with o-cresolphtaleine. Phosphorus is revealed by the reaction of inorganic phosphorus with ammonium molybdate in the presence of H_2SO_4 to form an unreduced phosphomolybdate complex. The concentration is determined with respect to a standard calcium-solution and phosphate-solution in a LKB spectrophotometer at 575 nm and 340 nm, respectively. The intensity of the optical density of the reacted complex is directly proportional to the concentration of Ca and P.

2.2. Cell culture tests

The cytotoxicity is studied through the viability test of osteoblasts and epithelial cells with exposure to bioceramic powder, and the rate of survival cell colonies allows the estimation of the cell viability. The cell vitality and the cell proliferation are studied on macroporous disc scaffolds, and the number of cells and the activity of mitochondria enzyme indicate the cell proliferation and cell vitality, respectively.

2.2.1. The cytotoxicity of bioceramics

The cytotoxicity is determined through viability tests according to the colony-forming method [14,15] *i.e.* the method of the relative plating efficiency (RPE) leading to the 50% lethal concentration (LC50 or RPE50) determination. Osteoblasts MC3T3-E1 cells (ATCC, CRL-2593) and epithelial cells L132 are used. MC3T3-E1 is an immortalized cell-line derived from mouse calvarium tissue. The osteoblastic phenotype of MC3T3-E1 cell line is regularly checked by the laboratory assays of alkaline phosphatase and osteocalcin. The cells are cultured in a minimum essential medium (alpha MEM, GlutaMAX®, *Gibco Invitrogen SARL*, *France*) supplemented with 10% fetal calf serum (FCS, *Gibco Invitrogen SARL*, *France*), 50 μg/mL aminoglycoside antibiotic (gentamicin, *Panpharma*, *France*) and 25 μg/mL amphotericin b antifungal agent (fungizone, *Gibco Invitrogen SARL*, *France*). All *in vitro* cell incubations are performed in a CO_2 incubator (CB 150/APT-line, *Binder*, *Germany*) at 37 °C in 5% CO_2 atmosphere (relative humidity: 100%). After three and six days, the cell cultures exhibit a growth or multiplication factor equal respectively to 5 and 20.

Cells are first cultured several passages (up to P4) for stabilization and the plated in dishes (100 colonies per dish are obtained on average). They are incubated for 10 days with continuous exposure to increasing concentration (0, 25, 50, 100, 200 and 400 μg/mL) of ceramic powders and PMMA balls (Ø<50 μm) without renewal of the medium. Pure Ni-powder (ref. NI006021, *Goodfellow*, *UK*) is used as a positive control. The concentration leading to the death of half the initial seeded cells is determined for each sample. At least eight experiments are performed for each material and concentration. Therefore, this test quantitatively determines an important criterion of cytotoxicity, the cell death or the cell survival. It is subsequently considered specific, reliable and easily reproducible for evaluating the cytotoxic effect of any chemical substance (powder suspensions or aqueous solutions) by the comparison of the 50% lethal concentration (LC50).

2.2.2. Cell vitality and proliferation

The cell vitality and proliferation tests are performed in the same conditions as the cytotoxicity study through osteoblast cells MC3T3-E1 culture on macroporous bioceramic scaffolds during three and six days without renewal of the culture medium according ISO 10993-5. The cell proliferation quantity is measured by cell counting (cell counter Z1, *Coulter Electronics*, *USA*). Simultaneous to the proliferation test, the cell vitality test gives a precise indication of the cell function via the reaction of the deoxidizing Alamar-Blue dye (*Interchim*, *France*) by the mitochondrial respiratory activity. Sterilized ceramic samples and Thermanox® (*Nunc*, *Germany*) are placed into 24-well cell tissue culture polystyrenes (TCPS) plates (*Nunc*, *Germany*). 10^4 cells are seeded in each well. A TCPS-well group containing only cells is used as a negative control. The culture medium is removed respectively after 3 and 6 days and replaced with 500 μL medium containing 10% fluorescent Alamar-Blue dye for 3 h. The solutions are then transferred into 96-well microtiter plates (*Nunc*, *Germany*). The fluorescence intensity is measured (fluorometer TwinkleTM LB 970, excitation: 530 nm, emission: 590 nm, *Berthold Technologies*, *Germany*). The cell vitality rates of test groups are then calculated as the ratio of their fluorescence intensity to that of TCPS control. The cells that remain on the ceramic samples or controls are trypsinized and the quantity of detached cells is counted. The relative proliferation rates of test groups are calculated as the ISO/EN standardized percentage of cell numbers to that of TCPS control. The final results are rated as the mean of six totally separate triplicate assays.

3. Results and discussion

3.1. Manufactured bioceramics

As described, aqueous suspensions from HA, β-TCP and calcite powders can afford to the direct manufacture of dense bioceramics (Table 2), while macroporous ones are manufactured from the impregnation of organics frames. The latter are produced from the forming process of PMMA balls carried out at 180 °C, by controlling the chemical parameters the size of interconnection is about 110 ± 12 μm in diameter (Fig. 2a). The distribution of the pore sizes is analyzed by scanning electron microscopy. Identical homogenous macroporous architectures are obtained for the three ceramic materials: 70% (±5%) porous volume, pores with a diameter of 250–

Table 2
Characteristics of the HA, TCP and calcite dense ceramics.

Dense ceramics	Sintering temperature	Relative density after sintering
HA	1250 °C	97.3%
TCP	1100 °C	98.1%
Calcite	890 °C(CO_2)	93.4%

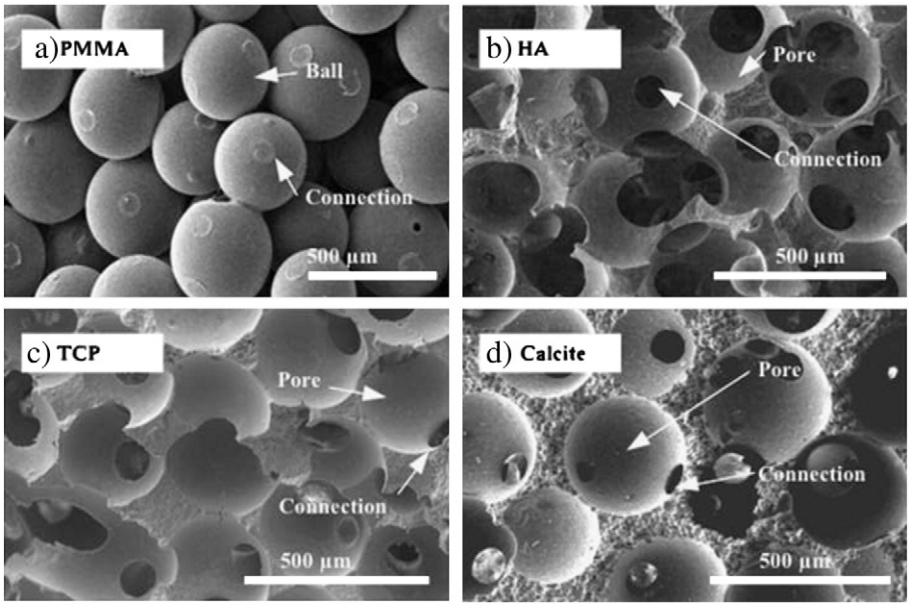

Fig. 2. Micrographs (SEM) of the organic scaffold (Ø 300–400 µm, interconnection Ø 100 µm) and the macroporous bioceramics (70% porosity, pores Ø 300 µm, interconnection Ø 100 µm). a/ PMMA balls frame, b/ HA, c/ TCP, d/ calcite.

350 µm and interconnection with an average diameter of 100 µm (Table 3 and Fig. 2b–d).

Being brittle materials, the major significant mechanical property for three macroporous ceramics is the compressive strength σ, which generally ranges from 5 to 15 MPa according to the literature [16–18]. Mechanical tests performed on the HA- and β-TCP-based macroporous materials (Fig. 3) have shown the compressive strength (σ value) from 10 to 16 MPa with total pore volume of about 70%. The compressive strength of calcite is shown significantly lower than those of HA and β-TCP.

Indeed, there is the study [19] that reported another method of fabrication of calcite ceramic, which is based on a mixture of synthetic aragonite grains and a porogen (naphthalene for example), and thereafter compacting the mixture and heating to obtain a macroporous calcium carbonate. However, this method cannot well control the systematic interconnection and their size, which is exactly overcome by our method; the PMMA beads in contact with each other make it possible establish a totally interconnected architecture and avoid the presence of isolated porosity [12].

Such ability to manufacture calcite scaffolds with controlled internal architecture along with the capacity to keep the manufactured scaffolds identical, allows whatever three chosen ceramics to be comparable toward the cells. It is also one of desired properties of bioceramic scaffold and indispensable to facilitate the cell colonization and total replacement by natural bone eventually.

The mechanical properties of synthetic materials used to fill bone defects are generally low and the compressive strength σ does not surpass 10 MPa when the macroporosity exceeds 40% [18]. Once being implanted, the material is subject to mechanical stresses, a good osteosynthesis in the material is essential to avoid deterioration of the bone substitute. Moreover, the mechanical properties of these ceramics depend on many factors, such as macroporosity, microporosity, the shape, the size and the distribution of the macropores and the size of the interconnections.

Table 3
Pore size distribution in the different scaffolds.

Material	Total porosity (%) ±1%	Pore diameter	
		250–350 µm (%)	<10 µm (%)
HA	67.7	65	2.7
TCP	66.9	65	1.9
CaCO3	71.6	65	6.6

3.2. Cellular evaluation

The viability or the cytotoxicity assessments are performed through osteoblastic culture in contact with ceramic powders and PMMA and Ni. The contact inhibitive nature of this osteoblastic cell line makes it uneasy to form the well distinguishable colonies (Fig. 4a). Thus, the culture condition must be adapted to the requirement of test. Eventually, the best colony formation is obtained with a 1/1 (v/v) mixture of alpha-MEM medium-GlutaMAX® and Dulbecco's medium supplemented with 10% newborn calf serum. 50 tests have shown rather satisfying cell colonies with the relative plating efficiency (RPE) superior to 20% (Fig. 5).

No significant reduction in the cell survival rate is observed and the results are quite close to that of control and that obtained from the human epithelial cell line L132 (Table 4, Fig. 4b), which usually used as the reference for the cell viability tests with a high RPE equal to 37 ± 3.1% [15]. The L132 cells appear to give some better results (*i.e.* higher though insignificant values of cell survival rate) on the bioceramics, but lower values for positive control (Ni powder). Thus, the osteoblastic MC3T3-E1 cell line seems to be well suited for the cell viability testing of bone substitute materials with the advantage of being cell type more relevant to bone than epithelial cells (L132) [14]. The test also shows the innocuousness from PMMA or

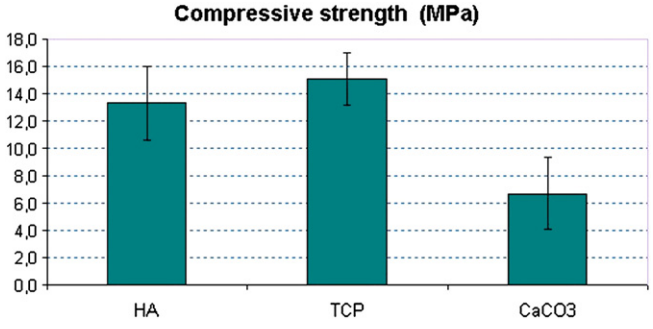

Fig. 3. Compressive strength of the macroporous bioceramics.

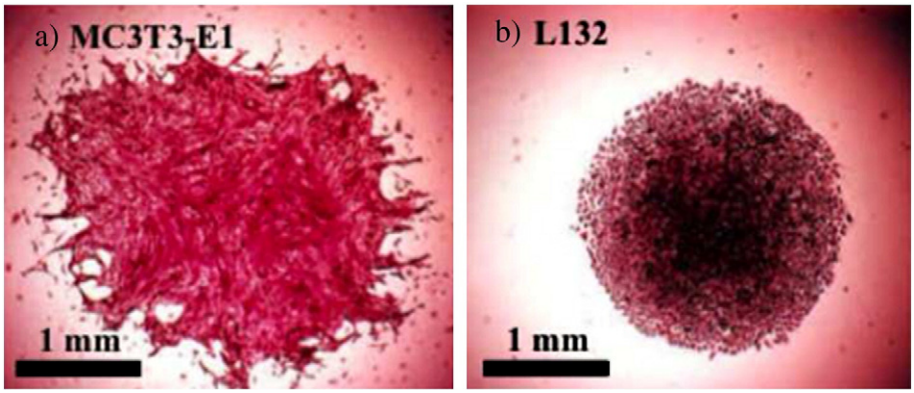

Fig. 4. Micrographs of mouse osteoblast like cells (MC3T3-E1) and human epithelial cells (L132) clones.

its potential pyrolysis residues, which is an important guarantee for the applied manufacturing process.

The cell proliferation of MC3T3-E1 cells is determined after 3- and 6-day culture on the macroporous ceramic discs (Fig. 6). The relative proliferation rates obtained for the investigated ceramics are expressed in % with regard to the negative control (TCPS). Thermanox® is another control material proposed by the ISO/EN 10993/30993 standard and used in particular for experiments such as SEM that require the sample to be removable after cell cultures for preparations or observation. Thermanox® is often considered equivalent to TCPS, though the cell growth rate on Thermanox® is generally 10% lower than on that on TCPS, which may sometimes affect the significance of result.

The result also shows that HA and TCPS have a similar level of cell proliferation rate. In all cases, the number of cells counted after 3- or 6-day culture is always higher than the cell number initially seeded, which shows a positive cell growth on the test substrate. Nevertheless, β-TCP and calcite induce the relatively lower proliferation rates, respectively 40% and 30% after 6-day culture, which are anyway consistent with the results, which are already known in literature [20–22]. Obviously, certain cytotoxicity of β-TCP and calcite materials to MC3T3-E1 osteoblasts, even slight, can be questioned from such finding.

With the similar non-cytotoxicity found from the cell viability test for all ceramics and controls, these lower cell growth rates may arise from some other factors related to the renewal rate of nutrients in *in vitro* culture environment, or the surface morphology, chemistry *etc*., which are difficult to be determined on the macroporous materials and also be considered as the cause of the lower proliferation

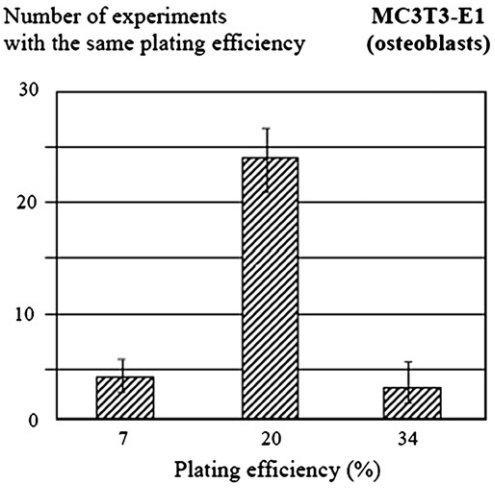

Fig. 5. Assessment of the relative plating efficiency with the MC3T3-E1 osteoblast-like cell line after specific adaptation of the culture conditions (n = 50).

rates by other authors working on phosphocalcic ceramics and coral [20,21]. Anyhow, the similar proliferation rates for both β-TCP and calcite at least confirm the similar interest of calcite material to the widely-used β-TCP material.

In parallel to the cell proliferation, the cell vitality is studied and also showed the reduced level for both phosphocalcic ceramics to a lesser extent. The cell vitality test uses Alamar-Blue, a colorimetric and fluorescent indicator of REDOX reaction, which is able to distinctly detect the changes arising from the metabolic activity of the cells. After 3 and 6 days of culture, the activity of the MC3T3-E1 osteoblasts is near 80% for the porous HA and β-TCP with respect to the TCPS (Figs. 6 and 7). The cellular response on calcite reaches near 60%, though lower than that on the phosphocalcic ceramics. For all three ceramics, the cell vitality is significantly higher than the cell proliferation rate, which might suggest an activation of the osteoblastic function.

To understand better the decrease of the cell proliferation on porous samples, the study of cell morphology on scaffold surfaces after 6-day culture is performed by SEM. On porous substrates, the cell number was clearly less than that on the Thermanox® (Fig. 8a), but the cells showed good spreading with many large pseudopodia on calcium phosphate ceramics (Fig. 8b and c). While, on calcite (Fig. 8d), osteoblasts were rare, isolated and in retracted form. The cell surface had fewer cytoplasmic extensions and appeared much smoother. But, once more, the cell morphology at least clearly proved calcite to be non-cytotoxic. Since cell adhesion is a crucial cell activity in cell-material interaction, as a prerequisite to promote cell expansion, proliferation, migration differentiation and other cell activities.

3.3. Surface properties

The surface properties, such as chemical composition, surface energy and topography, play a major role in the tissue response to the biomaterials. The affinity of a liquid to the substrate can be evaluated by macroscopic criteria characterizing surface wettability and the interfacial free energy. The measurements of contact angle performed on dense ceramics and the negative control (Thermanox®) from

Table 4
Survival rate induced by bioceramics, PMMA and nickel (400 μg/ml) on mouse Osteoblast-like cells (MC3T3-E1) and human epithelial cells (L132) after 8 and 10 days of exposure.

Material	MC3T3-E1 (%)	L132 (%)
HA	93 ± 7	98 ± 5
TCP	99 ± 5	105 ± 8
Calcite	98 ± 5	107 ± 4
PMMA	92 ± 6	96 ± 6
Ni	7 ± 2	0.6 ± 0.2

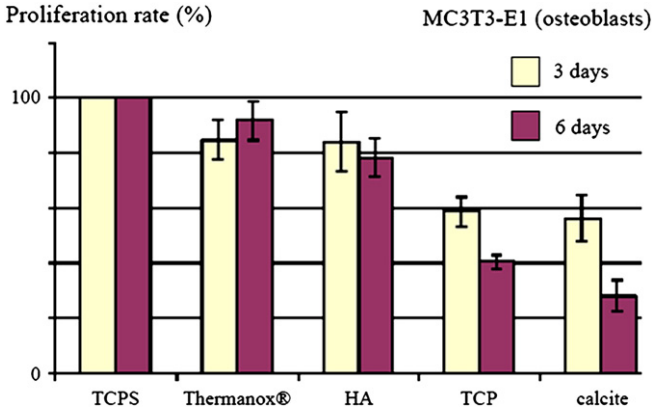

Fig. 6. Cellular proliferation on controls and macroporous ceramics during 3 and 6 days (n = 6).

standard liquids are shown in Table 5. β-TCP and Thermanox® were significantly more hydrophobic than calcite or HA.

The intrinsic properties of materials involve in the nature and the structure of the adsorbed proteins deposition, which can take different configurations. Since the physiological fluids *in vivo* are a complex mixture of various proteins, the culture medium, supplemented with 10% calf serum, appears to be a suitable alternative to simulate the complexity of the *in vivo* condition. For this reason, it seems interesting to perform contact angle measurements on different ceramics with the protein-enriched culture medium. The measurements of contact angle with culture medium *i.e.* the values of $\theta_{\text{culture medium}}$ were 57° (Calcite), 48.7° (HA), 47.6° (Thermanox®) and 44.6° (β-TCP), respectively, which revealed that the wettability of the culture medium on $CaCO_3$ was clearly lower than that on β-TCP. It implies a higher affinity of proteins for calcium phosphate ceramics and Thermanox®. The higher hydrophobicity presented by calcite might be relevant to the lower cell proliferation observed previously for this material.

A slight hydrophobicity could lead to a structural rearrangement of proteins for obtaining minimum energy, which may explain the affinity of proteins for β-TCP.

The adsorption of proteins on the material surface is a biological process crucial for cell-materials interactions, such as cell adhesion and cell proliferation. Yet, proteins are macromolecules with many functional groups, the positive or negative charges, which could involve in the mechanisms of adsorption by induced electrostatic interaction.

The surface wettability of each substrate was defined by the dispersive and the polar components of the surface energies (Table 6). The results obtained from measurements of the contact angle showed very similar surface energies for all three materials: HA (53.432 mJ/m^2), β-TCP (51.025 mJ/m^2), $CaCO_3$ (56.566 mJ/m^2) and Thermanox® (51.52 mJ/m^2). The dispersive component of the surface energy is predominant for all materials. However, osteoblast adhesion was generally modulated by the polar component of the surface energy [23], with regard to which, it is slightly lower for Thermanox® and β-TCP than that for calcite and HA.

Thus, as already mentioned by Redey et al. [23], the surface energy plays an essential role in the osteoblast adhesion, whereas the osteoblast spreading may depend on the surface chemistry, especially on the proteins adsorption and/or on the apatite layers formation. The proliferation may be limited to the solubility of the biomaterial.

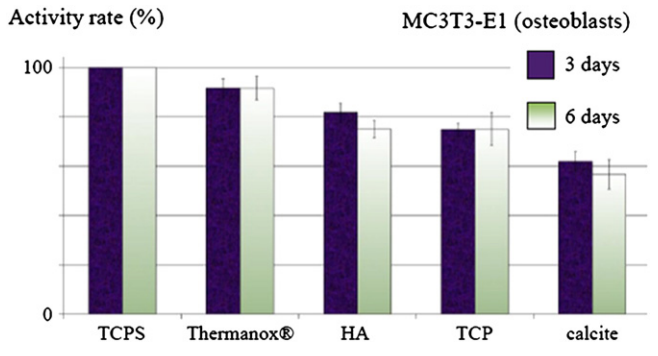

Fig. 7. Cellular activity (Alamar-Blue test) on controls and macroporous ceramics during 3 and 6 days (n = 6).

3.4. Degradation study

The β-TCP and particularly the calcite are ceramics known to degrade relatively rapid in contact with biological fluids. This biodegradation could influence the cell response (cytocompatibility) to implant materials. Calcium and phosphorus in the culture media in contact with the macroporous ceramic were quantified and SEM observation was performed to study the effects of the biodegradation under our experimental condition.

The level of calcium and phosphorus in the culture medium were measured with the ceramic pellets in contact with culture medium for 3 and 6 days without cell (Fig. 9). Calcium and phosphorus in control (the culture medium without ceramics) were stable at 3 and 6 days. The calcium concentration for HA decreased consistently for 3-day contact and increased after 6 days of contact. For β-TCP, the calcium level was also slightly lower than that of control. While unlike calcium phosphate ceramics, the calcium content for calcite remained stable at 3 days and superior to the control medium at 6 days. Correspondingly, the phosphorus level decreased significantly for HA and moderately for β-TCP. On the contrary, for calcite, the phosphorus level decreased very slightly. It is self-evidently due to the absence of the phosphate groups in calcite. The slight decrease of phosphorus could arise from a surface precipitation process.

From these results, the decrease in calcium and phosphorus for phosphate ceramics might imply the phenomena of dissolution–precipitation on the surface of ceramic [24]. On the contrary, for calcite, a moderate increase of the calcium level at 6 days may indicate the start of the degradation of ceramics.

However, it is difficult to quantify the degradation of ceramics from the overall concentrations of calcium, because many other factors are present, such as the different amount of calcium possessed by ceramics (HA: Ca/P = 1.666, β-TCP: Ca/P = 1.5), Moreover, the measured calcium level may not reflect the quantity used in the precipitation process.

Anyhow, the stronger degradation observed for calcite, according to our experimental condition, may explain the reduction of the quality of cell adhesion observed by SEM and the decreased cell proliferation rate.

For comparison, SEM observation of the ceramic surface microstructure, after 6-day culture with MC3T3-E1 osteoblasts and thereafter detached by trypsinization, revealed unmodified architectures for HA and β-TCP. However, for calcite, many ceramic grains were observed, which revealed an early debonding of the sintered ceramic (Fig. 10). Such alteration of surface topography may influence the cell adhesion, the bone matrix deposition and the cell proliferation rate (as described above).

No apatite formation is clearly demonstrated on the surface of calcite under the experimental condition of this study, which could be due to the much slower precipitation kinetics than that for calcium phosphate ceramics. Ohtsuki et al. [25] studied the calcite surface after different periods of immersion in SBF. Precipitation of apatite was detected on the surface of calcite only after a 120-day immersion [25].

Condition of 6-day culture without renewal of the culture medium, *i.e.* the worst experiment case, makes the cells suffer from the impoverished medium and from the ceramic degradation products induced by the slight pH variations. Such condition has strong

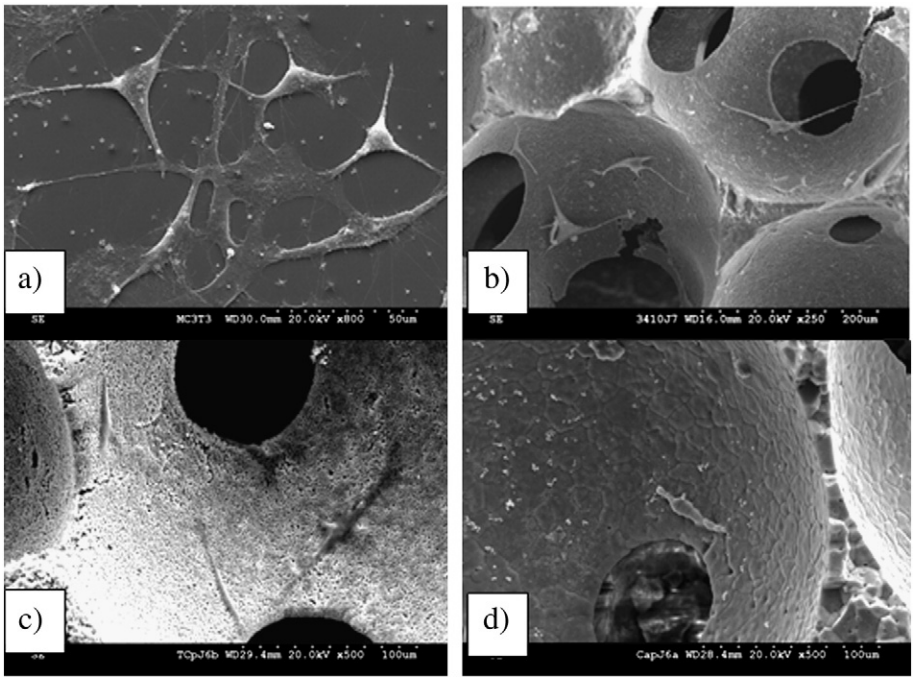

Fig. 8. Micrographs of mouse osteoblast like cells (MC3T3-E1) on scaffold surfaces after 6 days culture a/ Thermanox®, b/ HA, c/ TCP, d/ calcite.

influences in particular on the calcite, which has the highest degradation rate. Consequently, the proliferation of the osteoblasts is strongly affected by the degradation and the solubility of the ceramics. The cellular reaction is subject to the evolution of the pH and to the level of Ca and P in the culture media, which can interfere with the cellular division. The differences in proliferation observed between the three ceramics can arise from the early cell adhesion on the cell–substrate interface at the very first hours of culture. The studied materials are biodegradable and their surface properties are therefore continuously changing. The initial cellular attachment is influenced by the environmental characteristics of the original surface.

However, a surface allowing a good cellular attachment is not definitely able to promote the proliferation or the differentiation of the osteoblasts, particularly if this surface is continuously changing during the degradation process.

What emerges from the literature showed that any very slight modification of a ceramic (intrinsic properties such as the chemical nature, the grain size, the specific area or the density) or any variation of the process (such as the sintering temperature) may induce unforeseeable alteration in the resorption behavior. It highlights the interest of application of synthetic materials manufactured through a strict and reproducible process.

Table 5
Measurements of the contact angle (θ°) on different substrates depending on liquid characteristics.

	Thermanox®	HA	βTCP	$CaCO_3$
Water	63.2 ± 5.9	57.21 ± 5.2	62.33 ± 3.1	55 ± 6.0
Glycerol	61.3 ± 5.7	64.3 ± 1.9	65.7 ± 2.6	41.5 ± 3.9
Ethylene glycol	46 ± 1.8	46.0 ± 5.2	45.1 ± 4.1	43.0 ± 4.1
Diiodomethane	34.9 ± 5.3	47.6 ± 0.7	50.1 ± 6.3	38.0 ± 6.4
α-Bromonaphatalene	19.1 ± 2.0	24.6 ± 3.9	13.4 ± 4.7	17.0 ± 5.7
Tricresylphosphate	16.7 ± 2.1	20.4 ± 5.7	21.1 ± 5.1	5.0 ± 0.7
Mercury	160 ± 5.0	129.3 ± 5.0	134.5 ± 5.0	130.7 ± 3.2
Culture medium (with 10% serum)	47.6 ± 1.9	48.7 ± 2.1	44.6 ± 1.6	57 ± 3.0

4. Conclusion

A previously patented process developed and validated for manufacturing hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP) ceramics with a controlled internal architecture, is proved to be well suited to calcite macroporous ceramics manufacturing. Architecture similar to that of HA and β-TCP is also obtained for calcite: an open macroporous ceramic with macropores (300 to 400 μm in diameter) interconnected (100 μm in diameter), allowing the use as a scaffold for cell culture. Such similar architecture therefore also allows comparing calcite with the other ceramics as HA or β-TCP.

A model for cell colony-forming test is validated by using MC3T3-E1 osteoblast to evaluate cytotoxicity of bioceramics. By comparing with the results from L132 epithelial cells, it shows that, as HA and β-TCP, pure synthetic calcite does not present any cytotoxic character.

Weaker than on HA and β-TCP, the osteoblastic activity (vitality and proliferation) on calcite remains sufficient for cell culture, however.

The measurement of the contact angle showed that the wettability with culture medium supplemented with serum was significantly lower on $CaCO_3$ than those on β-TCP, thus reflecting a higher affinity of proteins for calcium phosphate ceramics and Thermanox®.

Easier to synthesize than aragonite, without the inconveniences of those natural but often animal-origin calcium carbonate, thermally stable, calcite is able to be formed into a bone substitute scaffolds with controlled internal architecture by simple slip-casting process. Indeed requiring further improvement, calcite can definitely be an interesting alternative not only to coralline aragonite but also to

Table 6
Surface tension expressed as a percentage.

Materials	$\gamma_{SV}^{P}(\%)$	$\gamma_{SV}^{D}(\%)$	$\gamma_{SV}(mJ/m^2)$
Thermanox®	19.78	80.22	51.52
HA	26.68	73.32	53.432
β-TCP	22.24	77.76	51.025
$CaCO_3$	24.94	75.06	56.566

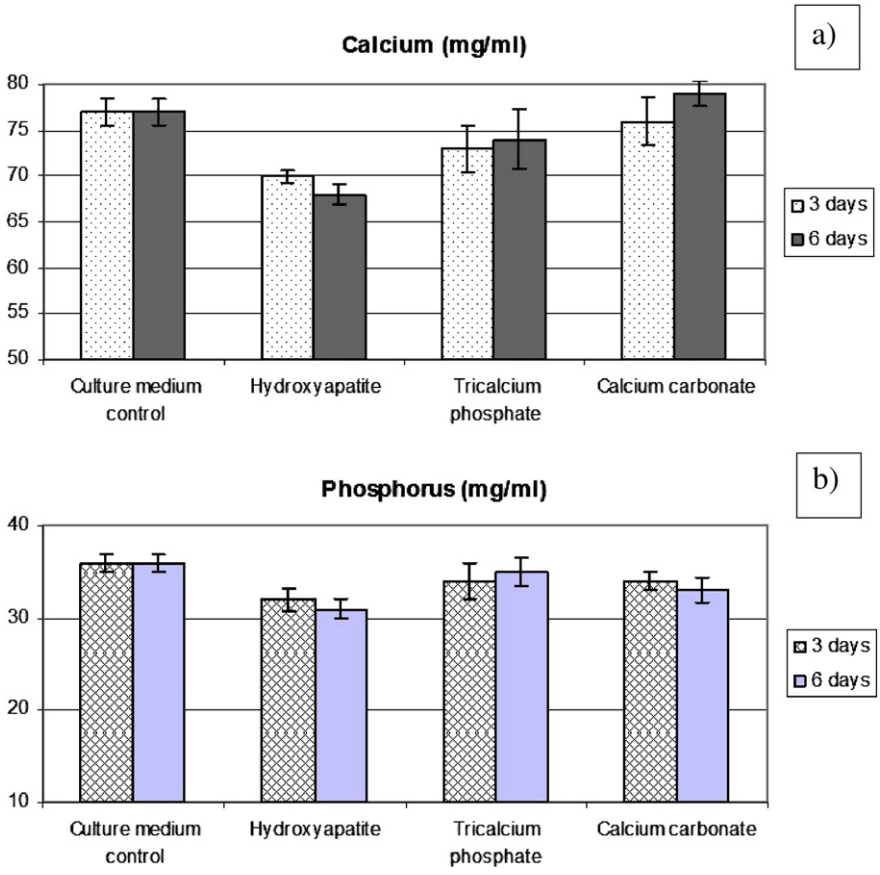

Fig. 9. Concentrations of a/ calcium and b/ phosphorus in culture media in contact with ceramics (after 3 and 6 days).

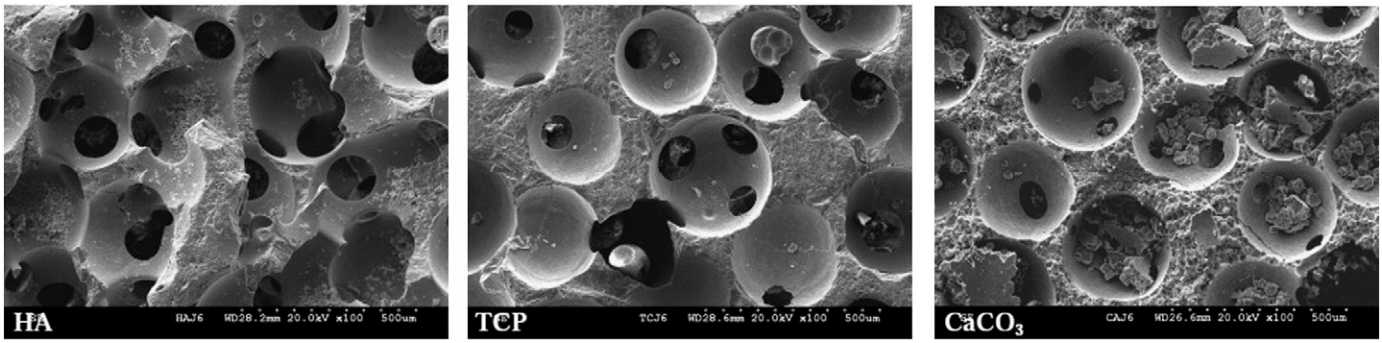

Fig. 10. Micrographs of porous architectures after 6 days of culture after trypsinization.

calcium phosphate ceramics, particularly in bone sites with the large bone remodelling.

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