Preparation, physical-chemical characterisation and cytocompatibility of calcium carbonate cements

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Abstract

The feasibility of calcium carbonate cements involving the recrystallisation of metastable calcium carbonate varieties has been demonstrated. Calcium carbonate cement compositions presented in this paper can be prepared straightforwardly by simply mixing water (liquid phase) with two calcium carbonate phases (solid phase) which can be easily obtained by precipitation. An original cement composition was obtained by mixing amorphous calcium carbonate and vaterite with an aqueous medium. The cement set and hardened within 2 hours at 37°C in an atmosphere saturated with water and the final composition of the cement consisted mostly of aragonite. The hardened cement was microporous and showed poor mechanical properties. Cytotoxicity tests revealed excellent cytocompatibility of calcium carbonate cement compositions. Calcium carbonates with a higher solubility than the marketed calcium phosphate cements might be of interest to increase biomedical cement resorption rates and to favour its replacement by bone tissue.

Keywords: bone cement, calcium carbonate, aragonite, FTIR spectroscopy, cytotoxicity

Introduction

Filling bone defects with materials generating resorption activity and leading to neoformed bone tissue is one of the main subjects of concern for orthopaedic and dental surgery specialists. Many surgeons use bone hetero- or autografts or implants of natural origin (coral for example) [1-6]. However, supply difficulty, biological variability and viral or bacterial contamination risks are major drawbacks [6-7]. In order to solve the problems inherent to substitution with natural biomaterials, synthetic materials have been developed as biomedical ceramics and cements [8].

The fast-setting biomedical ionic cements have emerged as an attractive concept essentially for bone filling and reinforcement biomaterials. Calcium phosphate (CaP) bone cements have developed considerably in the last few years due to their excellent biocompatibility and bioactivity properties and also ease of use especially as an injectable paste [9-17]. Several types of reaction can be involved in CaP biomimetic cements and most of them lead to apatite with varying degrees of crystallisation and carbonate contents [18]. However, one of the main challenges is to reach higher rates of resorption and an improvement of bone formation. Considering that the biological resorption of bone substitute materials is generally related to the solubility of their constituting phase(s), brushite cements or associations of CaP with more soluble mineral compounds (calcium sulfate for example) seemed to offer interesting cement compositions to improve and adapt the cement resorption properties [19-20]. Furthermore calcium carbonate could be an interesting candidate to prepare cement with improved biodegradation rates due to its higher solubility compared to apatite [21-23]. Although some CaP cements containing a low proportion of calcium carbonate have already been proposed, the feasibility of cement composition entirely consisting of calcium carbonates opens new possibilities which have not yet been exploited [11, 24-26].

Three crystalline and one amorphous phases of anhydrous calcium carbonates are encountered in nature: calcite (C), aragonite (Ar), vaterite (V) and amorphous calcium carbonate (ACC) [27-28]. The most thermodynamically stable phase is calcite followed by aragonite and vaterite [21]. Natural calcium carbonate from certain corals and nacre present the aragonite structure. Nacre and coral-based bone substitutes have been shown to be biocompatible and bioactive biomaterials and they have been used for more than 20 years as powders, porous ceramics or gels [1-6, 29-31]. Many *in vivo* studies reported that calcium carbonate-based macroporous bioceramics, prepared by physical-chemical treatment of coral, promote bone ingrowth and resorption [1, 32-33]. However a balance between osteogenesis and biomaterial

resorption is crucial to obtain good clinical results and parameters such as implant porosity, size and association with growth factor, bone specific protein and/or cells can be involved to adapt implant biodegradability [3, 5, 34-39]. The mechanical properties of coral-based graft substitute remain poor and implant rejection can occur probably due to the presence of organic matter residue [7, 40]. Although several attempts have been made to produce synthetic calcium carbonate bioceramics, sintering proves to be difficult and calcium carbonate cements, prepared by mixing calcium carbonate phases with an aqueous medium, offer an interesting way to prepare low-temperature bioceramics [41-42]. Cements, unlike sintered materials and natural materials can easily be associated with active biological molecules (specific proteins, antibiotics...) [43-44].

The feasibility of calcium carbonate-based cements has been recently demonstrated [45-46]. The work presented herein is a preliminary study on new type of bone cement made solely of calcium carbonate phases. The calcium carbonate cement concept is based on the reactivity of biphasic mixtures of calcium carbonate powders comprising metastable phases: one highly reactive amorphous phase and a metastable crystalline phase that can serve as seeds and orient their (re)crystallisation into a more stable calcium carbonate crystalline phase in the presence of small amounts of aqueous medium. Preparation of synthetic calcium carbonate-based cements thus requires the synthesis of pure calcium carbonate polymorphs that can be involved in initial, transient or final cement composition. Several protocols for calcium carbonate polymorphs (calcite, aragonite and vaterite) synthesis and studies on their formation and transformation have been reported [47-52].

FTIR spectroscopy and X-ray diffraction were used to check the purity of the initial calcium carbonate powders and also the composition of the calcium carbonate cements during setting and hardening. Setting kinetics and mechanical properties of calcium carbonate cements were also investigated. Finally, the cytotoxicity of each cement final composition was evaluated.

Materials and Methods

Preparation and characterisation of powders

Calcium carbonate crystalline phases were prepared by double decomposition between a calcium chloride solution and a sodium carbonate solution at 100°C giving aragonite and at 30°C giving vaterite.

In this preliminary work, one of the problems was to prepare pure amorphous calcium carbonate phase. Unlike amorphous calcium phosphate which can be easily obtained by

precipitation, amorphous calcium carbonate crystallises rapidly and, in the absence of stabilising ions (ACC1 preparation), a mixture of metastable calcium carbonates consisting of ACC associated with some vaterite and calcite is obtained (data not shown). A new synthesis protocol of pure amorphous calcium carbonate (ACC2) was set up which is based on the introduction of magnesium ions in the cationic solution used for ACC precipitation by double decomposition. Mg^{2+} is known as a crystallisation inhibitor and especially as amorphous calcium carbonate stabiliser [53]. Amorphous calcium carbonate was rapidly prepared from a calcium chloride solution including strontium (ACC1) or magnesium (ACC2) chloride and a sodium hydrogencarbonate solution at ambient temperature. After filtration and washing, the precipitates were lyophilised and the powders stored in a freezer.

Preparation of cement

The cement powders were made up of a mixture of metastable calcium carbonate phases (see Table 1). The cement paste was obtained by mixing the powders with an appropriate amount of liquid phase (either deionised water or a sodium chloride solution: 0.9% w/w). We chose to test the use of isotonic solution (0.9 % (w/w) NaCl solution) as it is already the liquid phase of some cements (like alpha-BSM®) and because it could open the possibility to use blood serum as liquid phase. Moreover, the possibility to prepare $CaCO_3$ cements with the patient's blood could have interesting advantages in adding active components to the cement (platelets, proteins, growth factors ...) [45].

The wet paste was placed in a sealed container saturated with H_2O at 37°C for setting and hardening. The maturation of cement *in vitro* in an atmosphere saturated with water can be compared to that occurring *in vivo* in contact with biological tissues.

All the powders synthesised and the cement prepared were characterised by transmission FTIR spectroscopy from KBr pellets (Perkin Elmer FTIR 1600 spectrometer) and X-ray diffraction (Inel CPS 120 diffractometer) using a Co anticathode. Observation of cements by scanning electron microscopy (SEM) was carried out using a Leo 435 VP microscope. Small pieces of hardened and dried cement samples were placed and fixed on a support with double faced carbon tape.

FTIR quantitative analysis for the study of cement maturation

When studying a novel cement, it is important to have qualitative and quantitative data to identify the reaction involved in the cement setting and hardening processes and to quantify the transformation kinetics. Several analytical investigations to simultaneously quantify

vaterite and aragonite polymorphs which are precursors of the most stable calcium carbonate crystalline phase, i.e. calcite, are reported in the literature [54]. According to the work of Dickinson and Mc Grath the quantitative detection limits in binary and ternary calcium carbonate mixtures were lower for powder X-ray diffraction analysis than for Raman and IR spectroscopic analysis whereas for Kontoyannis and Vagenas, lower detection limits were obtained for Raman spectroscopy compared to powder XRD analysis [55-56]. It should be noticed that the accuracy of X-ray diffraction analysis depends strongly on the crystallinity of the carbonate phases whereas vibrational spectroscopies are less sensitive to this parameter. FTIR spectroscopy appeared as a simple and accurate technique to identify and quantify calcium carbonate polymorphs. Most of the IR methodologies presented in the literature were restricted to binary mixture probably because of overlapping bands for vaterite, aragonite and calcite. Recently, Vagenas et al. presented a FTIR method to quantitatively discriminate between calcite, aragonite and vaterite in a ternary mixture [57]. Their methodology is based on the deconvolution of the various polymorphs ν_4CO_3 band but it should be noticed that the intensity of the absorption bands remains very low in this domain. However, a decrease of the broad band characteristic of vaterite at 745 cm^{-1} and an increase of the sharp bands at 713 cm^{-1} and 700 cm^{-1} assigned to aragonite were clearly observed on the spectra of the evolving calcium carbonate cements (data not presented).

We propose here a method for aragonite quantification in aragonite-vaterite mixtures based on the decomposition of the ν_2CO_3 band. The quantitative evaluation of aragonite content by FTIR spectroscopy was performed by curve-fitting in the ν_2CO_3 domain (940-800 cm^{-1}) using Grams/32 software (Galactic Industries Corporation). In this domain aragonite and vaterite showed a sharp and intense absorption band at 855 cm^{-1} and 875 cm^{-1} respectively. However, the method proposed cannot be used for simultaneous discrimination of the three calcium carbonate polymorphs and for aragonite quantification in ternary mixtures (i.e. calcite, aragonite and vaterite mixtures) as calcite and vaterite both show a band around 875 cm^{-1}. Further investigation into this method of analysis needs to be performed to determine the aragonite detection limit and relative error in the proportion of aragonite (in w/w %) calculated for the binary mixture.

All the IR spectra were decomposed with the same parameters according to the following protocol. First, a baseline correction was performed on each spectrum. Peak positions and some curve parameters (lorentzian peaks) were set and used as initial input in a curve-fitting program. Iterations were continued until the best fit was obtained. The output of this analysis

was expressed as relative peak area and peak position. From these decomposition results, we calculated the ratio of aragonite peak area to the total ν_2CO_3 band area. Figure 1a shows an example of decomposition of the ν_2CO_3 band into two main sub-bands at 875 cm^{-1} and 855 cm^{-1} respectively assigned to vaterite and aragonite.

A calibration curve was built from standard mixtures of aragonite and vaterite (15 %, 25 %, 50 %, 75 % aragonite w/w) whose spectra were decomposed according to the procedure detailed above, see figure 1b. A good correlation (r = 0.9993) between the experimental points (from 15% to 75 % (w/w) of aragonite) was obtained.

In order to follow up, step by step, the variations of the cement composition during setting and hardening, samples of (ACC2+V) cement prepared with water were freeze dried to stop their maturation after different periods of time. The samples were then analysed by FTIR spectroscopy and their spectra decomposed according to the procedure detailed above.

Setting time and compressive strength

The setting rate of the cement was followed with a TA–XT2 Texture Analyser fitted with a cylindrical needle (0.7 mm in diameter). It was considered that the cement had set when the paste developed a resistance to needle penetration of over 600 g/mm^2.

The compressive strength of the cement was evaluated using a Hounsfield Series S apparatus. The cement paste was placed in a cylindrical mould (height / diameter ratio ≅ 2 and diameter equal to 10.5 mm) and packed tightly to eliminate air bubbles trapped in the paste. After setting and hardening of the paste placed at 37°C in a sealed container saturated with water for 1 day, the hardened cement was withdrawn from the container and left to dry for 1 week at 37°C. The cement was then removed from the mould and the compressive test performed.

Indirect cytotoxicity study

Cytoxicity tests are essential before *in vivo* evaluation of new bioceramics. The indirect cytotoxicity evaluation of calcium carbonate cement compositions was assessed by an extraction method according to NFEN30993-5 ISO 10993-5 [58-59]. Osteoprogenitor cells obtained from human bone marrow, according to Vilamitjana-Amédée et al. with some modifications, were used for testing the extracts and cultured in Iscove Modified Dulbecco's Medium (IMDM, Sigma Aldrich, France) containing 10% foetal calf serum (FSC, Sigma, France) [60]. The cells were seeded at a density of 40 000 cells/cm^2 in 96-well microtiter plates (Nunc, Denmark) and the culture was maintained at 37°C for 96 h after cell plating. At

subconfluency the medium was replaced by the material extraction vehicle. To obtain extraction vehicles, fragments of sterile hardened cement were immersed in IMDM. The ratio of the sample surface area to the volume of the vehicle was 5 cm^2/ml. Extractions were performed in borosilicate glass tubes at 37°C for 120 h without stirring according to the standard procedures. Borosilicate tubes containing identical extraction vehicles with either no material or a solution of phenol at a concentration of 6.4 g/l (known to be cytotoxic) were processed under the same conditions to provide negative and positive controls, respectively. The medium was removed and replaced by control extracts at various concentrations (100% (v/v), 50% (v/v), 10% (v/v), 1% (v/v)) in the culture medium for 24 h at 37°C. At the end of the extract incubation period, tests were performed: cell viability (Neutral Red assay) and cell metabolic activity (MTT assay) [61-62]. The intensity of the colours obtained (red and blue respectively) is directly proportional to the viability and metabolic activity of the cell population and inversely proportional to the toxicity of the material. Indirect cytotoxicity tests were duplicated for each cement composition. The mean values of absorbance measurements obtained from colorimetric tests and their corresponding standard deviation (±SD) were calculated. The results are expressed as a percentage of the negative control (plastic) tested during the same experiment.

Results

Among the various powder mixtures associating reactive amorphous and crystalline $CaCO_3$ phases, the compositions leading to hardened and cohesive cement are reported in table 1. The liquid-to-solid ratio (L/S) ranges between 0.4 and 0.6 depending on the composition of the powder mixture probably due to powder particle size, specific surface area and thus to the powder mixture reactivity. We did not consider these parameters in this preliminary study, and the amount of solution was determined to allow the formation of a mouldable paste. For one cement composition, the L/S ratio can vary to some extent but the reported value corresponds to the optimum final mechanical resistance of the cement.

Of all the powder combinations tested, the ACC2 and vaterite powder mixture (in the proportion 1:2 respectively) appeared as the most promising formulation for calcium carbonate cements and was selected for further investigations on these novel calcium carbonate cements as it presented optimum cement cohesion and mechanical resistance. However the initial cement compositions are not limited to those presented in table 1 and other calcium carbonate powder mixtures ((ACC+V) with different L/S ratio and phase

proportions, (Ar+V) mixture and (ACC+Ar) mixture) were tested. They led to setting pastes but the compressive strengths remained very poor.

Figure 2 showed the X-ray diffraction (XRD) diagram of two hardened cements. It is interesting to note that the final phase of the (ACC1+V) mixture is calcite whereas that of (ACC2+V) is mostly aragonite. These results point out the importance of the composition of the initial mixture of metastable calcium carbonate phases in the orientation of the setting reactions and the final composition of the cement.

The FTIR spectra in the ν_2CO_3 domain of cements analysed after different periods of maturation at 37°C are presented in figure 3. As time elapses, we can clearly notice a decrease of the absorption band assigned to vaterite (around 875 cm^{-1}) and the appearance of the absorption band characteristic of aragonite at 855 cm^{-1} after 2h of maturation at 37°C. The aragonite band progressively increased for 24h when it reached maximum mechanical strength. After two days or more, no significant variation was noticed.

To further analyse setting and hardening of the cement evolution, a quantitative study based on FTIR spectroscopy data in the ν_2CO_3 domain was carried out. The proportion of aragonite (w/w) in the cement was determined using the calibration curve presented in figure 1 and is reported in table 2. The data show a strong increase of the proportion of aragonite during the first day at 37°C and especially during the first 8 hours. After 2 days, the final cement composition consisting of 90 % (w/w) aragonite was reached.

The steady increase in the proportion of aragonite was also revealed by SEM observations of (ACC2+V) cement fragments after various periods of maturation (see figure 4). Indeed, SEM micrographs indicated a visible change in crystal morphology during cement setting and hardening, especially between 2 h and 8 h, where the vaterite lentil-like morphology disappeared while entangled more elongated crystals are visible in figures 4c and 4d. In the latter micrographs, entangled small needle-like crystals were observed suggesting the presence of aragonite. These observations corroborated the FTIR spectroscopy and X-ray diffraction results. Figure 5 showed SEM micrographs of block of hardened cement prepared with sodium chloride solution (0.9 % NaCl w/w) as liquid phase. We can see the entanglement of small needles of aragonite (figure 5b) arranged around many spherical micropores.

The setting time and the cement compressive strength are reported in table 3. Just after mixing the powder with the liquid phase, the paste was viscous and easily moldable for several minutes then the cements set and hardened. Faster setting and higher mechanical resistance were measured for cement prepared with ACC1.

Results of the indirect biocompatibility study following incubation of cells with material extracts at different dilutions showed no cytotoxicity effect of cements prepared with (ACC1 + V) and (ACC2 + V) mixtures, see figure 6. Values of cell viability obtained for undiluted extract were (94 ± 13)% for (ACC1 +V) and (103 ± 10) % for (ACC2+V) which are close to the 100% value for the referenced control (plastic). The results of the metabolic activity measurement for undiluted extract were also close to the 100% value for the referenced control (plastic) ((108 ± 7)% for (ACC1 +V) and (93 ± 9) % for (ACC2+V)). In no cases, did we notice a significant difference for the two cement compositions or between the different extract dilutions (1 to 100 %).

Discussion

Cement compositions

The calcium carbonate cements presented herein and the trial composition (ACC2+V; 1:2) are the first biomedical cements entirely consisting of calcium carbonates ever reported. Several CaP biomedical cement formulations that incorporate a certain proportion of $CaCO_3$ have been designed, the $CaCO_3$ being present to improve cement mechanical properties, cement compliance or to create macroporosity but the proportion of calcium carbonate in such cements did not exceed 15% (w/w) and the final product did not contain only calcium carbonate phases.

The calcium carbonate cement compositions presented in this paper can be prepared straightforwardly by simply mixing water (liquid phase) with two calcium carbonate phases (solid phase) which can be easily obtained by precipitation. The initial composition of calcium carbonate cements comprising a biphasic powder mixture including amorphous calcium carbonate and a metastable crystalline phase can be related to the α-BSM® cement concept (ETEX Corporation). The latter involves an amorphous CaP phase and a crystalline metastable CaP phase (DCPD) acting as a template to facilitate apatite crystal nucleation and growth [63]. In this new type of calcium carbonate cement, the initial crystalline phase probably seeded the crystallisation of a more stable calcium carbonate phase. The presence of such a metastable crystalline phase appears essential for cement setting and hardening as upon admixture with water, ACC2 powder (pure ACC) did not harden whereas ACC1 powder (mixture of ACC, calcite and vaterite) set and hardened (data not presented).

The possibility of orienting the recrystallisation of a calcium carbonate polymorph during cement setting and controlling the phase(s) composition of the hardened cement appeared as

an interesting feature of these cements to adapt their biodegradation properties to the intended application.

Setting and hardening reaction

In a preliminary study of calcium carbonate cement compositions, especially mixtures of ACC1 and aragonite (in the proportion 3:1 respectively), FTIR spectroscopy analyses revealed that cement setting did not occur if vaterite, from the initial metastable $CaCO_3$ powder, remained in the final cement and/or if the proportion of aragonite in the resulting cement was lower than 50 % (w/w) (data not presented). These preliminary results suggested that the transformation of vaterite into aragonite was the key reaction of cement setting. In the present study, we determined the composition of the final phase (FTIR data) comprising mostly aragonite which indicated that the setting reaction of (ACC2+V) is the recrystallisation of vaterite into aragonite.

With regards to the FTIR quantification results, aragonite represented 90% w/w of the set cement and the remaining 10 % could be untransformed vaterite and calcite which are indistinguishable in the ν_2CO_3 infrared domain. As aragonite and calcite polymorphs are also difficult to discriminate in the ν_4CO_3 infrared domain, X-ray diffraction analysis for the identification of minor phase(s) possibly existing in the final composition of (ACC2+V) cement was complementary to FTIR spectroscopy characterisation. The presence of small amounts of calcite was confirmed as the most intense reflection peak (104) of calcite appeared very small on (ACC2+V) cement X-ray diagram. (see figure 2). This result suggests that our evaluation of aragonite proportion in the cement final composition, based on FTIR spectroscopic data for aragonite-vaterite mixtures, was approximative. Complementary FTIR analysis of a prepared binary mixture of aragonite and calcite in the proportion 90:10 % w/w, (data not presented) confirmed the presence of about 10 % (w/w) of calcite in (ACC2+V) cement. However, for future investigations, we will have to set a semi-quantitative method applicable to ternary mixture (vaterite, aragonite and calcite) to precisely determine the transient and final compositions of $CaCO_3$ cement.

Observations of the changing crystal morphology by SEM confirmed the recrystallisation of the metastable calcium carbonate phases (amorphous calcium carbonate and vaterite) mostly into aragonite during setting and hardening. Crystallisation of calcium carbonate polymorphs and entanglement of crystals are responsible for cement setting and hardening. These processes are also involved in the setting and hardening of all CaP cements [8]. However, the type of reaction responsible for setting and hardening these novel calcium carbonate-based

bone cements differs from the well-studied and -developed CaP-based cements in several aspects. CaP biomedical cements can be classified into two categories: apatite and brushite cements that respectively lead to hydroxyapatite and brushite (Dicalcium Phosphate Dihydrate: DCPD) which is converted into apatite *in vivo* [8, 64]. Two types of setting reactions can be distinguished for CaP cements: acid-base reaction and/or fast hydrolysis of a metastable CaP phase into apatite associated with respectively more or less pH variation of the paste during setting. In the case of calcium carbonate cements, the formation of a more stable crystalline phase (aragonite or calcite) is probably involved due to a dissolution-reprecipitation process of the unstable ACC presenting a high solubility. We have shown that aragonite crystallisation is progressive during the first 24 hours of cement hardening and no transient phase was detected by FTIR spectroscopy. We can assume that this type of $CaCO_3$ transformation does not lead to significant pH variation of the paste during cement formation. Moreover, no visible change in pH (solution colour) was observed when fragments of hardened cement were immersed and equilibrated in the IMDM solution used for the indirect cytotoxicity test. Thus, it means that the products of the setting reaction and/or the residues are, as expected, not highly acidic and/or basic compounds.

Studying the transformation mechanism of calcium carbonate in water, Ogino et al. reported that the gradual transformation of metastable polymorphs (ACC, V and Ar) to the stable form, calcite, depends on the temperature within the period of time ranging between 60 min to 1300 min [52]. Formation of all three polymorphs (C, Ar, V) was observed around 40°C and the authors showed that vaterite transformation to aragonite through dissolution-reprecipitation can be fast (1h) whereas aragonite to calcite transformation is the limiting step as it takes longer (15h). Even though the amount of water required for calcium carbonate cement preparation is very low (L/S = 0.6 w/w), water is very probably involved in the (re-)crystallisation of ACC2 and vaterite. We can consider that for the (ACC2+V) mixture the setting and hardening processes were too fast to allow complete transformation of metastable phases up to the most stable $CaCO_3$ polymorph (calcite).

Another parameter that could influence the final cement composition is the presence of significant amounts of magnesium ions in the ACC2 powder (2.5 % w/w of Mg, data not presented) which was precipitated from a cationic solution containing magnesium ions. Several authors reported that adsorption of magnesium ions inhibits the growth and the dissolution of calcite whereas crystallisation and dissolution processes of aragonite are less kinetically altered by the presence of Mg^{2+} [53, 65-68]. Thus, in the case of (ACC2+V), aragonite would crystallise rather than calcite due to the presence of magnesium ions.

The case of (ACC1+V) cement composition is different as calcite crystals were present in ACC1 powder and they can seed the recrystallisation of metastable calcium carbonate phases included in the initial powder mixture (ACC and vaterite).

Further studies need to be done to understand and optimise the setting and hardening reactions of these emerging calcium carbonate cement compositions.

As the setting and hardening reaction does not induce an increase of cement temperature or large pH variations in the cement, we can consider association of the paste with biologically active molecules (growth factors, specific proteins) promoting tissue repair.

Calcium carbonate cement properties

Even though the compressive strength of the calcium carbonate cement remained poor in all cases ($R_{comp} \leq 13$ MPa), such mechanical properties would not be a handicap for *in vivo* applications such as bone filling especially in low mechanical stress locations. Moreover, these properties can probably be improved by optimising the specific surface area of the reactive powders and thus the L/S ratio. Shorter setting times and higher compressive strengths were obtained for the (ACC1+V) mixture which led to cement mostly consisting of calcite (Table 3). The lower liquid-to-solid ratio can explain the differences observed in mechanical properties but the effect of calcite crystal entanglement should also be considered. However, as the preparation of such a cement involved the metastable ACC1 the complex composition of which is difficult to control during synthesis, we decided to discard this composition for future investigations on calcium carbonate cements. Furthermore, aragonite, which the (ACC2+V) hardened cement mostly comprises, has been used as a biocompatible and bioactive biomaterial for more than 20 years.

The excellent cytocompatibility of the calcium carbonate cement compositions revealed by indirect cytotoxicity evaluation is probably related to the stability of pH involved in the setting reaction and the low solubility of the calcium carbonate involved. Another advantage of such calcium carbonate cements is that their *in vivo* biodegradation would release calcium and carbonate ions and/or CO_2 which are non-cytotoxic metabolites under the metabolic control of the organism.

Observations of the hardened cement body by scanning electron microscopy showed that the cement prepared either with water or sodium chloride solution as liquid phase was microporous. However, the use of sodium chloride solution lead to a more structured cement with numerous spherical micropores that appeared interconnected. In future investigations we can consider introducing macroporosity into calcium carbonate cement based on the easy

release of gaseous CO_2 in slightly acidic conditions which could allow cell rehabitation and bone formation within the cement. An open macroporosity associated with this new type of cement would enable macroporous calcium carbonate blocks to be prepared at low temperature which can be very interesting for tissue engineering applications.

The expected higher rates of cement biodegradation and bone reconstruction after in vivo implantation of this cement compared to CaP cements would be a decisive advantage for the development of calcium carbonate cement formulations especially to improve bone regeneration in dental or orthopaedic surgery.

Conclusions

The feasibility of calcium carbonate biomedical cements composed of 100 % $CaCO_3$ has been demonstrated and an original cement composition was proposed. Following the phase composition during the setting and hardening periods evidenced the recrystallisation of the initial metastable calcium carbonate phases mostly into aragonite and/or calcite depending on the initial powder mixture. We did not detect any toxic effects for the human osteoprogenitor cells suggesting good cytocompatibility of $CaCO_3$ cement compositions.

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Figure 1: Quantitative analysis of aragonite weight proportion in aragonite-vaterite mixtures using FTIR spectroscopy data:

a) Example of decomposition of the ν_2CO_3 band (920-820 cm^{-1}) of (ACC2+V) cement

b) Calibration curve giving aragonite peak area on ν_2CO_3 band total area ratio versus the aragonite proportion (w/w) in aragonite-vaterite mixture.

Figure 2: X-ray diffraction diagrams of the ending phase(s) of calcium carbonate cements prepared with a) ACC1+V (1:1; L/S=0.5) and b) ACC2+V (1:2; L/S=0.55).

Figure 3: FTIR spectra in the ν_2CO_3 domain (920-820 cm^{-1}) of the cement (ACC2+V; 1:2; L/S=0.55) during setting and hardening.

Figure 4: SEM micrographs of the (ACC2+V) cement fragments during setting and hardening.

a) (ACC2 + V) powders initial mixture
b) cement after 2 hours of maturation
c) cement after 8 hours of maturation
d) cement after 2 days of maturation

Figure 5: SEM micrographs of the (ACC2+V) cement prepared with sodium chloride solution (0.9 % NaCl w/w) as liquid phase.

a) Magnification: x 1000
b) Magnification: x 2500

Figure 6: Indirect cytotoxicity evaluation of the two calcium carbonate cement compositions

a) Cell viability
b) Metabolic activity

Table 1: Calcium carbonate cements: initial compositions.

Powder mixtures	Powder weight ratio	L/S (w/w)
ACC1 + V	1:2	0.4
ACC2 + V	1:1	0.55
ACC2 + V	**1:2**	**0.6**
ACC2 + V	1:3	0.5

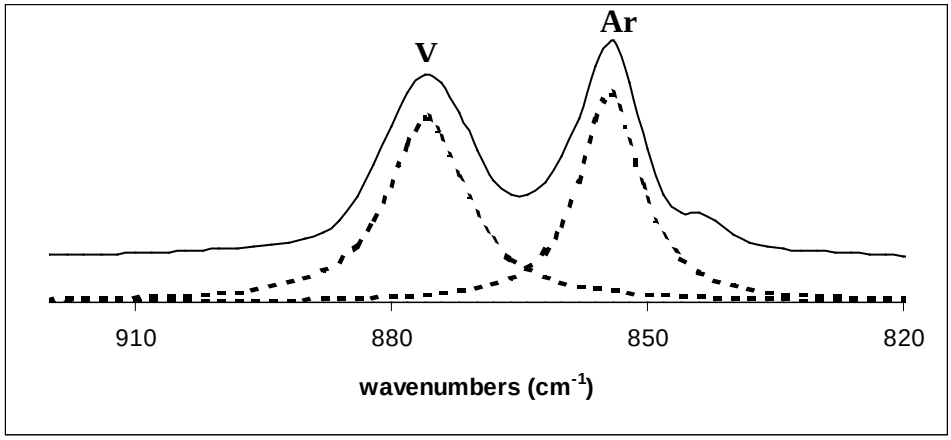
L = liquid; S = solid
V = vaterite
ACC1 and ACC2: amorphous calcium carbonate prepared in different conditions

Table 2: Proportion of aragonite (w/w) determined from FTIR spectroscopy data (calibration curve in figure 3b) of the (ACC2+V) cement during setting and hardening.

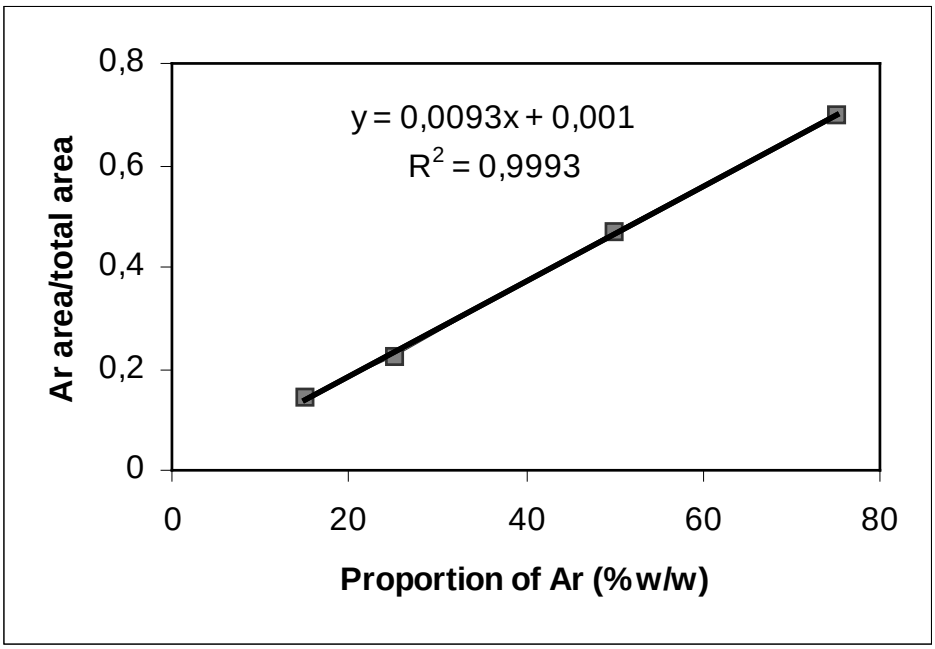
Time	**Area ratio (Ar / total area)**	**Proportion of Aragonite (w/w)**
5 min	0.11	12 %
2 h	0.20	22 %
4 h	0.28	31 %
8 h	0.52	58 %
1 day	0.73	81 %
2 days	0.81	90 %
6 days	0.82	91 %

Table 3 : Setting time and compressive strength of two calcium carbonate cement compositions.

Powder mixtures	**Powder weight ratio**	**L/S (w/w)**	**Major final phase**	**$t_{setting}$ (min)**	**R_{comp} (MPa)**
ACC1+ V	1:2	0.4	Calcite	30	13
ACC2 + V	1:2	0.55	Aragonite	112	3


Ar
V
910
880
850
820
wavenumbers (cm-1)

a)


0,8
0,6
0,4
0,2
0
y = 0,0093x + 0,001
R2 = 0,9993
Ar area/total area
0
20
40
60
80
Proportion of Ar (%w/w)

b)

Figure 1

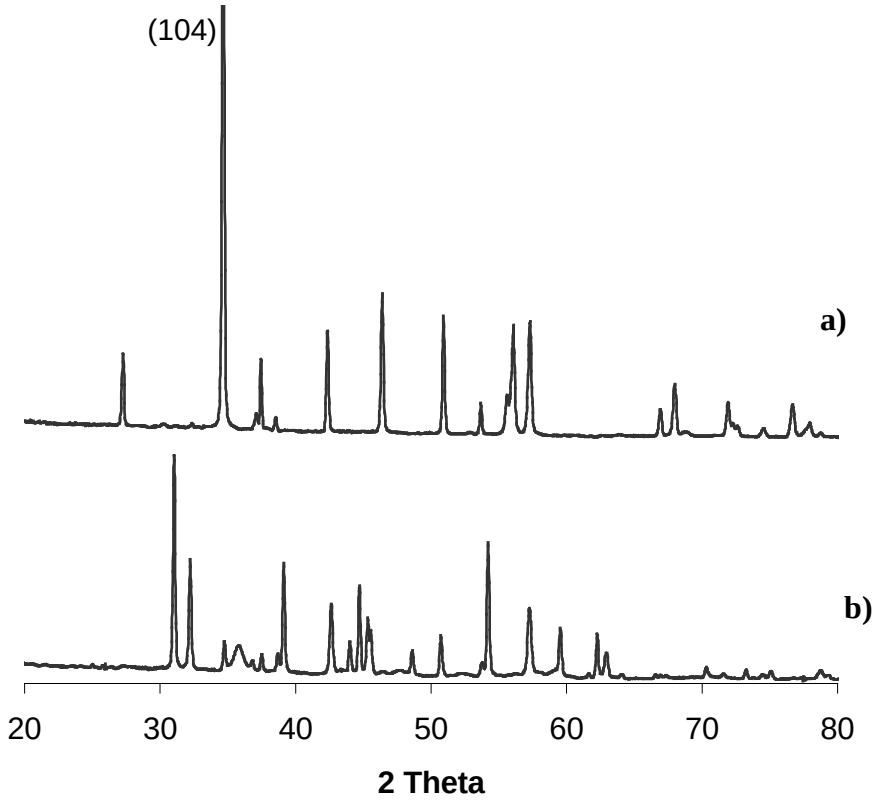


Figure 2

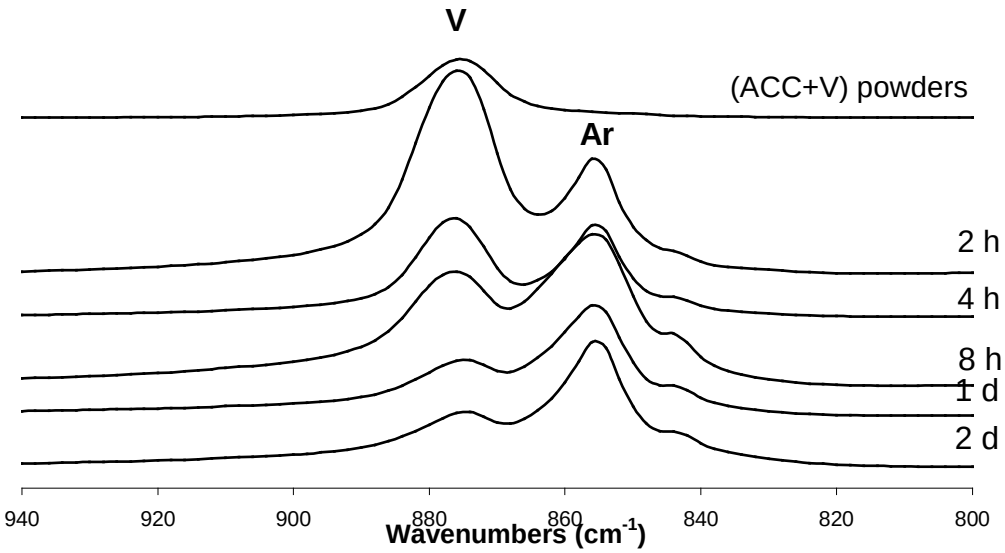
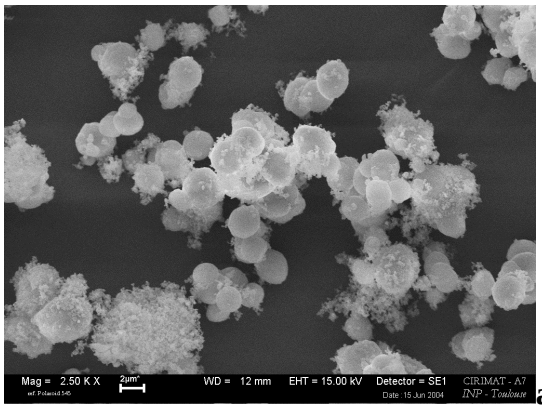
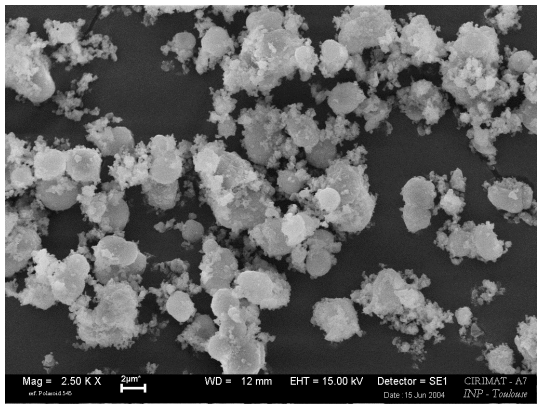

V
(ACC+V) powders
Ar
2 h
4 h
8 h
1 d
2 d
940
920
900
880
860
840
820
800
Wavenumbers (cm-1)

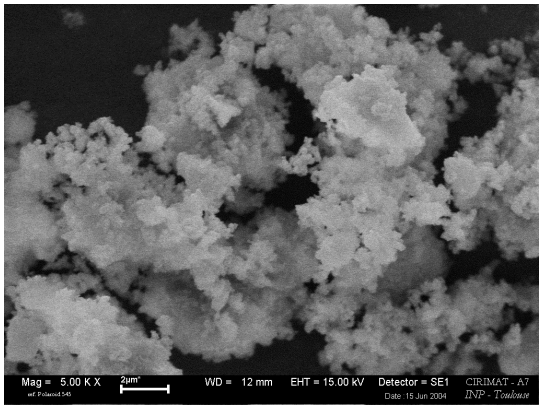
Figure 3



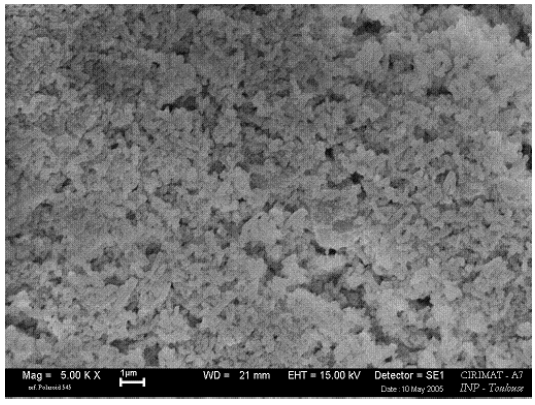
a)



b)

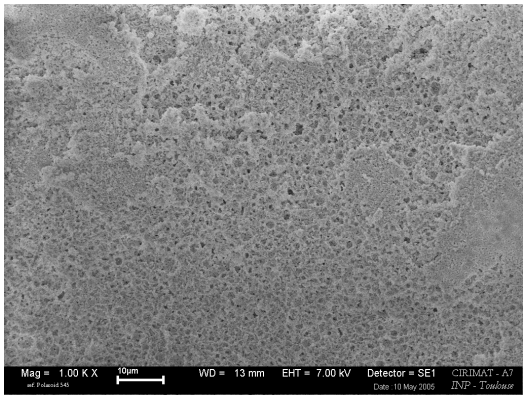


c)

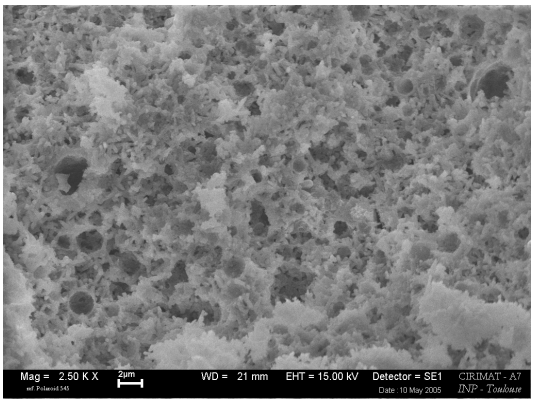


d)

Figure 4

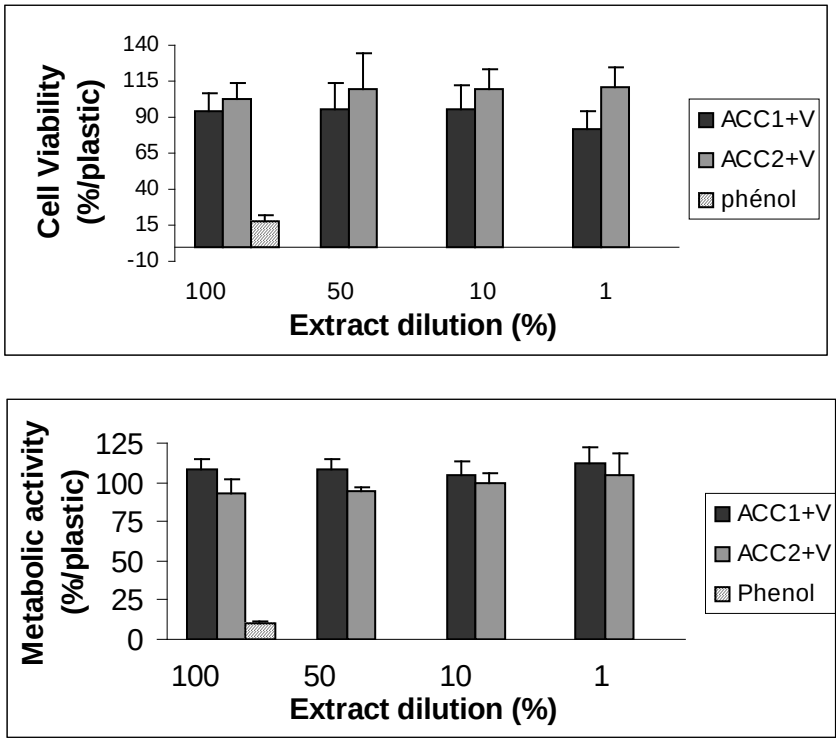

Mag = 1.00 K X
10µm
WD = 13 mm
EHT = 7.00 kV
Detector = SE1
CIRIMAT - A7
INP - Toulouse

a)


Mag = 2.50 K X
2µm
WD = 21 mm
EHT = 15.00 kV
Detector = SE1
CIRIMAT - A7
INP - Toulouse

b)

Figure 5


Cell Viability (%/plastic)
140
115
90
65
40
15
-10
100
50
10
1
Extract dilution (%)
ACC1+V
ACC2+V
phénol

a)

Metabolic activity (%/plastic)
125
100
75
50
25
0
100
50
10
1
Extract dilution (%)
ACC1+V
ACC2+V
Phenol

b)

Figure 6