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Development of composite tissue scaffolds containing naturally sourced mircoporous hydroxyapatite

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Abstract

The aims of this work were to investigate the conversion of a marine alga into hydroxyapatite (HA), and furthermore to design a composite bone tissue engineering scaffold comprising the synthesised HA within a porous bioresorbable polymer. The marine alga, *Phymatolithon calcareum*, which exhibits a calcium carbonate honeycomb structure, with a natural architecture of interconnecting permeable pores (microporosity 4–11 μm), provided the initial raw material for this study. The objective was to convert the alga into hydroxyapatite while maintaining its porous morphology using a sequential pyrolysis and chemical synthesis processes. Semi-quantitative XRD analysis of the post-hydrothermal material (pyrolised at 700–750 °C), indicated that the calcium phosphate (CaP) ceramic most likely consisted of a calcium carbonate macroporous lattice, with hydroxyapatite crystals on the surface of the macropores. Cell visibility (cytotoxicity) investigations of osteogenic cells were conducted on the CaP ceramic (i.e., the material post-hydrothermal analysis) which was found to be non-cytotoxic and displayed good biocompatibility when seeded with MG63 cells. Furthermore, a hot press scaffold fabrication technique was developed to produce a composite scaffold of CaP (derived from the marine alga) in a polycaprolactone (PCL) matrix. A salt leaching technique was further explored to introduce macroporosity to the structure (50–200 μm). Analysis indicated that the scaffold contained both micro/macroporosity and mechanical strength, considered necessary for bone tissue engineering applications.

Keywords: Composite tissue scaffold; Hydroxyapatite; Marine alga

1. Introduction

1.1. Bone tissue engineering

Bone tissue engineering is an increasingly important research area whose goal is to surpass the limitations of conventional treatments based on organ transplantation and biomaterial implantation [1]. There are three general approaches in tissue engineering [2]:

- the use of isolated cells or cell substitutes to replace those cells that supply the needed function;
- the delivery of tissue-inducing substances, such as growth and differentiation factors to targeted locations;
- growing cells in three-dimensional scaffolds.

The third approach has seen significant research developments. Porous matrices, known as scaffolds, to which cells attach and colonize, play a vital role in synthesising bone–extracellular matrix and associated biological molecules to facilitate the formation of functional tissues/organs. Scaffolds for bone regeneration should meet criteria to serve this function, these include [3]:

- a high porosity and appropriate pore size;
- a high surface area is required;

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- biodegradability is generally required and a degradation rate is needed to match the rate of tissue formation;
- mechanical integrity to maintain the pre-designed tissue structure;
- biocompatibility (i.e. non-toxic to cells);
- the scaffold should display a positive interaction with cells, that may include enhanced cell adhesion, proliferation, migration, and differentiation;
- scaffolds are also used as carriers for the delivery of growth and differentiation factors, e.g., the factors can be adsorbed on the surface of the scaffold.

These scaffold requirements are extremely complex and tissue-specific to the structure and function of the host tissue. Creating scaffolds and engineering bone tissues that meet the needs of specific repair sites in particular patients is a major challenge in the field of bone tissue engineering research.

1.2. Scaffold design

In bone regeneration, scaffolds serve as a template for cell interactions and formation of bone–extracellular matrix to provide structural support to the newly formed tissue. Therefore, it is necessary that the scaffolds should, in some respects, mimic host bone morphology in order to optimise integration into surrounding tissue.

Porosity is defined as "the percentage of void space in a solid and it is a morphological property independent of the material" [4]. Pores are necessary for bone tissue formation because they allow migration of endothelial cells: to promote osteoblasts or progenitor cells; to promote vascularisation of mesenchymal cells; or to facilitate osteoblast proliferation and differentiation (i.e. osteogenesis). In addition, a porous surface improves mechanical stability and interlocking at the critical interface between the implant biomaterial and the surrounding natural bone.

The minimum pore size required to regenerate mineralised bone is generally considered to be ~100 μm after the study of Hulbert et al. [5] where calcium aluminate cylindrical pellets with 46% porosity were implanted in canine femora. Large pores (100–150 and 150–200 μm) showed substantial bone in-growth. Smaller pores (75–100 μm) resulted in in-growth of unmineralised osteoid tissue. Smaller pores (10–44 and 44–74 μm) were penetrated only by fibrous tissue. These data were approximately 100–200 μm diameter in normal harvesian systems [5]. Research by Itali et al. investigated the effect of pore size on rabbit femoral defects under non-load bearing conditions and suggested that 100 μm may not be the critical pore size for non-load bearing conditions [6].

Relatively larger pores favour direct osteogenesis, since they allow vascularisation and high oxygenation, while smaller pores result in osteochondral ossification although the type of bone in-growth depends on the biomaterial and the pore geometry. Moreover, porosity and pore size properties influence mechanical properties. An increase in the void volume results in a reduction in mechanical strength and stiffness of the scaffold, which can be critical for regeneration in load-bearing bones. The extent to which pore size can be increased while maintaining mechanical requirements, is dependent on a number of factors including the nature of the biomaterial and the processing conditions used in the fabrication of 3D scaffolds.

Cylindrical synthetic porous HA implants with pore size of 400–600 μm and 80% porosity healed femoral defects in rats [7]. Porous particles of HA (average pore size of 150 μm, porosity 70%) and porous coral HA blocks (average pore size 230 μm, porosity 66%) were used for delivery of BMP-2 in a rat ectopic model and induced direct osteogenesis, without preceding cartilage formation [8]. In another study natural coral scaffolds moulded into the shape of a human mandibular condyle, with pore sizes 150–220 μm and 36% porosity, were seeded with rabbit marrow mesenchymal cells and induced ectopic bone formation in nude mice [9]. Porous biphasic ceramic of HA-tricalcium phosphate (TCP) with 50% porosity and 100–150 μm pore sizes have been shown to heal canine femoral defects [10].

In general, ceramic biomaterials are able to form bone apatite-like material or carbonate HA on their surfaces, enhancing their osseointegration. These materials are also able to bind and concentrate cytokines, as is the case with natural bone. However, brittleness and slow degradation rates are disadvantages associated with their use. Producing HA with highly interconnective pores is a great challenge for bone tissue engineering. Even though the HA material has excellent osteoconductivity (it's surface properties support osteoblastic cell adhesion, proliferation, and differentiation), it is mechanically brittle and often difficult to process into a highly porous structure. To overcome these advantages, composite materials with synthetic or natural polymers have been explored. Such composite scaffolds with a highly porous structure and strong mechanical properties are able to support three-dimensional new bone tissue formation.

Coating HA scaffolds (87% porosity and 150–200 μm pore size) with a hydroxyapatite/polycaprolactone [HA/PCL] composite has been shown to improve the mechanical properties: higher amounts of the composite coating (more polymer) increased compressive strength (maximum 0.45 MPa versus 0.16 MPa for no coating) and elastic modulus (maximum 1.43 MPa versus 0.79 MPa for no coating) [11]. In HA/chitosan–gelatin composites (with most pores between 300 and 500 μm), porosity can be increased by decreasing the chitosan–gelatin/HA ratio. These scaffolds supported the proliferation and mineralisation of rat calvarial osteoblasts *in vitro* [12].

1.3. Bone tissue derived from marine alga (coral)

Hydroxyapatite (HA) [$Ca_{10}(PO_4)_6(OH)_2$] is a mineral that contributes to strength and stiffness of bone. Several attempts have been made to artificially replicate the natural morphology of bone mineral using synthetic HA [13–15]. However, the interconnective architecture has proven extremely difficult to replicate, preventing the penetration of cells and thus prohibiting appropriate osteogenesis. For this reason, there has been significant interest in creating natural HA with acceptable porosity from marine coral derivates [16].

Marine coral has a natural architecture of interconnecting permeable pores, which serves as an osteoconductive structure promoting cell adhesion and proliferation. In addition, it is believed that the use of natural raw materials for the synthesis of HA enables it to be accepted better by the organism, without immunorejection, because of its similar physical–chemical characteristics [16]. Moreover, in 1974 Roy and Linnehan successfully converted the calcite structure of marine coral to HA by a hydrothermal reaction while maintaining its original morphology [17]. Since then, research has focussed not only on the choice of starting material (that possess a natural architecture of highly regular interconnecting permeable pores) but also on improving the conversion techniques that maintain the natural morphology of the starting material. Calcium carbonate used in the synthesis of HA can be obtained from several natural sources, but calcium carbonate originating from marine alga and corals uniquely display characteristic porosity and interconnectivity suited to human bone replacement. An aim of this work was to demonstrate the conversion of a marine coral into HA using a pyrolysis and hydrothermal exchange process and furthermore to design composite scaffolds using the naturally derived HA combined with a bioresorbable polymer (polycaprolactone).

2. Materials and methods

2.1. Phymatolithon calcareum

A red coralline alga, *Phymatolithon calcareum*, obtained from Castleward Strangford, Northern Ireland, was used in this study (Fig. 1). The *Phymatolithon calcareum* samples were dried and cleaned of macroscopic impurities such as sand and crustaceans using a high pressure distilled water jet. Scanning electron microscopy (SEM), X-ray diffraction (XRD), and TGA (thermogravimetric analysis) analysis were used to determine original chemical and physical characteristics. Initial characterisation indicated that *Phymatolithon calcareum* contains approximately 7% (w/w) organic mater (mass loss at 500 °C), 88% (w/w) $CaCO_3$ and 5% (w/w) $MgCO_3$ (ICP-MS). The pore size distribution of the alga was analysed by SEM image analysis and was found to range from 4 to 11 μm with a mean pore size of ≈7 μm (see Tables 1 and 2).

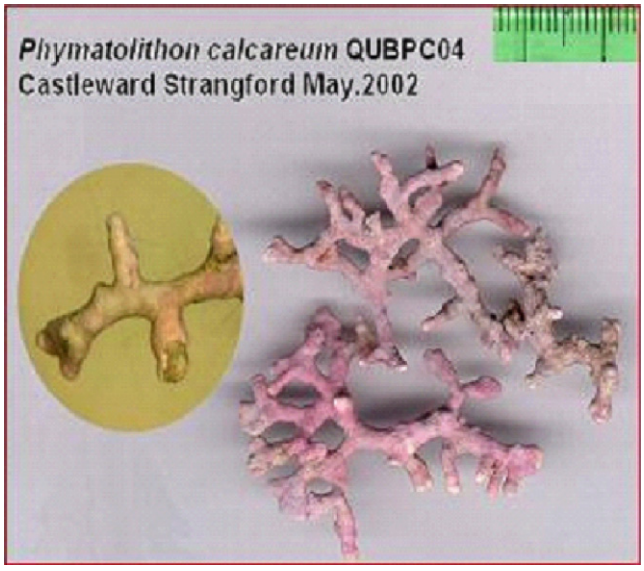

Fig. 1. *Phymatolithon calcareum*.

Table 1
Pore size distribution of *P. calcareum*

Pore size (μm)	Frequency (%)
3	0
4	6
5	19
6	29
7	17
8	17
9	8
10	2
11	2
12	0

2.2. Other materials

- Polycaprolactone (PCL) (Solway, CAPA 6505) is a high molecular weight linear polyester derived from caprolactone monomer. It has a molecular weight of 50,000 and melting point between 58 and 60 °C. PCL was used as an adhesive to bind the HA particles together, in addition to increasing the mechanical strength of the scaffold.
- Acetic acid. Glacial acetic acid, Fisher Scientific UK, was diluted to a concentration of 80% (v/v) and used to blend PCL with HA.
- Sodium chloride salt crystals (BDH labs) were used in scaffold processing to create porous structure. The salt crystals were ground and sieved into particle size ranges of 100–250 and 250–500 μm.

2.3. Conversion into HA

Before undergoing the conversion process, the marine coral was subjected to pyrolysis treatment to remove organic constituents. In this technique the marine coral was subjected to a heat treatment at temperatures from 700 to 850 °C, for 60 min to obtain a calcium carbonate [$CaCO_3$] and calcium oxide [CaO] material. A second processing technique was used to

Table 2
Statistical analysis for pore size and cell wall thickness of *P. calcareum*

Pore size	
Mean (m)	7.21
S.D.	1.53
Medium (m)	6.93
Min. pore (m)	4.08
Maximum pore (m)	11.01
Skew	0.18
Cell wall thickness	
Mean (m)	3.2
S.D.	0.97

convert $CaCO_3$/CaO to hydroxyapatite (HA) in a low-pressure hydrothermal conversion technique at 100 °C and ambient pressure.

(i) A solution of diammonium hydrogen phosphate [$(NH_4)_2HPO_4$] and water was prepared, $(NH_4)_2HPO_4$:water = 1:8 (w/w). Coral dry weight (10 g) was mixed in 80 mL of $(NH_4)_2HPO_4$ solution.
(ii) 2.5% magnesium nitrate, by weight relative to the solid content in the furnace, was added. For the batch described previously, 0.25 g of $Mg(NO_3)_2{\cdot}6H_2O$ was used.
(iii) The pH of the solution was adjusted by adding ammonium hydroxide [NH_4OH] until pH of 9.0–9.5.
(iv) The solution with pyrolised coral was placed in a reaction vessel with a glass stirring propeller and heated to ~100 °C for 6 h.
(v) Solution pH was verified on completion of the reaction. If the pH value was no longer in the range set at the beginning of reaction, the batch was rejected.
(vi) The resultant material was washed with water to remove traces of residual phosphate.
(vii) The material was then dried for 24 h at 90 °C.

During the hydrothermal conversion under favourable conditions, the exchange reaction shown in Eq. (1) is known to take place [17]:

$$10CaCO_3 + 6(NH_4)_2HPO_4 + 2H_2O \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 6(NH_4)_2CO_3 + 4H_2CO_3 \text{ hydroxyapatite} \quad (1)$$

2.4. Scaffold production

A salt leaching technique, in conjunction with the PCL hot platen pressing, was used to create a scaffold with pore sizes in the 100–500 μm. Salt particles of a desired size (100–500 μm) were used in combination with PPC/PCL (PPC [processed *P. calcerarum*]:PCL = 1:2, w/w). In this study, a ratio of 1:1 (w/w) between salt and total combined mass of PPC and PCL was used. After combining the PPC, PCL and salt, scaffolds were created using the hot platen press at 63 °C and pressure of 50 bar for 30 min. The salt was then leached out by soaking the scaffolds in water (30 °C) with constant agitation for a 72 h period [18].

2.5. Characterisation techniques

Scanning electron microscopy was carried out using JEOL 6500 FEG apparatus to examine the internal morphology of the material as well as the interconnectivity and porosity of the scaffold. SEM images (magnification ×500 μm) were imported to Lucia imaging software. An average of $n = 50$ point-to-point measurements were taken for each image to determine pores size and pore size distribution.

Thermogravimetric analysis was carried out using Netzsch STA Jupiter apparatus, using gaseous nitrogen over a temperature range of 20–1000 °C and heating rate of 10 °C/min. The samples were crushed and placed in a platinum crucible for analysis. Changes in weight/mass were recorded as a function of temperature and/or time. Performing TGA on the source material helped to determine the optimum processing conditions with respect to temperature and time before the material decomposed and lost its inter-connective porous structure.

For X-ray diffraction a Philips X'pert PRO diffractometer was used to determine the mineral content within the samples at each stage. The XRD trace was recorded in the range of $5° \leq \varphi \leq 100°$ and quantified using Philips X'Pert High Score software.

2.6. Biocompatibility assay

A 96-well-plate with falcon mesh inserts was used in this assay. Each mesh-well was covered with a layer deep of CaP particles. 200 μl of culture medium with a seeding density of ≤5000 or ≤10,000 cells was added to each well. The well plates were left in an incubator at 37 °C for 2 and 5 days. At each time point samples were taken for SEM analysis.

3. Results and discussion

3.1. Conversion of P. calcareum to porous hydroxyapatite

After an initial cleaning process to remove macroscopic impurities, *P. calcareum* was analysed using TGA to determine the optimum processing conditions to maintain the microporous structure from the coral (Fig. 2). A small mass reduction, about 2%, occurred at approximately 300 °C due to removal of volatile components such as moisture. A further gradual reduction occurred until 700 °C and can be attributed to the loss of organic matter. From 700 to 800 °C, there is a significant decrease in mass (40%) due to the decomposition of $CaCO_3$ to CaO, evidence of this exothermic reaction is further indicated by the decrease in the DSC analysis (Fig. 2). Therefore, it was assumed that charring at a temperature of 800 °C would have a detrimental effect on the inter-connective and porous structure of the coral, i.e., producing a CaO powder material. From this analysis, temperature of 700–750 °C were used in subsequent processing to pyrolyse *P. calcareum*. It was envisaged that this would allow removal of organic material and some breakage of the calcite crystal lattice, but preserve the inter-connective pore structure.

SEM images were obtained to characterise the porous structure and inter-connective channels of *P. calcareum*, and to determine if these were maintained following pyrolysis and subsequent hydrothermal processing. Fig. 3 illustrates the original external structure of *P. calcareum* prior to hydrolysis. It indicates that *P. calcareum* has a pore structure applicable for tissue engineering scaffolds, albeit with a pore size of approximately 10 μm. The interconnective porous structure would aid bioresorption and may provide cell and vascular support *in vivo* for tissue in growth. Maintaining this interconnective pore structure is the main challenge during the pyrolysis and hydrothermal processing stages of scaffold production. Fig. 4 illustrates a SEM of *P. calcareum* alga after pyrolysis and subsequent hydrothermal

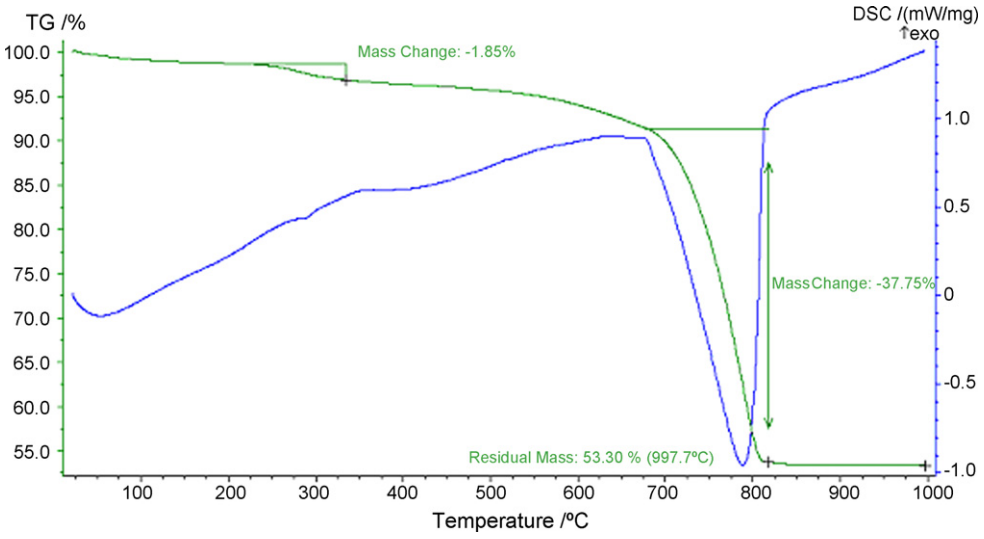

Fig. 2. TGA and DSC analysis of *P. calcareum*.

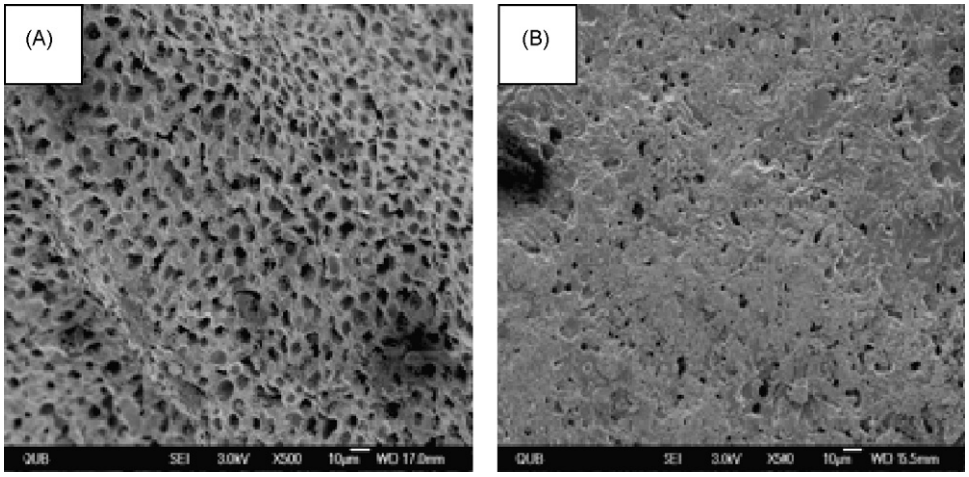

Fig. 3. SEM of *P. calcareum* prior to processing (A) cross-section and (B) longitudinal section.

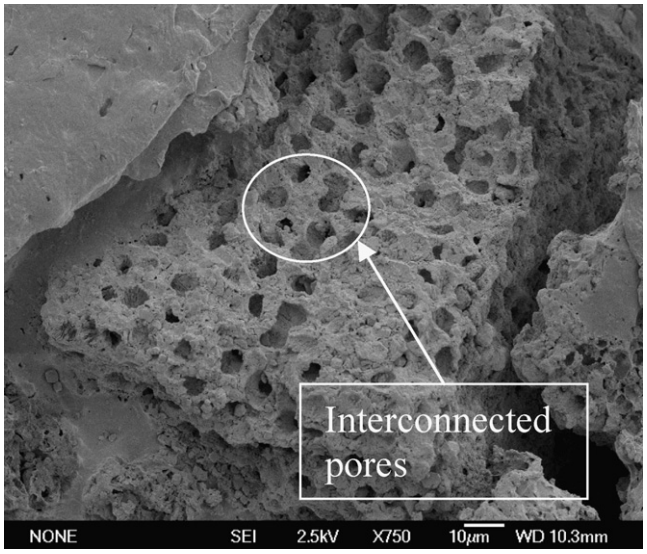

Fig. 4. SEM of *P. calcareum* after pyrolysis at 700 °C for 2 h and hydrothermal conversion to HA.

conversion processing and indicates that the porous structure with interconnective channels is still evident, although some of the "honeycomb" morphology has deteriorated.

The aim of the hydrothermal synthesis process is to convert the porous calcite structure into a porous hydoxyapapite structure, whilst maintaining the morphology of the original alga. Fig. 5a–c illustrates the XRD spectra for pyrolised *P. calcareum* alga after hydrothermal conversion. The single peak around $2\theta = 30^\circ$ corresponds to $CaCO_3$ while three peaks around $2\theta = 31.5^\circ$ correspond to HA. The $CaCO_3$ peak is observed in alga pyrolised at 700 and 750 °C for 60 min. For alga pyrolised at 800 °C for 60 min (Fig. 5c), no calcite peak was observed indicating that all of the calcite converted to CaO with the inevitable breakdown on the original porous alga structure For the 700 °C (Fig. 5a) material it is noted that the HA peaks at $2\theta = 31.5^\circ$ are quite weak for the *P. calcareum* material after synthesis, indicating that the calcite structure has been maintained although some HA formation has occurred For the 750 °C material (Fig. 5b) more HA has been formed via hydrothermal synthesis (58%, w/w, quantitative XRD) due to a higher conversion of calcite to

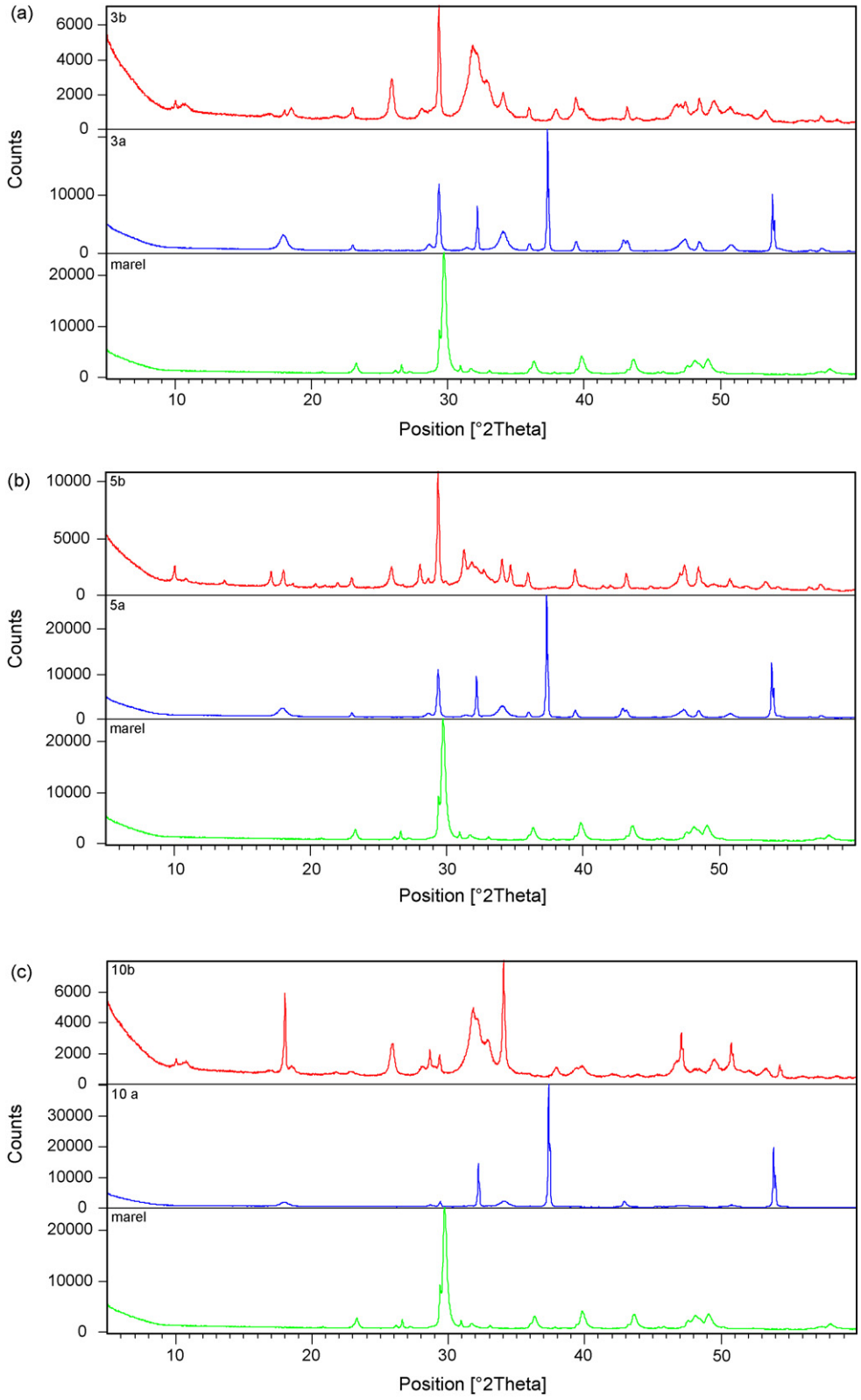

Fig. 5. (a) Comparative XRD spectra of alga. Pyrolysis conditions 700 °C 60 min (raw material = bottom spectra; material after pyrolysis = middle spectra, material after hydrothermal synthesis = top spectra). Calcite peak observed at $2\theta = 29$, after synthesis hydroxyapatite peak observed at $2\theta = 31.5$. (b) Comparative XRD spectra of alga. Pyrolysis conditions 750 °C 60 min (raw material = bottom spectra; material after pyrolysis = middle spectra, material after hydrothermal synthesis = top spectra). Calcite peak observed at $2\theta = 29$, after synthesis hydroxyapatite peak observed at $2\theta = 31.5$. Semi-quantitative XRD: calcite = 28% (w/w) [$CaCO_3$]; hydroxyapatite = 58% (w/w) [$Ca_{10}(PO_4)_6(OH)_2$]; whitlockite = 21% (w/w) [$Ca_{18}Mg_2H_2(PO_4)_{14}$]. (c) Comparative XRD spectra of alga. Pyrolysis conditions 800 °C 60 min (raw material = bottom spectra; material after pyrolysis = middle spectra, material after hydrothermal synthesis = top spectra). Calcite peak at $2\theta = 29$ not visible after pyrolysis, after synthesis hydroxyapatite peak observed at $2\theta = 31.5$.

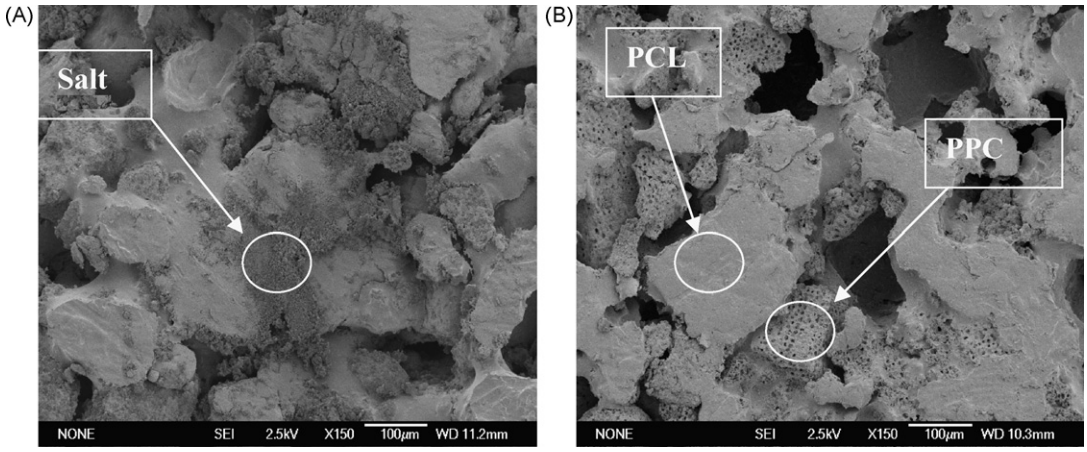

Fig. 6. SEM's for scaffold produced by hot pressing-salt leaching method (small salt crystals, 100–250 μm). The left hand side (A) and right hand side (B) pictures are scaffolds before and after salt leaching, respectively (porosity 0.3–0.35 using density analysis).

CaO in the pyrolysis process. At 750 °C however there appears to be is enough calcite remaining in the material (28%, w/w, quantitative XRD) to maintain the original alga morphology and interconnected pore structure. After the chemical synthesis process (prolysis temperatures 700–750 °C) it is probable that the material now consists of a calcium carbonate microporous lattice, with hydroxyapatite crystals on the surface of the micropores. Furthermore, research has indicated that the use of natural raw materials for the synthesis of HA enables the scaffold to be accepted better by the organism because of its similar physical–chemical characteristics [16].

3.2. Scaffold design

In order to improve the mechanical strength and to create a scaffold macro-structure the "processed *P. calcareum*" (PPC) material was blended in various ratios with polycaprolactone (PCL) and pressed into 2 mm sheets using a hot platen press technique (Section 2.4). Although the PPC contains inter-connective pores of approximately 10 μm, which may facilitate osteochondral ossification *in vivo*, optimum bone tissue engineering scaffolds require significantly larger pores (100–500 μm) to allow vascularisation and high oxygenation which will promote osteogenesis [5].

SEM analysis was performed on the scaffolds after the leaching (Figs. 6 and 7). An advantage of the salt leaching technique is that the macroporous structure of the scaffold can be easily tailored. It can be seen that the larger pores (100–250 and 250–500 μm depending on salt particle size) are formed as a result of removal of salt particles (Figs. 6 and 7). These larger pores are interspersed with particles of the PPC which are connected via PCL bridges. Furthermore, the PPC particles clearly maintain the morphology and pore inter-connectivity of the original *P. calcareum* alga.

3.3. Mechanical analysis of scaffolds

Mechanical testing was performed in both fine and coarse scaffolds. A small punch test technique was employed at room temperature (20 °C) and body temperature (37 °C). The mechanical behaviour determined by the small punch test was dependent on the test temperature and the size of salt particles used to create the porous structure in the scaffolds. The data illustrated in Fig. 8 and in Table 3 were determined from an average of at

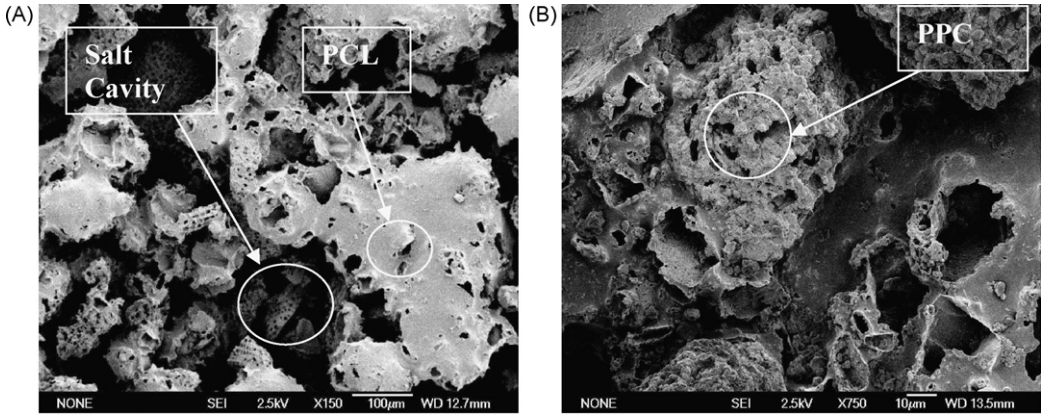

Fig. 7. (A and B) SEM's for scaffold produced by hot pressing-salt leaching method after salt leaching (large salt crystals 250–500 μm) (porosity 0.35–0.40 using density analysis).

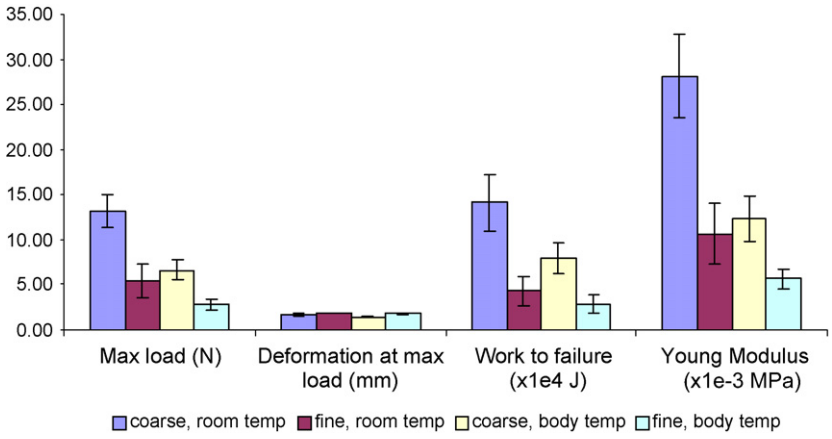

Fig. 8. Histogram of mechanical testing data.

Table 3
Composite scaffold mechanical analysis data (using small punch test)

	Maximum load (N)	Deformation at maximum load (mm)	Work-to-failure (J)	Modulus (MPa)
Coarse, room temperature	13.22 ± 1.90	1.73 ± 0.14	0.0142 ± 0.0031	2.81E+04 ± 4.72E+03
Fine, room temperature	5.39 ± 1.81	1.86 ± 0.08	0.0043 ± 0.0016	1.06E+04 ± 3.34E+03
Coarse, body temperature	6.67 ± 1.16	1.39 ± 0.11	0.0079 ± 0.0017	1.24E+04 ± 2.53E+03
Fine, body temperature	2.83 ± 0.61	1.83 ± 0.06	0.0029 ± 0.0010	5.68E+03 ± 1.06E+03

least three measurements of each sample. The data indicate that coarse scaffolds (250–500 μm salt particles) have higher maximum load compared to the finer scaffolds (100–250 μm salt particles). The data at room temperature are higher compared to body temperature. The variability in the tests was found to be 22% for maximum load, 6% for displacement at maximum load, 29% for work-to-failure and 22% for modulus.

The data on: deformation at maximum load; work-to-failure; and modulus, follow a similar trend, i.e., coarse scaffolds have higher mechanical properties compared to the finer scaffolds. As expected the analysis at room temperature gives higher mechanical properties than at body temperature as the main binding constituent, PCL, is nearer the melt temperature. However, the composite scaffolds have a mechanical strength which is adequate for bone tissue engineering applications.

3.4. Biocompatibility studies

After 14 days the CaP ceramic (i.e., the material post-hydrothermal analysis seeded) was found to be non-cytotoxic and displayed good biocompatibility when seeded with MG63 cells. The SEM micrograph images in Fig. 9 show CaP ceramic after 5 days in culture with 5000 MG63 cells. Although the cell coverage was relatively sparse, micro processes were observed indicating good cell attachment interactions. The same observations were made using seeding densities of 10,000

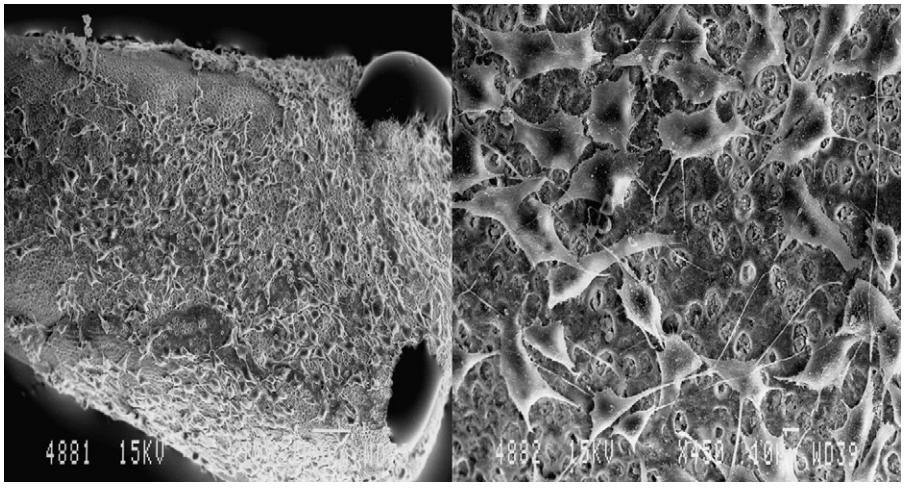

Fig. 9. CaP ceramic seeded with 5000 cells for 5 days with MG63 cells.

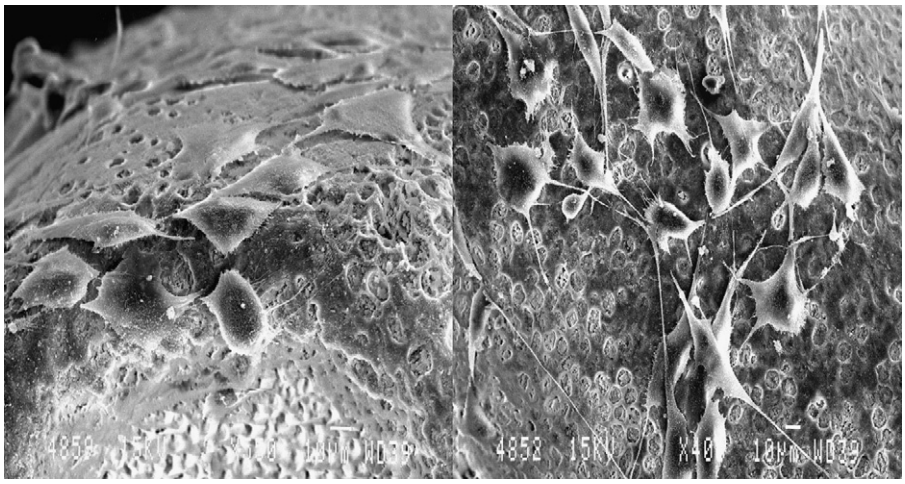

Fig. 10. CaP ceramic seeded with 10,000 cells for 5 days with MG63 cells.

MG63 cells/well, in Fig. 10. Furthermore, the CaP ceramics has remained intact after 5 days in culture. Despite the low seeding densities, the CaP ceramics have displayed good biocompatibility. Further work is required to quantify the cellular activity with higher seeding densities and longer culture periods.

4. Conclusions

Phymatolithon calcareum alga was partially converted to hydroxyapatite using a two-stage process. The *P. calcareum* was first pyrolised into $CaCO_3$/CaO. TGA was employed to determine the optimum pyrolysis processing conditions with respect to temperature and time. A second chemical synthesis step was performed using an aqueous phosphate solution with addition of magnesium ions at 100 °C and ambient pressure. The calcite–hydroxyapatite material produced still maintained the interconnecting porous morphology of the starting material under pyrolysis temperatures of 700–750 °C (60 min). Semi-quantitative XRD analysis of the material under these processing conditions indicated that the alga most likely consists of a calcium carbonate macroporous lattice, with hydroxyapatite crystals on the surface of the macropores. Cell visibility (cytotoxicity) investigations of osteogenic cells were conducted on the CaP ceramic which was found to be non-cytotoxic and displayed good biocompatibility when seeded with MG63 cells.

In scaffold design, the processed *P. calcareum* is combined with PCL to produce 1–5 mm scaffold sheets, using a hot platen press device. Scaffolds produced by the salt leaching technique demonstrate good porosity. These scaffolds have a range of larger pores as a result of leaching off salt particles (100–500 μm) and small pores (10–50 μm) from the original morphology of the alga.

Overall, this study successfully demonstrates that processed *P. calcareum* and PCL can be designed into a three-dimensional scaffold that may guide cell growth and enhance bone formation *in vivo*. Moreover, scaffolds produced using the natural morphology of alga offer promising alternatives for bone tissue engineering applications.

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References

[1] R. Langer, J.P. Vacanti, Tissue Eng., Science 260 (1993) 920–926.
[2] P.X. Ma, Scaffolds for tissue fabrication, Mater. Today 7 (5) (2004) 30–40.
[3] D.W. Hutmacher, T. Schantz, I. Zien, K.W. Ng, S.H. Teoh, K.C. Tan, Mechanical properties and cell cultural response of polycaprolactone scaffolds designed and fabricated via fused deposition modelling, J. Biomed. Mater. Res. 55 (2001) 203–216.
[4] V. Karageorgiou, D. Kaplan, Porosity of 3D biomaterial scaffolds and osteogenesis, Biomaterials 26 (27) (2005) 5474–5491.
[5] S.F. Hulbert, F.A. Young, R.S. Mathews, J.J. Klawitter, C.D. Talbert, F.H. Stelling, Potential ceramic materials as permanently implantable skeletal prostheses, J. Biomed. Mater. Res. 4 (3) (1970) 433–456.
[6] A.I. Itali, H.O. Ylanen, C. Ekholm, K.H. Karlsson, H.T. Aro, Pore diameter of more than 100 microns is not requisite for bone in growth in rabbits, J. Biomed. Mater. Res. 58 (6) (2001) 679–683.
[7] E. Damien, K. Hing, S. Saeed, P.A. Revell, Preliminary study on the enhancement of the osteointegration of a novel synthetic hydroxyapatite scaffold in vivo, J. Biomed. Mater. Res. A 66 (2) (2003) 241–246.
[8] Y. Kuboki, H. Takita, D. Kobayashi, E. Tsuruga, M. Inoue, M. Murata, et al., BMP-induced osteogenesis on the surface of hydroxyapatite with geometrically feasible and nonfeasible structures: topology of osteogenesis, J. Biomed. Mater. Res. 39 (2) (1988) 190–199.
[9] F. Chen, T. Mao, K. Kao, S. Chen, G. Ding, X. Gu, Bone graft in the shape of human mandibular condyle reconstruction via seeding marrow-derived osteoblasts into porous coral in a nude mice model, J. Oral. Maxillofac. Surg. 60 (10) (2002) 1155–1159.
[10] C. Zhang, J. Wang, H. Feng, B. Lu, Z. Song, X. Zhang, Replacement of segmental bone defects using porous bioceramic cylinders: a biomechanical and x-ray diffraction study, J. Biomed. Mater. Res. 54 (3) (2001) 407–411.
[11] H.W. Kim, J.C. Knowles, H.E. Kim, Hydroxyapatite/poly(epsilon-caprolactone) composite coatings on hydroxyapatite porous bone scaffold for drug delivery, Biomaterials 25 (7–8) (2004) 1279–1287.
[12] F. Zhao, Y. Yin, W.W. Lu, J.C. Leong, W. Zhang, J. Zhang, et al., Preparation and histological evaluation of biomimetic three-dimensional hydroxyapatite/chitosan–gelatin network composite scaffolds, Biomaterials 23 (15) (2002) 3227–3234.
[13] C. Bong-Soon, L. Choon-Ki, H. Kug-Sun, Y. Hyuk-Joon, R. Hyun-Seung, C. Sung-Soo, P. Kun-Woo, Osteoconduction at porous hydroxyapatite with various pore configuration, Biomaterials 21 (2000) 1291–1298.

[14] J. Dong, T. Uemura, H. Kojima, M. Kikuchi, J. Tanaka, T. Tateishi, Application of low-pressure system to sustain in vivo bone formation in osteoblast/porous hydroxyapatite composite, Mater. Sci. Eng. C 17 (1–2) (2001) 37–43.
[15] K.J. Lilley, U. Gbureck, J.C. Knowles, D.F. Farrar, J.E. Barralet, Cement from magnesium substituted hydroxyapatite, J. Mater. Sci. - Mater. Med. 16 (5) (2005) 455–460.
[16] K. Christian, E. Rolf, S. Bruno, 1990-07-03, Process for the preparation of hydroxylapatite material, C01B25/32, Bremen, US.
[17] D.M. Roy, S.K. Linnehan, Hydroxyapatite formed from coral skeletal carbonate by hydrothermal exchange, Nature 247 (438) (1974) 220–222.
[18] F. Kusmanto, G. Walker, P. Walsh, F. Buchanan, 12th BinI Conference, Royal Academy of Medicine in Ireland, 2006 (Paper 108).