Chapter 17
Calcified Algae for Tissue Engineering

Gina Choi and Louise A. Evans

Abstract Extensive research has been conducted on hydroxyapatite as a bone tissue engineering scaffold due to its low toxicity, biocompatibility, bioactivity and chemical similarity to bone. Hard coral species as well as red and green calcified marine algae have naturally porous skeletons that resemble cancellous bone. Under controlled hydrothermal conditions, these materials can be converted to hydroxyapatite with their porosity and interconnectivity preserved. The availability of hard coral species is limited due to the damage caused by harvesting procedures and decline in coral reefs. As an alternative, hydroxyapatite can be produced from red and green algae species. Currently, red algae derived Algipore® grafts are commercially available for maxillary sinus bone augmentation. Long term clinical studies have confirmed the bone regenerating capabilities of Algipore® when mixed with autologous bone debris and blood, but research on the use of Algipore® tissue scaffolds seeded with mesenchymal stem cells is still ongoing. This chapter reviews the synthesis of hydroxyapatite derived from marine algae and gives background to clinical studies as well as the characterisation techniques used to analyse these materials.

Keywords Hydroxyapatite · Aragonite · Calcified algae · Scanning electron microscopy · X-ray diffraction analysis · Fourier-transform infrared spectroscopy · Bone regeneration · Hydrothermal conversion · Mesenchymal stem cells · Algae-derived hydroxyapatite · Bone tissue engineering

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Table 17.1 Biominerals produced by living organisms and their functions (Adapted from [3])

Mineral	Formula	Organism or location	Function
Calcite	$CaCO_3$	Trilobite eyes	Optical imaging
Aragonite	$CaCO_3$	Coral	Exoskeleton
Amorphous calcium carbonate	$CaCO_3 \cdot nH_2O$	Plant leaves	Calcium storage
Hydroxyapatite	$Ca\text{-}_{10}(PO_4)_6(OH)_2$	Vertebrate bone	Endoskeleton
Octacalcium Phosphate	$Ca_8(HPO_4)_2(PO_4)_4 \cdot 5H_2O$	Vertebrate bone	Precursor phase
Whewellite	$CaC_2O_4 \cdot H_2O$	Plants	Calcium storage
Barite	$BaSO_4$	Chara	Gravity receptor
Celestite	$SrSO_4$	Acantharia	Exoskeleton
Silica	$SiO_2 \cdot nH_2O$	Plant leaves	Protection
Magnetite	Fe_3O_4	Chiton teeth	Grinding
Ferrihydrite	$5Fe_2O_3 \cdot 9H_2O$	Animal ferritin	Iron storage

17.1 Biomineralization

Biomineralization is the controlled process by which living organisms form minerals [1, 2]. Biominerals are produced by species from all five living kingdoms, with over 60 different biological minerals discovered [1]. Among these, 50% of known biogenic minerals contain calcium while 25% contain phosphate.

Biominerals provide a variety of functions for living organisms, including structural support, protection, motion, grinding, magnetic navigation and storage [3] (Table 17.1). Depending on the application, each species produces unique mineral structures with specific size, geometry, crystallinity and organisation [2, 4]. Biominerals may also be classified as crystalline or amorphous materials. Crystalline biominerals have ordered structures and morphologies which provide mechanical strength. On the other hand, amorphous biominerals have the ability to fill spaces, and also provide mechanical properties owing to the lack of fracture planes. In comparison to crystalline minerals, amorphous materials have high solubility and low density [5].

The process of biomineralization may be divided into two categories. The first category is biologically induced mineralization, where the precipitation of minerals is induced in an open environment on the surface of cells with minimal biological control [1, 2, 6]. As a result, biologically induced minerals are polycrystalline with irregular orientations. Furthermore, the precipitated mineral depends on the chemical conditions of the external environment, leading to the heterogeneity of the mineral between organisms of the same species [1, 2]. Examples of organisms that possess this ability include monerans, fungi and green algae [1, 6].

The second category is biologically controlled mineralization. This process is controlled genetically, producing crystalline minerals with ordered geometry and

shape [6]. In contrast to biologically induced mineralization, mineral precipitation occurs in a closed space and is mediated by an organic matrix [1]. The organic matrix is composed of macromolecules such as proteins, polysaccharides and acidic glycoproteins [1, 2], which provide a three-dimensional (3D) framework for the nucleation and growth of biominerals. The resulting biomineral is a composite material comprised of organic and mineral components with unique mechanical properties [2, 4]. Biologically controlled mineralization is used to produce mineralized tissue in animals such as bone, teeth and shells [1].

17.2 Bone

Bone is a hard mineralized tissue found in animals. It serves a variety of functions in the body such as structural support, movement, protection of organs, accommodation of bone marrow and as a mineral reserve for homeostasis [7–9]. Bone is a composite material with a predominately flexible Type 1 collagen organic matrix and a substituted hydroxyapatite mineral phase [9]. As a result of the composite nature, bone has excellent mechanical properties where the collagen provides elasticity, and the mineral provides strength [8].

17.2.1 Mineral Phase

The first X-ray diffraction studies of bone conducted by de Jong revealed that the mineral was an apatite-like material [10]. The mineral phase is now widely described as hydroxyapatite (HAp; $Ca_{10}(PO_4)_6(OH)_2$) [8, 9]. Stoichiometric HAp has a hexagonal unit cell structure, with a calcium phosphate ratio of 1.67 [11, 12] (Tables 17.2 and 17.3).

However, further studies have shown that biogenic HAp minerals are non-stoichiometric and are often substituted with other ions [15, 16]. LeGeros et al. described the mineral as a carbonate-substituted apatite, where carbonate (CO_3^{2-}) ions displace hydroxide (OH^-) or phosphate (PO_4^{3-}) ions [15]. Carbonate substitution is not only common in bone, but is characteristic of all biogenic apatites [12, 17]. Further substitution within the crystal lattice can occur with magnesium (Mg^{2+}) and acid phosphate groups (HPO_4^{2-}) [8]. Therefore, the biogenic mineral in bone can be described as a non-stoichiometric carbonate-substituted apatite.

In bone, carbonated HAp crystals have a uniform, thin, plate-like morphology [12]. These crystals are small with an average length of 50 nm and width of 25 nm [1, 8, 12]. As a consequence, they are poorly crystalline with higher solubilities compared to other biogenic apatite minerals such as tooth enamel [8, 16].

Table 17.2 Calcium phosphate compounds with their chemical formula and Ca/P ratio (adapted from [13, 14])

Name	Formula	Ca/P	pH stability
Monocalcium phosphate monohydrate	$Ca(H_2PO_4)_2{\cdot}H_2O$	0.5	0.0–2.0
Dicalcium phosphate dihydrate	$CaHPO_4{\cdot}2H_2O$	1.0	2.0–6.0
Amorphous calcium phosphate	$Ca_xH_y(PO_4)_z\ nH_2O$ ($n = 3$–4.5)	1.2–2.2	5.0–12
Octacalcium phosphate	$Ca_8(HPO_4)_2(PO_4)_4{\cdot}5H_2O$	1.33	5.5–7.0
α-Tricalcium phosphate (α-TCP)	α-$Ca_3(PO_4)_2$	1.5	–
β-Tricalcium phosphate (β-TCP)	β-$Ca_3(PO_4)_2$	1.5	–
Calcium-deficient hydroxyapatite	$Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$ ($0 < x < 1$)	1.5–1.67	6.5–9.5
Hydroxyapatite (HAp)	$Ca_{10}(PO_4)_6(OH)_2$	1.67	9.5–12
Tetracalcium phosphate	$Ca_4(PO_4)_2O$	2.0	–

Table 17.3 Selected crystal classes of calcified systems

Crystal class	Unit cell dimensions	Example
Hexagonal	$a = b \neq c$; $\alpha = \beta = 90°$ $\gamma = 120°$	Hydroxyapatite
Orthorhombic	$a \neq b \neq c$; $\alpha = \beta = \gamma = 90°$	Aragonite
Trigonal	$a = b = c$; $\alpha = \beta = \gamma \neq 90°$	Calcite

17.2.2 Organic Phase

The organic matrix is primarily composed of Type 1 collagen (85–90%), in addition to collagenous proteins such as proteoglycans, and non-collagenous proteins including osteocalcin and osteonectin [7, 8, 12, 18].

To form collagen fibrils, three polypeptide chains arrange into a triple helix that is 80–100 nm in diameter [12, 18]. They provide a 3D matrix on which HAp crystals nucleate and grow. The crystals are embedded across and between collagen fibrils in organised layers, forming a composite material [12]. Following crystal formation, the mineralized collagen fibrils align together in bundles to produce collagen fibres that subsequently bind to one other through an organic phase and form a fibril array [12, 18].

17.2.3 Bone Architecture

The two types of bone tissue are cortical and cancellous bone. Cortical bone is the dense, compact outer wall of bone while cancellous bone is the spongy structure found at the ends and centre of long bone as well as the middle of vertebrae [7, 9].

Cortical bone provides mechanical strength, and contains pores that range from 100 nm to 50 μm [9, 19]. It is composed of organised arrays of cylindrical mineralized structures called osteons [8, 12]. Each osteon is constructed by concentric cylindrical layers, called concentric lamellae. The concentric lamellae enclose a narrow channel in the centre called the central canal that accommodates blood vessels and nerves [9]. Transverse canals are located between adjacent osteons to connect the central canals. Other microscopic features that form pores include capillary channels called canaliculi, and small spaces between the concentric layers called lacunae which house osteocyte bone cells [12].

In contrast, cancellous bone is highly porous with poor mechanical strength [9]. Cancellous bone is formed by a network of interconnecting beams called trabeculae [7, 9]. The pores within trabeculae range from 200 to 600 μm, and are used to accommodate cellular structures and bone marrow [9, 19].

17.2.4 Bone Remodelling

Bone is a regenerative tissue that undergoes continuous remodelling throughout a person's lifespan [8]. Bone remodelling is the process in which old bone tissue is removed and replaced by new, regenerated bone. The three bone cells involved are osteoclasts, osteoblasts and osteocytes.

Remodelling begins with the resorption of old bone tissue by osteoclast cells [7–9]. Osteoclast cells secrete hydrogen ions and enzymes to digest the mineral and organic matrix, resulting in the formation of cavities. Following this, bone forming cells called osteoblasts produce a new organic matrix by synthesising collagen molecules and non-collagenous proteins. Osteoblast cells mediate HAp mineralization, leading to the formation of new bone tissue. Osteoblasts cease bone formation once entrapped within the mineralized tissue, and are then termed osteocyte cells [9, 20].

Bone remodelling is an important process, as it preserves the mechanical strength of bone. Ageing and physical activity result in the formation of microfractures in bone, leading to a decline in mechanical strength [8]. By replacing older bone tissue with newly regenerated bone, the quality of bone, as well as calcium homeostasis is maintained.

17.2.5 Bone Grafts

A unique characteristic of bone is its ability to repair and regenerate when damaged [21]. Despite this, permanent damage of bone may occur due to large trauma fractures, infections or tumour resections [21, 22]. In addition, the density and regenerative ability of bone decrease with age. The condition where cancellous bone thins out and increases bone fragility is called osteoporosis [7]. In 2000, an estimate of 9.0 million fractures occurred worldwide as a consequence of osteoporosis [23]. Osteoporotic

fractures can cause morbidity and disability, and are expected to increase drastically as the population ages [7, 23].

Surgical procedures are required to aid regeneration when bone fails to repair. To treat defects, bone grafts are typically implanted. Bone grafts are the second most common transplant procedure, with over 2.2 million surgeries performed annually worldwide [21]. Ideal graft materials are biocompatible, osteogenic, osteoinductive and osteoconductive. These characteristics are defined by Keating and McQueen [24] and Williams [25] as:

- ***Biocompatibility***: the ability of a biomaterial to perform the desired function in medical therapy by generating a cellular or tissue response without causing undesirable effects.
- ***Osteogenic***: the capacity of a material to regenerate new bone through osteocytes and osteoblasts cells.
- ***Osteoinductive***: stimulation of bone regeneration by inducing the differentiation of osteoblast cells from mesenchymal stem cells (MSCs).
- ***Osteoconductive***: the capacity of a material to provide an inert scaffold for bone ingrowth and subsequently, regeneration.

Bone grafts are traditionally harvested from the pelvic crest of the patient's or donor's tissue [26]. These are known as autologous and allogenic grafts, respectively. Autologous grafts are referred to as the "golden standard" due to their biocompatible, osteogenic, osteoinductive and osteoconductive properties [24, 27]. Although clinically successful, they present issues such as persistent pain, morbidity, limit of supply and the cost of surgical procedures and recovery [26, 27]. In comparison, allogenic grafts are biocompatible, osteoinductive and osteoconductive but lack osteogenic properties. The disadvantages of allogenic bone grafts are similar to those of their autologous counterparts, with the additional risk of disease transmission [26, 27]. For these reasons, it is important to develop substitute materials to be used in place of these grafts.

17.2.6 Bone Substitute Materials

Biomaterials are defined by Williams as substances that have been engineered to direct or interact with components of a living system for any therapeutic or diagnostic procedure [28]. The primary purpose of a biomaterial is to restore the functions of a living organ or tissue to improve the quality of human health [29]. Example applications include artificial joints, dental implants, artificial heart valves and contact lenses [30]. For biomaterials to be successful in restoring physiological functions, they must be biocompatible, pharmacologically acceptable, and have appropriate mechanical properties and design that are suitable for its application [29].

A wide variety of materials can be used to construct implants. Currently, the four classes of synthetic materials used for biomedical applications are metals, polymers, ceramics and composites [29, 30] (Table 17.4).

Table 17.4 Synthetic biomaterials with their respective advantages and disadvantages (adapted from [29, 30])

Class	Materials	Advantages	Disadvantages
Metals	Titanium alloys and stainless steel	Strong and ductile	Corrosive and bioinert
Polymers	Silicon, polylactic acid and nylon	Resilient	Poor mechanical strength
Ceramics	Alumina, zirconia and hydroxyapatite	Biocompatible	Brittle and poor elasticity
Composites	Carbon-carbon and ceramic coated metals	Strong and customisable	Difficult to make

Upon implantation, there are four ways biomaterials can react with surrounding tissue [31]:

- ***Biotoxic***: pathological change or rejection by surrounding tissue;
- ***Bioinert***: coexistence between material and tissue with minimal change;
- ***Bioactive***: biochemical adhesion between material and tissue; and
- ***Bioresorbable***: gradual dissolution and displacement of material by new tissue.

Among the four classes of biomaterials, ceramics have the potential to be used as orthopaedic implants and grafts due to their bioactive and bioresorbable properties [29]. Calcium sulphate and calcium phosphate materials are two examples of bioceramics that have been successfully used as bone grafts [27]. However, a major disadvantage of ceramics is that they are brittle with poor mechanical strength [26, 29, 31]. To compensate, ceramics can be manufactured as composites or coatings [31], but further developments are required to improve the overall design of ceramic implants.

17.2.7 Bone Tissue Engineering

Tissue engineering is an emerging field of research that aims to develop biological substitute materials to restore, maintain or improve tissue function using living cells [32, 33]. Unlike traditional biomaterials, a tissue engineering implant has a living function that helps to restore the biological function of the replaced tissue or organ [34].

The two fundamental components of a tissue engineering implant are the living cells and the extracellular matrix [33, 35]. The cells used may be either autologous or

allogenic, and are specific to the organ or tissue being replaced [35]. In particular, stem cells have been used extensively in this field as they can be differentiated into a desired type of cell such as bone or cartilage. Growth factors can also be supplied to support the growth and differentiation of stem cells [33]. The extracellular matrix provides a scaffold that accommodates the cells as well as the 3D structure of the tissue in which the cells are grown and proliferated [33, 35]. The scaffold may be produced using natural materials such as starch [36] or chitosan [37], or synthetic materials such as calcium phosphate bioceramics [38] or polymers such as polyglycolic acid [39] depending on the application.

In the application of bone tissue engineering, the scaffold should ideally mimic the natural structure of bone [34]. Additionally, it must be biocompatible, osteoconductive, osteoinductive and bioresorbable to allow the formation of new bone tissue at the implanted site [34, 40, 41]. Another property of the scaffold that is crucial for bone tissue engineering is its porosity. Scaffolds must contain a large volume of interconnected pores to allow the ingrowth of bone cells, tissue and blood vessels. Rough surface structures can also promote osteoconduction by supporting the attachment and proliferation of bone cells. Scaffolds are required to have sufficient mechanical properties that are similar to the surrounding bone tissue at the implanted site.

Following the development of a scaffold, the appropriate cell must be selected and cultured. The two that are commonly chosen for bone tissue engineering are osteoblast cells or stem cells [34, 40]. Osteoblast cells are involved in the process of bone regeneration, and may be isolated and grown from the patient's own bone tissue to reduce the possibility of a negative immune response [42]. However, according to Salgado et al. [34], there are a limited number of osteoblast cells available in the patient's tissue and the process involved to isolate and proliferate these cells is slow. Alternatively, they suggest the use of stem cells as they are readily available in the patient's tissue and have high ability to differentiate. Among the different types of stem cells, MSCs are gaining popularity in bone tissue engineering as they are available within bone marrow and demonstrate osteogenic characteristics [34, 43, 44]. In 1998, Bruder et al. first isolated and cultured human MSCs and loaded them into a ceramic scaffold to demonstrate their potential to heal bone defects [45]. Bone regeneration was observed within four weeks of implantation, and new bone tissue was found within the porous ceramic at the end of the 12 week study. Through this, Bruder et al. were able to demonstrate that human MSCs were capable of differentiating into osteoblast cells which form new bone tissue within the ceramic implant. Although research into the use of MSCs for tissue engineering is ongoing they show promising potential in the application of bone regeneration.

Another addition that can be made to a tissue engineering implant are growth factors [46]. In bone tissue engineering, growth factors play an important role in the adhesion and proliferation of cells within the scaffold. They are secreted by cells in the form of cytokines and are involved in cell signalling and communication [46]. Some examples of growth factors that have been used in bone tissue engineering include the osteoinductive bone morphogenetic proteins [47], osteogenic fibroblast growth factors [48] and platelet derived growth factors [49]. Growth factors may be

added to help enhance bone regeneration and improve the osteogenic properties of tissue engineering implants.

17.3 Calcium Phosphate Bioceramics

HAp-based bioceramics are of particular interest due to their chemical similarity to the mineral in bone [8, 17, 31, 50]. HAp materials are non-toxic, biocompatible and bioactive, allowing them to attach and adhere to surrounding bone upon implantation [13, 27, 50]. Furthermore, they are osteoconductive, providing a scaffold in which bone cells can integrate to enable the formation of new bone tissue [51, 52]. These characteristics allow synthetic HAp to be applied as a substitute bone graft material.

HAp is part of a collective group of related phases known as calcium phosphate compounds (Table 17.2). These compounds consist of calcium, phosphorous and oxygen atoms arranged in amorphous or crystalline structures [13]. Each compound has a characteristic calcium to phosphorous molar ratio (Ca/P), pH solubility and crystallinity (Table 17.2) [13, 53].

Calcium phosphate compounds are bioresorbable to different degrees and undergo dissolution after implantation, resulting in the formation of HAp in new bone tissue [31]. The rate of dissolution of the compounds decreases as the Ca/P ratio increases [31, 50, 52]. Compounds with Ca/P molar ratios greater than 1 are ideal for orthopaedic applications, as fast dissolution rates may lead to destructive effects [54, 55].

Although calcium phosphate bioceramics lack osteoinductive properties, the structure of the ceramics may be manipulated to induce the regeneration of bone. Animal studies conducted by Yuan et al. demonstrated the osteoinductive properties of microporous HAp implants compared to HAp implants without pores [55]. According to LeGeros [19], micro- and macropores in calcium phosphate implant materials entrap bioactive proteins that promote osteoinduction. Additionally, porous implant materials mimic the microstructure of bone, stimulating attachment, tissue ingrowth and vascular integration [13, 19, 52]. Overall, these studies provide evidence that the design of HAp bioceramics play a major role in the biological response.

17.3.1 Synthesis Methods

A variety of methods for the synthesis of HAp and other calcium phosphate materials have been developed (Table 17.5). These methods produce HAp with different crystallinity, geometry, and size [54]. For wet chemical procedures such as chemical precipitation, hydrothermal, sol-gel and hydrolysis methods, control over the pH, temperature and concentration of reagents are necessary to synthesise HAp of desired physical and mechanical properties [56, 57]. Wet chemical procedures have been well characterised and optimised by a number of researchers [56–60].

A challenge associated with the synthesis of HAp for bone replacement applications is the incorporation of pores that mimic the microarchitecture of bone. Porous HAp structures have large surface areas that enhance the bioactivity and osteoinductivity of the material, and allow the incorporation of bone cells and blood vessels [19, 61]. Various values have been suggested for the optimum pore size, which range between 10 and 500 μm [62, 63]. However, the addition of pores decreases the mechanical strength of ceramics, creating further challenges [61].

A common procedure employed to produce porous ceramics is the incorporation of volatile additives, known as porogens. Porogens such as polyvinyl butyral leave pores within the materials when sintered of uniform size [73]. However, a major limitation of their use is the random distribution of pores and their lack of interconnectivity. An alternative technique called gel cast foaming produces ceramics with high mechanical strength, but the method can be complicated and result in a large pore size distribution and lack of pore interconnectivity [61, 74]. The polymer sponge method or combination procedures are additional techniques that produce porous HAp ceramics with high porosity and interconnectivity [61, 75]. In these processes, a polymer template is utilised, allowing the controlled production of porous structures [75]. However, the method is relatively complicated and involves long procedures.

Table 17.5 Synthesis methods used to produce HAp

Method	Reagents	Reference(s)
Chemical Precipitation	$Ca(OH)_2$ and H_3PO_4 or $(NH_4)_2HPO_4$	Santos et al. [59]
	$Ca(NO_3)_2{\cdot}4H_2O$ and $(NH_4)_2HPO_4$	Mobasherpour et al. [64]
	$CaCl_2{\cdot}2H_2O$ and Na_2HPO_4	Mekmene et al. [57]
Hydrothermal	$Ca(OH)_2$ and $CaHPO_4{\cdot}2H_2O$	Liu et al. [56]
	$Ca(NO_3)_2{\cdot}4H_2O$ and $(NH_4)_2HPO_4$	Earl et al. [60]
Sol–Gel	$Ca(C_2H_3O_2)_2$ and $PO(OC_2H_5)_3$	Jillavenkatesa and Condrate [58]
Hydrolysis	DCPD ($CaHPO_4{\cdot}2H_2O$) and $CaCO_3$	Shih et al. [65]
Solid State	$Ca(OH)_2$ and $Ca_3(PO_4)_2$	Rao et al. [66]
Mechano-chemical	CaO and $CaHPO_4$	Yeon et al. [67]
Combustion	$Ca(NO_3)_2{\cdot}4H_2O$ and $(NH_4)_2HPO_4$	Ghosh et al. [68]
Synthesis from biogenic sources	Coral ($CaCO_3$) and $(NH_4)_2HPO_4$	Roy and Linnehan [69], Hu et al. [70]
	Seashells ($CaCO_3$) and $(NH_4)_2HPO_4$	Vecchio et al. [71]
	Egg shells ($CaCO_3$) and $(NH_4)_2HPO_4$	Raihana et al. [72]

In recent studies, HAp scaffolds have been produced using 3D printing technology [76–78]. The scaffold is designed using computer software, printed by depositing HAp granules layer by layer then sintered to produce the final structure. 3D printing is advantageous as implants may be customised for each patient [77]. Additionally, it allows the controlled manufacture of complex, porous structures with high precision and resolution. For example, Fierz et al. [77] designed scaffolds with longitudinal channels and interconnecting pores to encourage vascularisation of implants and improve osteoconduction. Although 3D printed HAp scaffolds are typically cylindrical to improve their loading capacities, material testing conducted by Cox et al. demonstrated their poor mechanical strength [78]. With further development, 3D printed ceramics of complex structures may be used to aid bone regeneration.

17.3.2 Synthesis from Biogenically Derived Minerals

In previously discussed methods, HAp is initially synthesised as powders or granules before being moulded or shaped into a porous ceramic. An alternative approach to produce porous HAp materials is to convert porous calcified structures derived from living organisms.

Calcium carbonate ($CaCO_3$) biominerals are found in many marine organisms and egg shells as exoskeletons or endoskeletons [3, 79]. The porous microarchitecture of these natural materials closely resembles the microarchitecture of bone, which can be preserved when converted to HAp under controlled experimental conditions [69, 70]. These pores promote the ingrowth of bone cells and tissue that subsequently leads to the regeneration of bone. Furthermore, HAp derived from biogenic sources exhibits physiochemical similarity with bone, improving biocompatibility [79].

Several examples of biogenically derived mineralized structures or organisms that have been converted to HAp include egg shells [72] cuttlefish bone [80], starfish [81], seashells [71], coral [69, 70, 82] and red algae [14, 83, 84] (Fig. 17.1). Among these, extensive research has been performed on coral as a precursor material. It is important to note that the organic or cellular material must be removed using thermal or chemical treatment before the mineralized structure is converted to HAp in order to avoid biotoxic rejection of implant material.

17.3.2.1 Coral

Coral is a marine organism distributed in tropical regions in shallow waters. Corals are built up from colonies of individual animals called polyps that form an inorganic skeleton [85]. The mineral component of the biomineralized skeleton is the $CaCO_3$ polymorph aragonite which has an orthorhombic unit cell structure. In comparison, calcite is the thermodynamically stable $CaCO_3$ polymorph which has a trigonal unit cell structure (Table 17.3). Although calcite is stable at standard temperature and

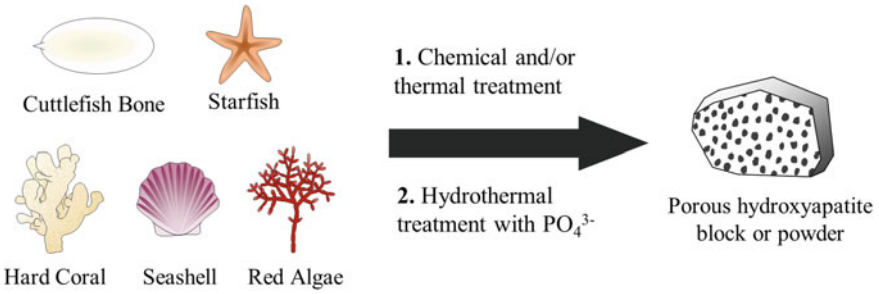

Fig. 17.1 Schematic diagram showing synthesis of porous or powdered HAp bioceramics derived from marine exoskeletons. Step one is the removal of organic material and step two is the conversion of calcium carbonate biominerals to HAp

pressure, many marine organisms produce aragonite due to the high ionic strength of sea water [86].

Coral has a unique structure that is highly porous. Many species of hard coral have interconnecting porous networks that are almost identical to the microarchitecture of bone [87]. The use of coral as a raw material for the synthesis of porous HAp was first demonstrated by Roy and Linnehan [69]. These authors developed a method for the hydrothermal exchange of $CaCO_3$ from the coral *Porites*' skeleton with diammonium hydrogen phosphate ($(NH_4)_2HPO_4$) solution to produce HAp. The reaction took place in a sealed gold tube heated at 270 °C with a pressure of 103 MPa over 24 h. The equation below describes the hydrothermal exchange:

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

According to Roy and Linnehan [69], the high temperatures and pressures used in hydrothermal conversion accelerate the process of ion exchange and sterilise the product. The interconnecting porous structure of coral was successfully preserved after conversion, showing its potential use as an implant material. However, further development of reaction conditions was required, as the pressure and temperatures reported by the authors were extremely high.

Later, Sivakumar et al. further developed a method to convert $CaCO_3$ derived from Indian *Goniopora* coral [82]. *Goniopora* species have interconnecting pores that closely resemble cancellous bone. The coral was initially cleaned and subjected to heat treatment at 900 °C for 2 h to remove organic matter and impurities. Afterwards, the heat treated coral was placed in a pressure vessel with $(NH_4)_2HPO_4$ solution at elevated pressures for several hours. The final product was termed "coralline hydroxyapatite". Although heat treatment allowed the removal of impurities and the organic phase, the primary limitation of this method is the transformation of aragonite into brittle calcium oxide (CaO) during thermal treatment at elevated temperatures. As a result, the final coralline HAp produced by Sivakumar et al. was powdered, and could not be used as an implant without further processing. In addition, the conditions used for hydrothermal conversion were not specified.

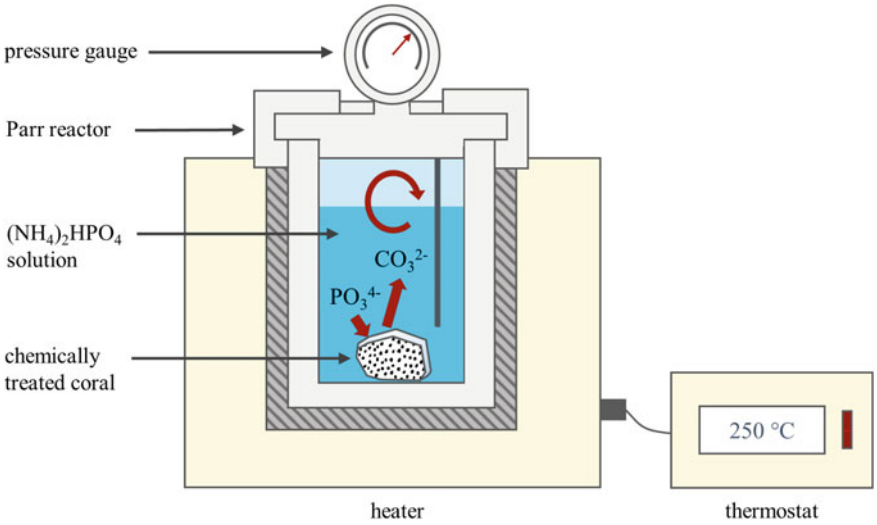

Fig. 17.2 Diagram of the hydrothermal apparatus typically used to convert aragonitic coral to HAp. The sealed Parr reactor is subjected to a high temperature which increases the pressure within the reactor. As a result, phosphate ions from the solution exchange with carbonate ions in the coral structure to produce HAp

Further method development using Australian *Goniopora* coral was reported by Hu et al. [70]. These authors used boiling water and 5% sodium hypochlorite (NaOCl) solution to remove the organic matter chemically instead of using the thermal treatment procedure reported by Sivakumar et al. A Parr reactor with a Teflon liner was used to convert aragonite to HAp (Fig. 17.2). The reaction took place at 250 °C at a pressure of 3.8 MPa with excess $(NH_4)_2HPO_4$ over 36 h. Hu et al. successfully produced carbonate substituted coralline HAp that retained the interconnecting porous structure of coral (Fig. 17.3). Note that the pressure used was lower relative to that of Roy and Linnehan's method given above. The pore size of coralline HAp was reported as 200–250 μm, which is ideal for tissue ingrowth.

Although hydrothermal conversion has been used successfully to produce porous HAp of high crystallinity, these methods involve high pressures and temperatures. Furthermore, these processes are costly due to the complexity of the reaction vessels and conditions required.

An alternative method to produce biphasic CaP and $CaCO_3$ composite scaffolds from coral using microwave processing was developed by Pena et al. [88]. In their procedure, a domestic microwave was utilised at different powers over a period of 5 h. Microwave processing allows the uniform heating of particles, removal of volatile compounds and reduction in thermal stress, resulting in the preservation of the porous microarchitecture [88]. As a result, chemical or thermal treatments are not required for the removal of the organic component. Although pure HAp was not produced,

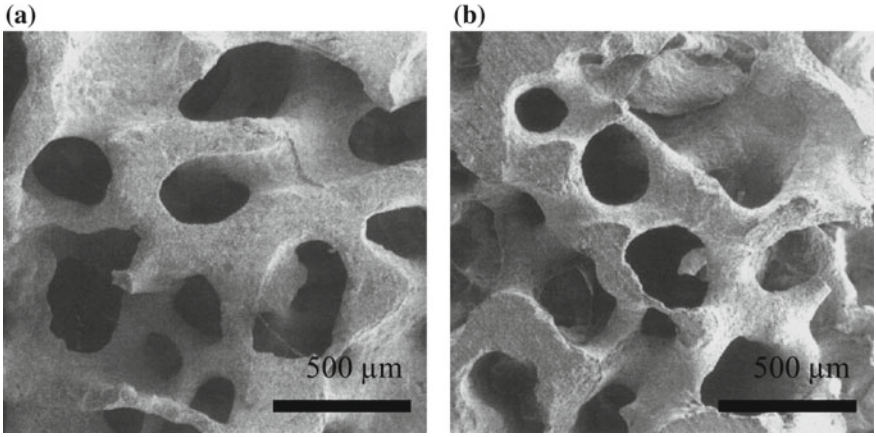

Fig. 17.3 SEM micrographs of Australian *Goniopor*a coral. **a** Prior to conversion; and **b** after hydrothermal conversion demonstrating the preservation of interconnecting pores (adapted from [70])

the composite mixture of $CaCO_3$ and CaP was shown to improve the resorption and bioactivity of the porous scaffold.

Mechano-chemical methods provide another alternative in preparing coralline HAp. This procedure was used by Cegla et al. [89] and Macha et al. [90] for the conversion of Australian coral. To prepare coralline HAp, coral samples were cleaned using 2% NaOCl and ground with an aluminium ball mill. Afterwards, the coral powder was suspended in 150 mL distilled water on a heated magnetic stirrer plate at 200 rpm and 80 °C. Phosphoric acid (H_3PO_4) or ammonium dihydrogen phosphate ($NH_4H_2PO_4$) was added to the suspension drop wise, and the reaction was left for 24 h.

In both studies, the effect of pH on the final product was examined. According to Cegla et al. $CaCO_3$ dissolves in H_3PO_4 due to the acidic conditions, resulting in the precipitation of calcium phosphate once supersaturation has been achieved [89]. Furthermore, the primary phase formed was found to be monetite, with HAp produced as a minor phase. Results presented by Pena et al. under varied pH conditions support these observations [88]. However, under basic conditions, $CaCO_3$ does not dissolve, and HAp is produced through ion exchange. Owing to this, Cegla et al. suggested treatment of coral at high pH conditions to allow the preservation of its unique microarchitecture.

Mechano-chemical methods provide an inexpensive, simple procedure in the production of coralline HAp in comparison to hydrothermal conversion [89]. As low temperature and pressure conditions are used, the reaction can be monitored over time by taking aliquots, which cannot be reproduced in hydrothermal methods. Despite this, mechano-chemical methods have only been applied to coral powders, so it is unknown if the procedure could also be applied to 3D coral structures.

Coralline HAp is an excellent bone tissue engineering scaffold because of its characteristic porosity and chemical similarity to bone. Coral species such as *Porites* and *Goniopora* are composed of 99% aragonite and can be sliced to the desired shape before conversion [91]. However, a significant limitation regarding the use of coral for biomedical applications is the lack of supply and damaging effects of harvesting procedures [87]. Coral reefs are declining on a worldwide scale due to warming sea waters, overfishing and ocean acidification, with over 20% of reefs destroyed as of 2004 [92]. As coral plays a significant role in marine ecosystems, preservation and conservation are crucial for the environment.

17.3.2.2 Calcified Macroalgae

Algae are a group of plant-like organisms that come in diverse sizes and environments [93]. Certain species of freshwater and marine algae deposit $CaCO_3$ along their cell walls, forming a calcified skeleton that provides mechanical support and protection [86, 93]. With the exception of some red algae species, marine algae produce $CaCO_3$ as the polymorph aragonite [86].

Calcified macroalgae may be used as an additional source of biogenic $CaCO_3$ because of their porous skeletons. Similar to coral, calcified algae contain interconnecting micropores that allow the transport of nutrients between cells [94]. According to Felício-Fernandes and Laranjeira, harvesting algae will not cause extensive damage to surrounding seabeds such as coral [14]. Furthermore, they are easy to maintain and are widely available.

The first study on algae-derived HAp was conducted by Kasperk and Ewers [83]. In their study, the red algae species *Corallina officinalis* was converted to HAp and compared with coralline derivatives [94]. Kasperk et al. found that *C. officinalis* species contained longitudinal micropores that were 10 μm wide and 30 μm long which formed a honeycomb pattern on the surface. As a result of these micropores, the surface area of the algae was found to be greater than coralline HAp. Furthermore, they reported that the small crystal size of algae-derived HAp further improved the osteogenesis of the implant compared to coralline HAp. This algae-derived HAp is commercially available under the product name Algipore® [95].

Later, Felício-Fernandes and Laranjeira developed a hydrothermal method for the conversion of $CaCO_3$ from the red algae *Rodophycophyta* [14]. These algae have a unique porosity and contain a high amount of $CaCO_3$ as the polymorph calcite [86]. The red algae were initially washed with water and 10% NaOCl to remove the organic matter. Afterwards, hydrothermal conversion was achieved in a sealed reaction vessel with a stoichiometric amount of $(NH_2)_4HPO_4$ at 200 °C for a period of 24–48 h. The final product was characterised as non-stoichiometric carbonated HAp. Although pure HAp was not synthesised, Felício-Fernandes and Laranjeira argue that the chemical similarity between algae-derived HAp and bone is advantageous. Furthermore, scanning electron microscopy (SEM) revealed the interconnecting pores which were preserved after the conversion of the algae, similar

to the work of Kasperk and Ewers [83]. The average pore diameter was reported as 20 μm.

Another method reported by Walsh et al. produced HAp derived from red coral species *C. officinalis* using a low temperature hydrothermal conversion [84]. The mineral phase of *C. officinalis* is a magnesium-rich calcite. To remove magnesium and organic matter, the algae were first washed and subjected to heat treatment to 700 °C with a slow ramp of 0.5 °C/min to prevent structural decomposition. To synthesise HAp, stoichiometric $NH_4H_2PO_4$ solution was mixed with the algae in a reaction flask stirred at 100 rpm at a temperature of 100 °C for 12 h. In this procedure, the porous microstructure of the algae was preserved after conversion. One major drawback of this approach is the thermal decomposition of $CaCO_3$ into brittle CaO when pre-treated at 700 °C as CaO produces HAp with poor mechanical properties. Also, the pores had an average diameter of 10 μm and formed long canals that lacked interconnectivity. Regardless of these issues, the conversion procedure is simple compared to the one reported by Felício-Fernandes and Laranjeira [14], as it utilises equipment that is readily available, and allows hydrothermal synthesis at atmospheric pressure and low temperatures. However, pre-treatment to remove the organics at a lower temperature would be preferable.

In a recent study, the green calcified algae *Halimeda cylindracea* was converted to HAp [96]. *Halimeda* species are one of the most heavily calcified algae that are abundant in tropical regions and coral reefs [86, 97]. The structure of these species is described as an expanded, branched thallus of calcified segments that are joined by a network of flexible organic fibres [93, 97] (Fig. 17.4). The mineralized skeleton is composed of the $CaCO_3$ polymorph aragonite [98] that is porous in structure, allowing it to be a suitable precursor for the synthesis of porous HAp scaffolds.

H. cylindracea was initially treated using 2% NaOCl solution to remove the organic material and impurities from the ocean. To convert the $CaCO_3$ skeleton to HAp, the hydrothermal method of Hu et al. [70] was followed over a slightly lower temperature and time (220 °C over 24 h, lowered to 160 °C overnight). Chemical characterisation confirmed the successful conversion of aragonite to HAp. In addition, SEM micrographs revealed the porous surface of clean *H. cylindracea* that was preserved after conversion with the removal of the plate-like coverings (Fig. 17.5a, c). These pores were 7–18 μm in diameter, which is similar to the values reported for red algae species [14, 84, 94]. Unpublished SEM micrographs demonstrated that chemical treatment was insufficient in removing the fibrous organic material that ran throughout the centre channel of *H. cylindracea* (Fig. 17.5b). However, the high temperature and pressure conditions used during the conversion procedure resulted in their removal (Fig. 17.5d). Cross sectional SEM micrographs reveal the interconnected microporous channels that run throughout the mineralized skeleton from the outer surface towards the inner cortex that were preserved after conversion (Fig. 17.5d, f). The diameters of the channels increase as they merge towards the inner cortex. Further work should be undertaken to characterise the physical, chemical and mechanical properties of *H. cylindracea* derived HAp as results so far are promising for orthopaedic applications.

Fig. 17.4 Image of green calcified macroalgae *H. cylindracea* showing branched, beaded thallus

Various authors have demonstrated that $CaCO_3$ derived from calcified red and green algae can be converted through different methods to produce porous HAp scaffolds. Algae-derived HAp can provide an alternative implant material to minimise the extensive damage caused by harvesting coral. However, a limitation of red algae is its porosity. The average pore diameter for red algae reported by various authors range from 10 to 20 μm [14, 84, 94], while pore diameters for Australian *Goniopora* coral were reported as 200 to 250 μm [70]. Though the microporosity of algae promotes the attachment of bone cells and tissue, the macroporosity of coralline HAp is similar in dimension to cancellous bone, allowing the ingrowth of bone tissue [79, 94].

17.4 Clinical Studies on Algae-Derived HAp for Bone Tissue Engineering

The first histological study conducted by Kasperk et al. [94] demonstrated the osteogenic properties of *C. officinalis* algae-derived HAp under implantation in a rat femur defect. In comparison to coralline derivatives, bone regeneration and vascularisation occurred more prominently in the algae-derived HAp. The authors suggested that the interconnected microporous architecture of the algae provided a large surface area (50 $m^2\ g^{-1}$) that allows for attachment of osteoblast cells which promote bone regeneration. Furthermore, HAp crystals within the converted algae were small in size and were chemically similar to the mineral in bone which may have favoured

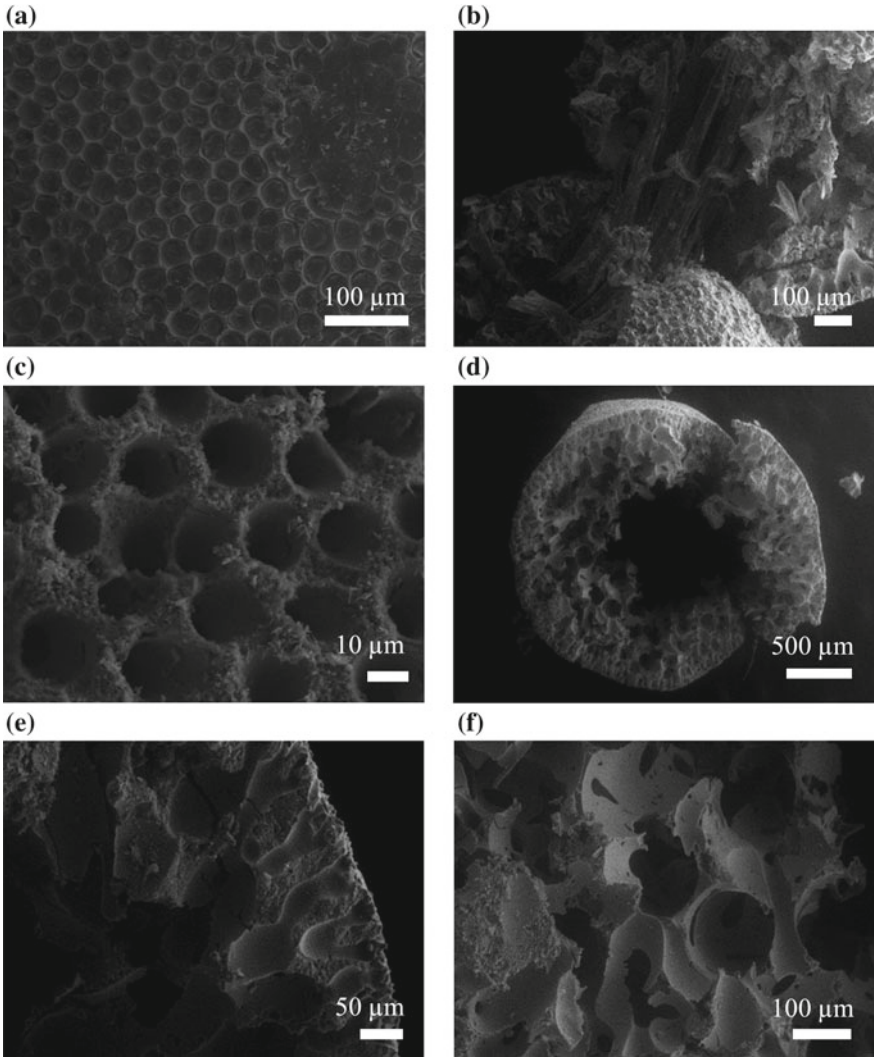

Fig. 17.5 SEM micrographs of green algae *H. cylindracea* **a** surface after cleaning; **b** longitudinal cross-section after cleaning; **c** surface after conversion; **d** and **e** transverse cross-section after conversion, and **f** inner surface after conversion

bone regeneration upon implantation. It is now available commercially under the name Algipore®, and is commonly utilised to reconstruct the maxillary sinus before dental implant surgery. This procedure is conducted to ensure that there is sufficient bone height and volume to surgically create sockets that secure dental implants [99].

In 1999, Schopper et al. conducted the first clinical trial on Algipore® for maxillary sinus bone augmentation on 70 patients [100]. Before implantation, Algipore® was mixed with bone debris (1:5–1:10 ratio) and venous blood obtained from the patient during surgical procedures to provide the osteoinductive proteins and growth factors that aid in bone regeneration. This method eliminated the need to harvest autologous bone from the patient, reducing donor site morbidity and pain. After 6 months of implantation, histological evaluation revealed the formation of new bone tissue and partial resorption of Algipore® at the implanted site. Osteoblast cells and unarranged collagen fibrils were also found in this region which are indicative of formation of new bone tissue. Bone tissue and vascular ingrowth were also observed within the porous structure of the material. A later clinical trial conducted by Ewers et al. on a single patient showed similar results [101].

In a recent study, Poeschl et al. evaluated the effects of the addition of platelet-rich plasma (PRP) in Algipore® implants for bone regeneration [102]. PRP can be extracted from the patient's own blood and contain a high concentration of platelets and proteins. It provides an autologous source of platelet derived growth factors which enhance vascularisation and bone regeneration [102–104]. In Poeschl's study, the method reported by Schopper [100] was followed for a control group of 11 patients. In a test group consisting of 14 patients, PRPs were processed from autologous blood and were added to the Algipore®/bone debris mixture (1:10 ratio) in place of venous blood. After 6–9 months of healing, histological and histomorphometric results showed an increase in the formation of new bone with the addition of PRP to Algipore® and bone debris indicating positive effects. However, an animal study conducted by Klognoi et al. [105] failed to find an improvement in bone regeneration with the addition of PRP to Algipore®. Marx suggested that imprecise preparation methods may cause the inefficiency of PRPs in bone regeneration [104]. As a result, further research must be conducted into the effects of PRP.

The short term clinical studies conducted by Schopper et al. [100], Ewers et al. [101] and Poeschl et al. [102] showed the positive effects of mixing Algipore® with autologous bone debris for maxillary sinus bone augmentation. Despite this, further research into the long term effects of these implants as well as a control using Algipore® alone was required. In 2005, Ewers published results of a long-term clinical study on the use of Algipore® for maxillary sinus grafting [95]. Over the duration of 14 years, 614 Algipore® implants were grafted in 209 sinus sites of 118 patients following the surgical method reported by Schopper et al. [100]. The implanted bone graft was composed of Algipore® granules (90%), autologous bone debris (10%) and either autologous blood or PRP. As with the results of Schopper et al. [100], new bone tissue was formed within 6 months of grafting, and the Algipore® graft underwent slight resorption as given by the 14% loss in volume. Implants that contained PRP were found to improve bone formation, with a 5% increase in the volume of new bone in comparison to implants with venous blood. Overall,

only 12% of cases experienced local infection and 27 implants were lost during the 14-year study, resulting in an implant survival rate of 95.6%. The long-term study demonstrated the successful use of the calcified marine algae-derived HAp Algipore® in the clinical application of maxillary sinus grafting.

Algipore® can also be applied as a scaffold for bone tissue engineering applications due to its chemical similarity to bone and interconnected porosity. Turhani et al. first showed that Algipore® was capable of supporting the adhesion and proliferation of osteoblast cells that were isolated from the human mandible bone [106]. Furthermore, it confirmed the biocompatible and osteogenic properties of Algipore® as shown by previous clinical trials. Afterwards, Malicev et al. successfully constructed a 3D bone-like tissue by seeding human alveolar osteoblast-like cells into Algipore® in a rotating bioreactor to allow the even distribution of cells and nutrients [107].

In addition to osteoblast cells, many studies have demonstrated the capacity of Algipore® to support and differentiate stem cells [108–111]. It was first demonstrated by Turhani et al. [108] who seeded mesenchymal cambial layer precursor cells into a 3D Algipore® scaffold. These cells underwent osteoblast differentiation within the algae-derived HAp scaffold and initiated mineralization, showing potential in producing a 3D bone tissue-engineered implant. Further in vitro studies conducted by Sollazzo et al. [110] and Girardi et al. [111] using MSCs also confirm the osteoinductive properties of Algipore® which is crucial in producing a tissue-engineered bone implant.

Animal studies have shown that the addition of MSCs and PRP to Algipore® scaffolds improved bone formation and osteogenesis when used for sinus grafting [112] and mandibular ridge implantation [113] in minipigs. In both studies, a control graft using Algipore® alone showed signs of bone formation after implantation, but was significantly slower than MSCs/PRP/Algipore® composites. These studies confirm that MSCs have osteogenic properties which help enhance bone regeneration, however further research is required into the contribution of MSCs and PRP to Algipore® scaffolds in bone regeneration before human clinical trials take place. Additionally, a comparison between traditional Algipore® grafts developed by Schopper et al. [100] and tissue engineered Algipore® composites should be drawn to see if tissue engineering can improve bone regeneration.

17.5 Techniques for Analysing Biogenically Derived HAp Materials

17.5.1 Fourier Transform Infrared Spectroscopy

Fourier transform infrared (FTIR) spectroscopy is a useful tool for the characterisation of organic and inorganic compounds. This characterisation technique is based on the molecular vibrations of atoms which arise from the stretching and bending of molecular bonds [114]. These vibrations have a characteristic frequency that absorbs

Table 17.6 Characteristic absorption bands for synthetic HAp [16, 115]

Chemical group	Absorption band (cm^{-1})	Description
OH^-	3500	Sharp peak of low intensity
	630	Water liberation
Adsorbed H_2O	3600–2600	Broad peak of low intensity, may be removed using heat treatment
	1650	Low intensity peak
Adsorbed CO_2	2300	Low intensity peak, adsorbed from atmosphere
CO_3^{2-}	1530	ν_3 type A substitution with OH^-
	1450	ν_3 type B substitution with PO_4^{3-}
	870	ν_2 vibration of weak intensity
HPO_4^{2-}	870–880	Medium intensity peak, characteristic of non-stoichiometric HAp
PO_4^{3-}	1000–1100	ν_3 vibration, large peak of high intensity
	960	ν_1 vibration, low intensity peak
	560–602	ν_4 vibration, doublet of medium intensity
	460	ν_2 vibration

IR radiation at a corresponding energy. For a molecule to be IR active, it must have a dipole moment caused by the asymmetric vibrations of the bonds.

FTIR spectroscopy is valuable for the characterisation of calcium phosphate materials, as each phase has characteristic vibrational bands for PO_4^{3-} ions. FTIR has the ability to characterise the phase composition of calcium phosphate mixtures and identify substituted ions in the lattice, namely, CO_3^{2-} or phase impurities [115]. Additionally, it can examine the crystallinity and purity of the material. Table 17.6 gives the characteristic absorption wavelengths for synthetic HAp and carbonated apatite.

FTIR spectroscopy has been used in many publications to characterise HAp derived from biogenically derived $CaCO_3$ [14, 70, 82, 94, 96]. It has also been used to characterise impurities such as adsorbed carbon dioxide (CO_2) from the atmosphere and residual $CaCO_3$ that did not undergo complete conversion to HAp [70, 82]. Macha et al. used FTIR to identify the intermediate product and phase composition by characterising sample aliquots throughout conversion [90].

Figure 17.6 displays the FTIR spectrum of *H. cylindracea* derived HAp. The synthetic product was characterised as a carbonated apatite due to the presence of the strong the PO_4^{3-} band at 1024 cm^{-1} as well as CO_3^{2-} vibrational bands between 1400 and 1500 cm^{-1}. The spectrum also display bands contributed by the adsorption of H_2O and CO_2. Felício-Fernandes and Laranjeira [14] suggest their removal using heat treatment up to 650 °C. However, caution must be taken to prevent the porous microarchitecture from decomposing. The bands in the FTIR spectrum are well

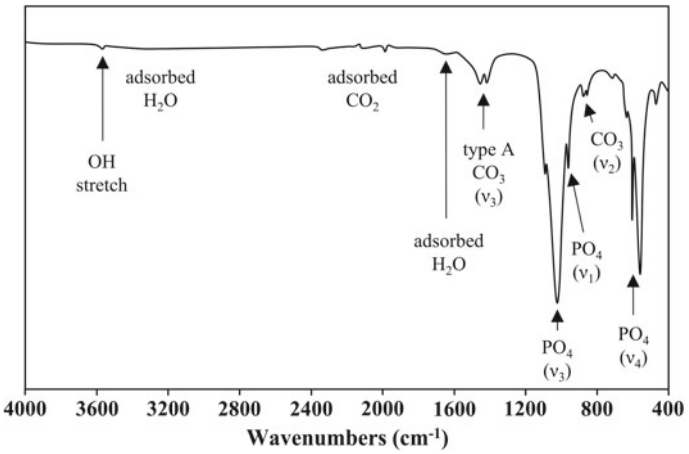

Fig. 17.6 FTIR spectrum of HAp derived from green algae *H. cylindracea* (Choi, unpublished data)

resolved, suggesting that the hydrothermal method developed by Hu et al. produced HAp of high crystallinity owing to the high temperature and pressure conditions [70].

17.5.2 X-ray Diffraction

X-ray diffraction (XRD) is another characterisation tool used for the analysis of HAp and other inorganic minerals. In powder XRD, an incident beam is deflected by the lattice planes in a crystal, forming a diffraction pattern that is characteristic to each mineral [116]. Like FTIR, powder XRD can be used to characterise HAp, determine purity and identify additional phase impurities. The lattice parameters of minerals may also be determined.

For the synthesis of HAp from biogenically derived minerals, a number of studies used powder XRD analysis to characterise the biogenic mineral, examine the effects of heat treatment on the phase composition and to characterise the final converted HAp [14, 70, 82, 84]. Sivakumar et al. [82] and Hu et al. [70] both used power XRD to characterise the initial $CaCO_3$ polymorph of coral as aragonite, and monitored its phase transformation to CaO under thermal treatment (Fig. 17.7a).

Powder XRD is also used to characterise HAp by assigning the Miller indices of the crystal lattice (Fig. 17.7b). Additional peaks may also identify impurities or minor phases within the pattern. For example, additional peaks found in the XRD spectrum of algae-derived HAp by Felício-Fernandes and Laranjeira [14] were attributed to the formation of minor CaP phases such as OCP and TCP.

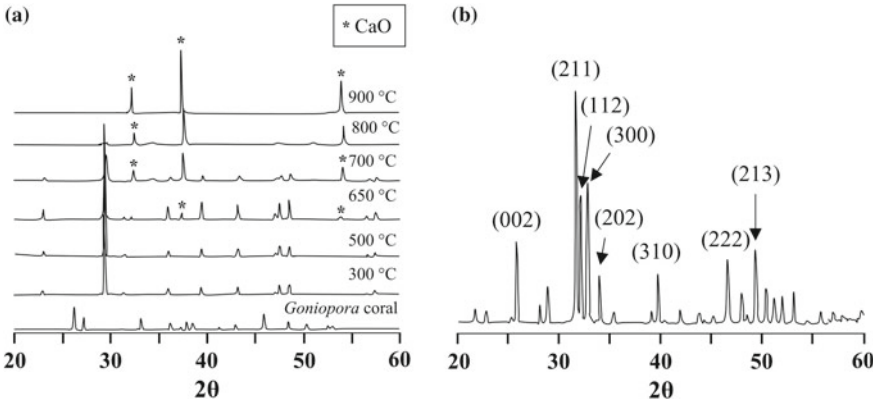

Fig. 17.7 XRD patterns of **a** *Goniopora* coral with heat treatment at various temperatures resulting in the phase transformation of aragonite to calcite then CaO and **b** coralline HAp with significant Miller indices (adapted from Hu et al. [70])

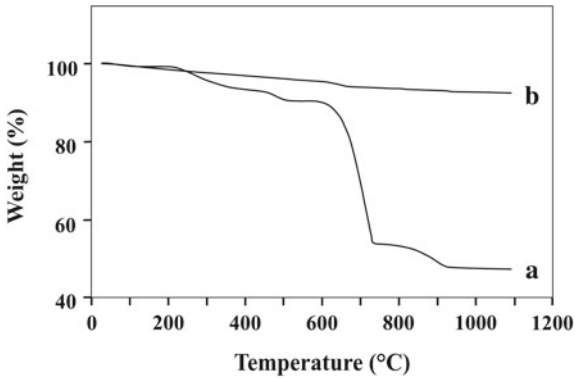

Fig. 17.8 TGA traces of **a** calcified algae *H. cylindracea* and **b** *H. cylindracea* derived-HAp (Choi, unpublished data)

17.5.3 Thermogravimetric Analysis

In thermogravimetric analysis (TGA), a material is subjected to high temperatures to investigate its physical and chemical behaviour [70]. Before the conversion of biogenic $CaCO_3$, thermal analysis may be conducted on coral or algae to find the optimal temperature to remove the organic components using thermal treatment without the decomposition of $CaCO_3$ [70, 82]. For synthetic HAp materials, TGA is used to determine the appropriate temperature for thermal treatment and the removal of adsorbed water or carbonate impurities [82]. TGA traces of calcified algae *H. cylindracea* as well as *H. cylindracea* derived-HAp are given in Fig. 17.8, and a summary of the typical decompositions and phase transformations that occur are compiled in Table 17.7.

Table 17.7 Thermal decomposition of coral, calcified algae and synthetic HAp in air [14, 70, 82]

Sample	Temperature (°C)	Description
Coral and calcified algae	50–140	Removal of adsorbed H_2O
	150–450	Decomposition and removal of organics
	600–750	Thermal decomposition of $CaCO_3$ to CaO ($CaCO_3 \rightarrow CaO + CO_2$)
Synthetic HAp	50–250	Removal of absorbed H_2O
	880–930	Removal of CO_3^{2-} and OH^-
	1470	Phase transformation of HAp to α-TCP (theoretical)

17.5.4 Scanning Electron Microscopy

Scanning electron microscopy (SEM) is a valuable technique for the analysis of the microarchitecture of biominerals and biogenically derived HAp. An image is formed by the emission or scattering of electrons from an incident beam that moves across the surface of the sample [117]. SEM has the capability to magnify beyond what traditional light microscopes can achieve, as electrons are used instead of light waves. It allows for microscopic structures such as the unique morphology of minerals and crystals to be examined [117, 118].

SEM has been used extensively by many authors to examine the porous microarchitecture of calcified marine organisms and biogenically derived HAp [14, 69, 70, 84, 88, 94, 96]. The interconnectivity between the pores has also been examined as it is crucial for bone grafting and bone tissue engineering applications. Pore sizes were estimated by the authors using SEM analysis, although complementary techniques such as nitrogen gas adsorption techniques have also been utilised to estimate the pore size and volume [14, 84].

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