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## **Designs from the Deep: Marine Organisms for Bone Tissue Engineering**

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## Abstract

Current strategies for bone repair have accepted limitations and the search for synthetic graft materials or for scaffolds that will support *ex vivo* bone tissue engineering continues. Biomimetic strategies have led to the investigation of naturally occurring porous structures as templates for bone growth. The marine environment is rich in mineralizing organisms with porous structures, some of which are currently being used as bone graft materials and others that are in early stages of development. This review describes the current evidence available for these organisms, considers the relative promise of each and suggests potential future directions.

## Introduction

Bone grafting is used routinely in orthopaedic and dental surgery for procedures such as spinal fusion, foot and ankle arthrodesis, impaction grafting during revision surgery, stimulation of healing in fracture non-unions and void filling following tumour resection. In the reconstruction of bony deficits, autologous or allogenic bone has proven most effective, however, their use is not without problems. Autograft harvest has associated significant donor site morbidity and restrictions on the amount of graft material available, particularly if the patient is elderly and bone stock is deteriorating. Allograft also has limited stocks (Galea *et al.*, 1998), a clinical failure rate of 20-30% and concerns about immunogenicity (Louisia *et al.*, 1999; Summers and Eisenstein, 1989; Wooley *et al.*, 1996). Furthermore, it is likely that with increasing concerns over transmissible spongiform encephalopathies the use of allograft material will be more tightly regulated and restricted in the near future.

Currently, the majority of clinically available and experimental synthetic bone graft materials are calcium phosphate (CaP) ceramics. Approximately 60% of bone consists of the mineral phase hydroxyapatite (HA), therefore structures containing HA or related CaPs, (e.g. beta-tricalcium phosphate) are natural choices as bone substitutes and scaffolds for bone tissue engineering (Rezwan *et al.*, 2006). Although the biological performance of CaPs is well proven and they possess excellent biocompatibility and osteoconductive properties, clinical results continue to be inferior to those seen with autograft or allograft. Therefore, development of new or modified synthetic bone substitutes continues apace (Bohner *et al.*, 2005).

It is the general consensus among the scientific and orthopaedic communities that the ideal material for bone tissue engineering should provide initial mechanical strength and support, with gradual resorption and replacement by newly synthesized host tissue. When calcium phosphates were first investigated as bone graft substitutes, dense material in granule or particulate form was often implanted but as these ceramics are only partially resorbable, replacement by new bone is incomplete

and the biomaterial remains *in situ* for many years. Development of advanced production methods and a desire to mimic more closely the natural structure of bone led to the production of porous materials that are more easily infiltrated by host tissue (Shors, 1999). Porous materials allow improved interdigitation with host bone, improved vascularization and fluid exchange. Several studies have suggested that the optimum pore size for neovascularization and bone ingrowth is between 200  $\mu\text{m}$  and 500  $\mu\text{m}$  (Green *et al.*, 2002; Wiesmann *et al.*, 2004) and that good connectivity of the pores throughout the scaffold is essential (Shors, 1999). This advancement in design, coupled with the principles of biomimetics and an interest in *ex vivo* tissue engineering of bone has led to a search for naturally occurring scaffolds that could be used for the purpose of bone regeneration.

The marine environment is a rich source of organisms with unique bone-like structures. Coral has been the most widely used – in the early 1970s, White (White, 1997) suggested that a variety of corals and echinoids with unique 3-D structures, described by Weber in the 1960s, may have a potential application for orthopaedics – but in the last five years, calcified materials from a diverse range of marine invertebrates have been investigated for use in bone tissue engineering. This review will compare the published results for each of these sources and will consider the relative promise of each organism, taking into consideration concerns of sustainability and conservation.

## **Coral**

Corals belong to the phylum Cnidaria, and most belong to class Anthozoa (Castro and Huber, 2008). Many soft corals have an internal structure that features calcium carbonate spicules but it is the hard, or stony, corals which are used for bone tissue engineering (Shors, 1999). Stony corals secrete an external calcium carbonate skeleton, mainly in the form of aragonite with some calcite (Abramovitch-Gottlib *et al.*, 2006; Allemand *et al.*, 1998). This skeleton, combined with an open, highly interconnected porous structure, make them of interest as scaffolds for bone tissue engineering. Most of the corals that have been investigated thus far for use as scaffolds for bone growth have pore sizes

between 100  $\mu\text{m}$  and 500  $\mu\text{m}$  diameter with a high degree of connectivity (Abramovitch-Gottlib et al., 2006; Geiger et al., 2007; Green et al., 2002; Mygind et al., 2007; Shors, 1999; Wiesmann et al., 2004).

Clinically, coral scaffolds or coral-derivatives have been used for spinal fusion (Coughlin et al., 2006; Griesshaber et al., 2007; Im et al., 2005), in maxillofacial surgery (Chen et al., 2008b; Green et al., 2003; Laza et al., 2007; Oliveira et al., 2007), dental surgery (Lahaye and Robic, 2007; Martina et al., 2005) and for other orthopaedic applications (Bachle et al., 2006; Coughlin et al., 2006; Hou et al., 2007; Kujala et al., 2002), either alone (Chen et al., 2008b; Coughlin et al., 2006; Green et al., 2003; Hou et al., 2007; Im et al., 2005; Lahaye and Robic, 2007; Laza et al., 2007; Oliveira et al., 2007), or as part of a composite material (Bachle et al., 2006; Coughlin et al., 2006; Griesshaber et al., 2007; Martina et al., 2005) with reported success and low complication rates, an exception being a clinical study of scaphoid non-unions in which autograft was supplemented with coral/BMP implants and poor results were blamed on poor vascularity (Kujala et al., 2002).

Experimentally, *in vitro* testing of corals directly as scaffolds to support osteogenic cell attachment, growth and differentiation has shown that primary human bone-derived osteoblasts (Doherty et al., 1994) and a mouse mesenchymal cell line (CRL-12424) attached and spread on the coral (*Porites lutea*) surface displaying a fibroblast-like morphology (Gravel et al., 2006). A similar study using the same cell line demonstrated cell proliferation on the surface of two corals (*Millepora dichotoma* and *Porites lutea*) with temporal expression of alkaline phosphatase and osteocalcin (measures of osteogenic differentiation) and evidence of mineralization (Abramovitch-Gottlib et al., 2006). Cui et al showed similar results *in vitro* with adipose-derived stem cells from dogs (Cui et al., 2007). *In vivo*, an early study reported successful healing of cortical and cancellous, non-critical and critical sized bone defects in dogs using four different corals; *Porites*, *Goniopora*, *Favites* or *Lobophyllia* (Guillemin et al., 1987) and Gao et al reported superior healing of a segmental tibial defect in sheep with coral compared to tricalcium phosphate (Ohgushi, 1997). However, these early successes have been

followed by reports of the inability of coral alone to support effective bone healing (Galea et al., 1998; Laza et al., 2007; Mygind et al., 2007).

In common with other synthetic CaP bone substitutes, corals have been shown to have limited osteoinductive capacity and it is only with the addition of osteogenic cells onto the coral construct that ectopic bone formation is demonstrated (Chen *et al.*, 2007; Ohgushi, 1997; Zhang *et al.*, 2007b). Therefore many studies investigating the ability of coral to support bone healing *in vivo* have included the use of osteogenic growth factors or cells to enhance the osteoinductive capability of the construct. In a rabbit model of spinal fusion, a Biocoral/collagen scaffold with bovine-derived bone protein extract resulted in solid fusions compared to scaffold alone (Mygind et al., 2007): a bone morphogenetic protein (BMP) loaded coral scaffold also improved healing in a canine long bone defect (Harris and Cooper, 2004) and rabbit calvarial defect (Hou et al., 2007). Cui *et al* showed that enhanced healing occurred at 12 and 24 weeks for a cranial defect in dogs when adipose-derived autologous stem cells were added to the coral scaffold (Cui et al., 2007). In the group which received coral alone, there was minimal new bone formation and much of the coral had been resorbed by 24 weeks leaving an empty defect. Similar results were reported by Geiger *et al* in a rabbit radial defect (Geiger et al., 2007) and Louisia *et al* in a rabbit ulnar defect (Louisia et al., 1999) with coral alone showing minimal bone formation but healing enhanced by the addition of autologous bone marrow stromal cells. Both studies used Biocoral® which is a commercially available bone substitute material from purified coral (*Porites sp.*) exoskeletons (Table 1). The coral is chemically treated to remove amino acids, artefacts and foreign bodies, but there is no alteration to its chemical or crystalline structure (Cirutteau, 1979; White, 1997). Geiger *et al* (Geiger et al., 2007) and Louisia *et al* (Louisia et al., 1999) both also reported substantial resorption of the coral scaffold after several weeks - a finding that is supported by others (Bensaid *et al.*, 2005; Guillemin et al., 1987; Ohgushi, 1997).

Based on these results, Louisia *et al* suggested that resorption of the coral scaffold could be one of the main benefits of its use as a bone tissue construct compared to other, more frequently used ceramics

such as hydroxyapatite. One of the perceived disadvantages of using CaP ceramics clinically is the relatively slow resorption rate. Although some formulations, such as those with a greater proportion of  $\beta$  tri-calcium phosphate, have a faster resorption rate, in general CaP resorption is slow and hydroxyapatite (the most commonly used synthetic bone graft material) can remain at the implantation site for several years (von Doernberg *et al.*, 2006). One of the goals of bone tissue engineering research is to produce a scaffold that is removed at the same rate as new bone is laid down so that it supports, but does not impede, bone repair. Some synthetic bone substitutes such as calcium sulphate (Cook and Cook, 2009) are resorbed too quickly to support bone cell attachment and bone growth, so could the calcium carbonate skeleton of coral be the optimum material?

Although these early results of Louisia *et al* and others showed promise, as with many *in vivo* studies, they lacked meaningful control groups and favoured inclusion of an empty defect group over the more useful positive control. This was rectified in the group's subsequent studies which compared the cell-seeded coral scaffolds to clinically relevant control groups such as coral mixed with fresh bone marrow (Petite *et al.*, 2000) and defects filled with autograft (Viateau *et al.*, 2007). Further study by this group, and others, showed that resorption rate of the coral scaffold is very finely balanced. Tuominen *et al* reported that adding BMP to the coral scaffold accelerated the rate of resorption (Tuominen *et al.*, 2000). Furthermore, the addition of fresh bone marrow (Petite *et al.*, 2000) or vascular endothelial growth factor (VEGF)-producing bone marrow stromal cells (Geiger *et al.*, 2007) caused the scaffold to resorb too quickly resulting in bone formation rates lower than those seen with cell-seeded constructs. This is in direct contrast to the enhanced bone formation rates found when bone marrow or VEGF was added to a CaP scaffold (Clarke, 2007) and suggests that, with further development, it may indeed be possible to engineer materials with resorption rates tailored to a specific clinical application. Certainly, coral scaffolds seeded with osteogenic cells showed promise in these studies and may provide a more appropriate scaffold for cell-based therapies than CaP ceramics. When compared with autograft control, cell-seeded coral scaffolds showed similar bone formation rates, however, radiographically, complete bridging of the defect was superior in the autograft group



(Viateau et al., 2007) and this may affect clinical outcomes. Furthermore, Bensaïd *et al.*, who also investigated the ability of MSC-loaded coral scaffolds to repair large bone defects in sheep, found that the coral scaffold resorbed too quickly to support bone formation in most instances, even with the cell-loaded constructs (Bensaïd et al., 2005). Differences in experimental protocols may account for the discrepancy between these and the results of Petite's group (Louisia et al., 1999; Petite et al., 2000; Viateau et al., 2007) as Bensaïd *et al.* loaded their scaffold with 640 times more cells and so may have provided too much stimulus for resorption similar to that seen with whole bone marrow and growth factors.

An alternative to using coral directly as a scaffold to support bone growth was first investigated by White and Shors in 1972, when they successfully fabricated replicas of the coral structure from  $\text{TiO}_2$ ,  $\text{Al}_2\text{O}_3$  and HA into bone-like plugs (White *et al.*, 1972). In 1974, Roy and Linnehan took a simpler biomimetic approach, employing the direct conversion of coral calcium carbonate skeletons into HA (termed coralline HA) (Roy and Linnehan, 1974). The years of scientific research that followed have resulted in the development of coral-based commercially available products for bone repair (Table 1). These products (Pro-Osteon™ 200R, Pro-Osteon™ 500R) are only partially converted and so have an outer HA layer (2-5  $\mu\text{m}$  thick) with an inner aragonite core (White and Shors, 1986; White and Shors, 1989). This may provide a solution to the problem of expeditious resorption of the coral scaffold when too much of a stimulus is given: the outer HA layer may slow down the resorption rate, yet the more soluble inner core will counter the slow remodeling typical of bone substitutes produced from unadulterated HA. One concern with HA coated corals *in vivo* is that, when the HA coating is breached, fast resorption of the core will result in a massive increase in localized extracellular calcium and this, as much as the lack of a physical scaffold, may impede bone growth. There is some evidence that, although small amounts of calcium will enhance bone cell proliferation and differentiation, too much may inhibit both osteoblast (Maeno *et al.*, 2005) and osteoclast (Hall, 1994; Yamada *et al.*, 1997; Zaidi *et al.*, 1989) function.

In practice, however, the coralline HA scaffold has shown very little ability to resorb. Bensaïd *et al* reported that there was little evidence of resorption of a Pro-Osteon™ Pro-Osteon™ 200R scaffold in a sheep long bone defect even after 16 weeks when the majority of an unconverted coral scaffold had been resorbed (Bensaïd et al., 2005) and in a clinical trial of Pro-Osteon™ 500R as an alternative to autograft for hind foot arthrodesis in ten patients, the Pro-Osteon™ was still visible on x-ray more than six years after surgery (Coughlin et al., 2006). Furthermore, others have shown that Pro-Osteon™ 500R did not support ectopic bone formation even when seeded with osteogenic cells, unlike conventional CaP scaffolds (Harris and Cooper, 2004). A review by Shors suggests resorption rates for coralline HA in a canine model of 2-5% per year in cancellous sites and 25% per year in cortical defects compared to rates of 65% in 2 weeks for the natural coral graft (Shors, 1999). It is thought that the inability of coralline HA to be resorbed *in vivo* is due to the highly crystalline nature of the HA coating, which is less soluble (Harris and Cooper, 2004). Therefore, although Pro-Osteon™ can conduct bone formation and has been shown to be an effective bone graft for several applications (Coughlin et al., 2006), it should be considered a permanent or slow-resorbing biomaterial and does not provide the half-way house of resorption rate between coral and CaP that might have been expected.

Current commercially available materials use high pressure-temperature synthesis to achieve the HA coating on the coral surface. Several other fabrication techniques, such as replication and microwave synthesis, have also been investigated (table 2). Recently, research into the use of corals as bone substitutes has focused on the hydrothermal conversion of alternative species such as a coral from the Gulf of Mannar, off the coast of India (Sivakumar *et al.*, 1996); modifications to the conversion process such as the addition of a second coating step to improve the mechanical properties (Hu *et al.*, 2001); or the use of alternative, more sustainable species (Abramovitch-Gottlieb et al., 2006). The slow growth rate of corals makes sustainability a major concern for their continued use in bone tissue engineering. To meet the market demand, BiometInterpore (manufacturers of Pro-Osteon™) requires 2-4 tons of coral per year from the Pacific and Indian Oceans (Ritter, 1997).

Since 1990, all species of stony coral have been listed under the Convention on International Trade in Endangered Species (CITES) treaty Appendix II (vulnerable to exploitation but not yet at risk of extinction) (Harriott, 2003). Compared to the damage inflicted on coral reefs by environmental stresses such as pollution and storm damage and by intensive fishing methods such as dynamite fishing, commercial coral harvesting probably has a relatively low impact. However, extensive farming of accessible reefs may lead to localized habitat destruction and overfishing of one or two target species may mean the reef overall is maintained but individual species are lost (Harriott, 2003).

Options for farming corals include strict management and control over the reef such as occurs in Australia, on the Great Barrier Reef in particular, or culturing corals in aquaria – one of the benefits cited by Abramovitch-Gottlieb *et al* for using the hydrocoral *Millepora dichotoma* in their recent studies (Abramovitch-Gottlieb *et al.*, 2006) For some of the branching corals, small pieces can be harvested grown in aquaria to commercially suited sizes and sold, by which time stocks in the wild have also replenished. In general, there is a location- and species-specific answer to the question of whether coral can be harvested sustainably (Harriott, 2003). Some algal species also have a calcareous skeleton and with a growth rate of 2.6cm/year, compared to an average of 1cm/year for coral species (Harriott, 2003), they may provide a more sustainable alternative for bone tissue engineering (Felicio-Fernandes and Laranjeira, 2000).

## **Algae**

There are three types of algae – red, brown and green, but it is species of red algae (phylum Rhodophyta), specifically a group of coralline algae, that have been used in bone tissue engineering. Coralline algae deposit calcium carbonate, in the form of calcite, in their cell wall and, in warm marine environments, have been shown to participate in reef building (Castro and Huber, 2008).

Unlike the corals, coralline algae have not been used in the native form as a scaffold or bone substitute but have instead been converted to calcium phosphate using similar hydrothermal conversion methods as those used to synthesise Pro-Osteon™. This was first described using *Corallina officinalis* by Roy and Linnehan in 1974 (Roy and Linnehan, 1974) and later developed by Kasperk *et al* in the late 1980's (Kasperk *et al.*, 1988). This latter research group has published an extensive body of work in the intervening decades. Despite the fact that the pores of this algae, although interconnected, are approximately 5-10 µm (figure 1) (Schopper *et al.*, 2005; Walsh *et al.*, 2008) and therefore considered suboptimal for tissue and vascular ingrowth (Felicio-Fernandes and Laranjeira, 2000), the coralline HA scaffold has been shown to support the attachment, proliferation and differentiation of both primary human bone-derived cells (Turhani *et al.*, 2005a; Turhani *et al.*, 2005c) and mesenchymal cambial-layer precursor cells (Turhani *et al.*, 2005b). Furthermore, a commercially available CaP product derived from *C. officinalis*, AlgiPore (also marketed as C GRAFT, Algisorb and AlgOss) has a relatively long history of clinical use in maxilla sinus grafting: in 2005, Ewers reported the long term follow-up (up to 13 years) of over 200 sinus grafts with a 95.6% survival rate (Ewers, 2005).

As the algal-derived HA is produced by similar methods to coral-derived HA, there are similar concerns about the resorptive capacity of this graft. Ewers reported that there was only a 14% volume loss of the material after six months in his clinical study of AlgiPore and some graft material was still visible even after six years (Ewers, 2005). In an attempt to increase the resorption rate, biphasic CaP materials were produced from the algal template, with various ratios of HA to β tricalcium phosphate (TCP) (Spasova *et al.*, 2007). In a cortico-cancellous defect model in sheep, biphasic materials with HA:TCP ratios of 50:50 and 30:70 did show a statistically significant decrease in residual graft volume and an increase in bone volume after 6 months compared to 100% HA but there was no further reduction in the material volume of any of the materials at 12 months and considerable amounts of even the 30:70 graft remained (Schopper *et al.*, 2005).

Another strategy for counteracting the lack of resorption of the coralline HA grafts may be to use alternative production techniques. In 2008, Walsh *et al* described production of an HA bone graft from *C. officinalis* using low temperature hydrothermal synthesis that resulted in semi-amorphous HA rather than the highly crystalline material produced by previous methods (Walsh et al., 2008). In theory, this material should be more resorbable *in vivo* but we must await further publications confirming both this and biocompatibility of the graft.

## **Cuttlefish**

Although there are tens of thousands of marine unicellular and multicellular organisms that produce some form of mineral, species from only four further invertebrate phyla have been investigated for bone tissue engineering, namely Mollusca, Arthropoda, Echinodermata and Porifera (Table 3). Of these, cuttlefish (Phylum Mollusca, Class Cephalopoda) have been the most extensively studied. Four groups have described the conversion of a single species of cuttlefish, *Sepia officinalis*, from the natural aragonite calcium carbonate mineral to carbonated HA (Ivankovic *et al.*, 2009; Kasiopas *et al.*, 2010; Lee *et al.*, 2007; Rocha *et al.*, 2005b). The resulting structure (figure 1) has a high percentage porosity (80%-94%) and with pore sizes in the range of 200-600  $\mu\text{m}$  (Kim *et al.*, 2008) may provide a more optimized scaffold for bone tissue ingrowth and revascularization than either coral or coralline algae.

To date, however, this remains untested as the current *in vitro* and *in vivo* testing published is of a very limited nature (Rocha *et al.*, 2006; Rocha *et al.*, 2005a) or has only been presented in short paper form (Kim et al., 2008). *In vitro* studies were short-term (up to 72 hours) and were concerned with initial biocompatibility of the material and therefore tested proliferation and alkaline phosphatase expression of primary osteoblasts *in the presence of*, and not directly attached to, powdered converted cuttlefish bone (Rocha et al., 2005a) or whole constructs (Rocha et al., 2006). No cytotoxic results were reported. *In vivo*, no inflammatory response to converted cuttlefish (*Sepia esculenta*) implanted in a rabbit femoral condylar defect was reported but no quantification of healing or bone formation

was performed (Kim et al., 2008). One study has suggested that cuttlefish "bone" can directly support bone formation, without requiring hydrothermal conversion to HA, and may be suitable as a potential xenograft for bone healing as *Sepia officinalis* was tested in a bone defect model against currently available synthetic graft and xenograft materials (Okumus and Yildirim, 2005). Again, this requires further investigation and there are some concerns about the mechanical strength of the raw material as it is malleable and easily shaped, but in general this represents a potential new direction for bone tissue engineering. Carbonated HA is closer to the chemistry of natural human bone than stoichiometrically pure HA (Bigi *et al.*, 1997) and has been shown experimentally to have enhanced biocompatibility (Ellies *et al.*, 1988; Landi *et al.*, 2003). The methods described to convert cuttlefish aragonite seemed to result in substitution of carbonate ions in a similar location within the HA structure to bone (Ivankovic et al., 2009; Kasioptas et al., 2010) and with cuttlefish in relative abundance in tropical and temperate seas (Okumus and Yildirim, 2005), sustainability may not be a concern making this resource of significant interest.

### **Arthropoda and Echinodermata**

Ion substitution of HA has been a strategy employed extensively to improve the biological performance of calcium phosphate and two groups of the phylum Echinodermata, sea stars (class Asterozoa) and sea urchins (class Echinozoa), have ossicles and spines made of Mg-rich calcite. Ossicles from the seastar, *Pisaster giganteus*, have been shown to support human bone-derived osteoblast and human bone marrow stromal cell attachment directly to the porous structure (Martina et al., 2005) whereas spines from two species of sea urchins, *Heterocentrotus trigonarius* and *Heterocentrotus mammillatus*, were converted to CaP before use (Vecchio *et al.*, 2007b) The biogenic Mg-rich calcite spines resulted in Mg-substituted tricalcium phosphate (TCP) rather than HA when converted. The structure had pores in the range of 20-50  $\mu\text{m}$  with trabecular struts of 100  $\mu\text{m}$  and showed no inflammatory reaction and bony apposition when implanted in a rat femoral defect model (Vecchio et al., 2007b). These are very early results but using an ion-rich biogenic source of calcite or aragonite as a starting material may provide scaffolds with optimal biocompatibility using simple

processing methodology, avoiding the need for complex reactions to obtain ion-substitution, such as those employed by Kannan *et al* to obtain fluorine ion substitution during conversion of cuttlefish bones (Kannan *et al.*, 2007). However, most sea urchin spines are too small to be of practical use and with only two species currently identified as having spines large enough to provide useful scaffolds, sustainability must be a concern.

Conversely, seashells and the exoskeletons of many arthropods such as crab and lobster are large enough to provide structures for any application. In a study examining the conversion of Giant clam (*Tridacna gigas*) and conch (*Strombus gigas*) shells, Vecchio *et al* showed that even when partially converted to HA (i.e. the structure has an outer HA shell and inner CaCO<sub>3</sub> core) the mechanical strengths were similar to that of human bone perhaps allowing this particular material to be used for load bearing applications (Vecchio *et al.*, 2007a). A further advantage of this biogenic source is that the large pieces can be machined into any shape. The authors give screws as an example and potential application, however, the material is non-porous and even though initial *in vivo* studies demonstrated bone apposition in a rat femoral defect model, with no evidence of material resorption at 6 wks, long term removal of the material may be a problem.

Crustacean exoskeletons may provide a solution to this. Studies of lobster (*Homarus americanus*) (Raabe *et al.*, 2005) and the sheep crab (*Loxorhynchus grandis*) (Chen *et al.*, 2008b) showed that their mineralized chitin exoskeletons have excellent material properties, high degrees of strength due to the 'twisted plywood' structure and, in the case of the lobster, a porous honeycomb structure at the micron level. Use of these exoskeletons as graft materials has not been investigated, however, as CaCO<sub>3</sub> has been shown to support bone growth in some of the studies outline previously, and chitin has been extensively studied as part of composite materials for biomedical devices (Khoushab and Yamabhai), there is no reason to suppose that crustacean exoskeletons will not support bone formation. Even if, however, they are not directly suitable as xenografts, studying their complex

architecture and morphology may provide inspiration for synthetic nanocomposite material design (Chen *et al.*, 2008a; Giraud-Guille *et al.*, 2004).

## **Porifera**

The final group of marine invertebrates investigated for the ability to support bone formation is the sponges (Porifera). Some sponges have a CaCO<sub>3</sub> skeleton but the majority has a protein skeleton made from spongin with or without additional support from siliceous spicules (Castro and Huber, 2008). Spongin is comparable to vertebrate collagen and as such has been extracted for use as a functional additive to composite biomaterials. In one study, sponge (*Chondrosia reniformis*)- derived collagen was used in conjunction with silica templating to produce hydrogels which supported attachment and growth of an osteoblast-like cell line (Heinemann *et al.*, 2007). Sponges themselves have also been examined as suitable scaffolds for bone tissue engineering. In two separate studies, six different “unidentified” sponges were found to support human BMSC (Green *et al.*, 2003) and mouse calvarial-derived primary osteoblasts (Zheng *et al.*, 2007) attachment and differentiation demonstrated by alkaline phosphatase expression or activity. Green *et al* subsequently identified their sponge as *Spongia officinalis*, a Mediterranean bath sponge, and showed that it also supported ectopic bone formation when implanted in a subcutaneous pouch athymic mouse model (Green, 2008).

More recently, three sponges from the genus *Spongia* (*S. officinalis*, *S. zimocca*, and *S. agaricina*) were used as precursors, or templates, to produce porous HA scaffolds by a replication technique (Cunningham *et al.*, 2010). Of the three sponges examined, *Spongia agaricina* produced the most promising replicated scaffold for bone tissue engineering as it had approximately 60% porosity with pore sizes in the range of 100-500 μm and 99.9% interconnectivity (figure 1). Therefore sponges could prove to be useful on a number of fronts, either as a source of collagen for hydrogel production or as an additive to synthetic CaP or polymer scaffolds, as templates for producing biomimetic ceramic scaffolds or directly as osteoconductive grafts. There is one serious drawback to the continued use of sponges as biomaterials, however, and that is one of sustainability. Sponges have been used for



centuries for numerous applications, most notably in the cosmetics industry. Those species that produce bioactive compounds are also increasingly being sought for use in biomedical fields. Demand now far outweighs supply of some species, particularly Mediterranean bath sponges, and has resulted in their inclusion on endangered and protected species lists (Baldacconi *et al.*, 2010; Corriero *et al.*, 2004). Currently, methods of farming these sponges (Corriero *et al.*, 2004; Muller *et al.*, 1999), or of transplanting them back into protected areas from which they have become extinct (Baldacconi *et al.*, 2010) are being investigated. Unless these studies prove successful or alternative species of sponge which are not endangered are found, these animals may not prove to be suitable for orthopaedic applications in the long term.

### **Future Directions**

The philosophy of biomimetics has provided a new direction for biomaterial design. Early forays in this area began with researchers trying to recreate the natural structure of bone on a microscale by mimicking the trabecular porous structure and collagen fibre/mineral composite chemistry and has now progressed to mimicking cortical bone on a macroscale, producing materials with a dense outer layer and inner cavity (Zhang *et al.*, 2007a). Reproducing the porosity of human bone has provided some degree of success but may be limited by production methods, for example the use of synthetic performs, which often result in partially closed porous structures with limited connectivity or low tortuosity (the ease of a path through the porous structure). This has driven a search for naturally occurring porous structures which can either be used directly as scaffolds or to provide templates for producing novel biomaterials.

We have seen above that the seas and oceans are a rich source of mineralized organisms that may be of benefit for this purpose. Research into these sources is in its relative infancy and there remains much work to be done with those already identified and many hundreds more that are yet untapped. As we progress towards 'smart' biomaterials that are application specific and have tailored resorption and osteoconductive profiles, it may be that we can utilize many of these resources – using the more

resorbable CaCO<sub>3</sub> structures directly for short term support, using sources rich in Mg, Si or other ions to produce ion-substituted CaP with intermediate resorption profiles, or converting porous CaCO<sub>3</sub> skeletons with optimal pore size and connectivity to provide long term scaffolds for *ex vivo* bone tissue engineering. The opportunities in the marine environment are almost limitless – in fact, as long as we ensure from the beginning that the resources we use are truly sustainable, the seabed is the limit.

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## Figure Legends

Figure 1. Scanning electron micrographs of (a) cuttlefish (*Sepia officinalis*), (b) sponge (*Spongia agaricina*), (c) red algae (*Corallina officinalis*) and (d) coccolithophores (*Emiliana huxleyi*) demonstrating a range of macro and microporous structures. *E.huxleyi* micrograph courtesy of Katherine Fee.

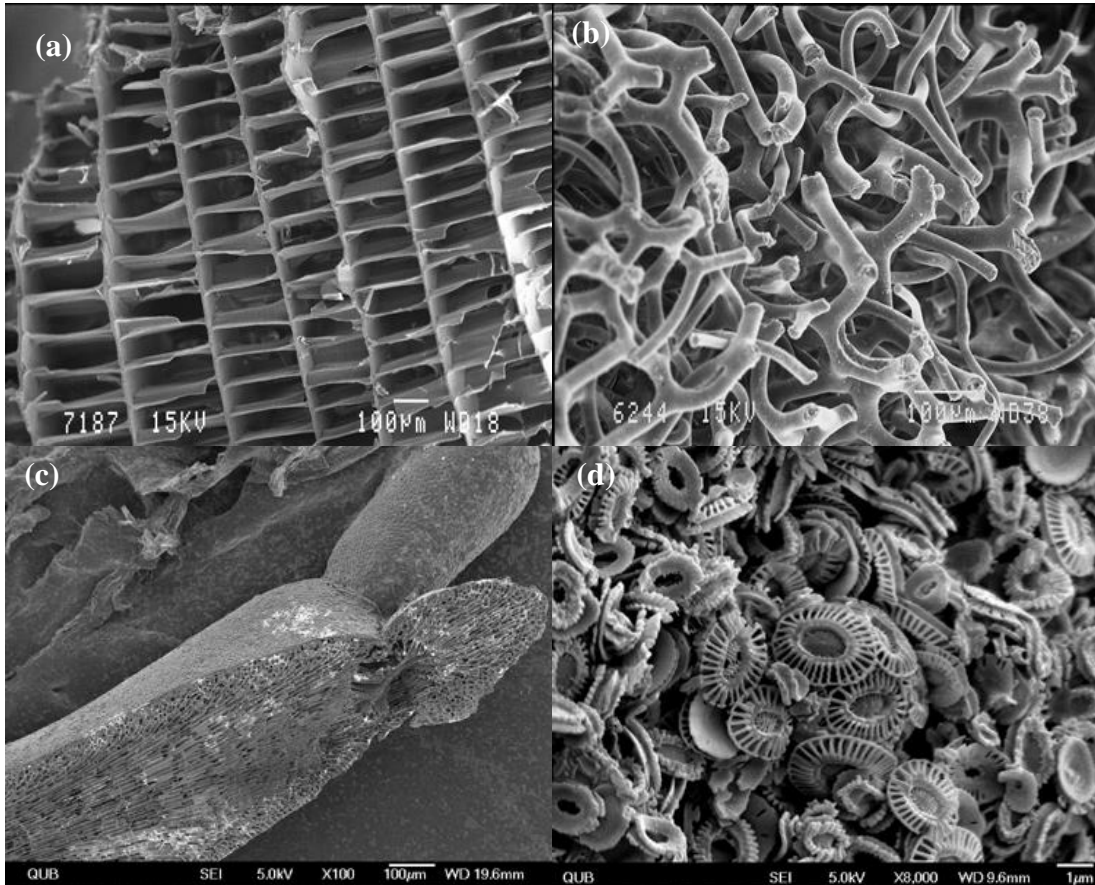


Table 1. Commercially available bone graft products from marine sources.

Product	Manufacturer	Country of Origin	Genera	Chemistry
Biocoral	Inoteb	France	<i>Porites</i> , <i>Acropora</i> , <i>Lobophyllia</i> , <i>Montastrea</i> , <i>Dichocoenia</i>	CaCO <sub>3</sub>
ProOsteon 200R	Biomet	US	<i>Porites</i>	HA/CaCO <sub>3</sub>
ProOsteon 500R	Biomet	US	<i>Goniopora</i>	HA/CaCO <sub>3</sub>
Algipore	DENTSPLY Friadent	Germany	<i>Corallina officinalis</i>	HA

Table 2. Examples of manufacture processes used to fabricate marine-derived/inspired bone grafts. Key: CS = Closed System, autoclave OP = Open System, reaction vessel HPT – High Pressure-Temperature HT – High Temperature  $\beta$ TCP = Tri-calcium phosphate  $\beta$ TCMP = Mg-substituted tri-calcium phosphate

Common Name	Genera/Species	Source	Fabrication Technique	Chemistry	Yr	Ref
Clams	<i>Tridacna gigas</i>	Unspecified	HT Synthesis with superheated steam (CS) - 180°C (20 days)	Complete transformation to HA	2007	(Veccchio et al., 2007a)
Corals	<i>Goniopora</i>	Gulf of Mannar, India	Microwave irradiation - 800 W for 40 mins at 2.45 GHz	Complete transformation to biphasic CaP with HA: $\beta$ TCP Phases	2003	(Murugan and Ramakrishna, 2004)
	<i>Porites</i>	Unspecified	HT Synthesis with vapour pressure (CS) - 160 - 200°C at 1-2 MPa (8-14hrs)	Complete transformation to HA	2002	(Jinawath et al., 2002)
	<i>Goniopora</i>	Great barrier Reef, Australian	(1) Acid digest (5%NaClO); (2) HPT Synthesis (CS) - 250°C at 2.8MPa	Complete transformation to a monophasic HA	2001	(Hu et al., 2001)
	<i>Goniopora</i>	Gulf of Mannar, India	(1) Pyrolysis 900°C (12hrs) (2) HPT Synthesis	HA – Process destroyed structure	1996	(Sivakumar et al., 1996)
	<i>Goniopora; Porites</i>	Unspecified	HPT Synthesis (CS) - 270°C at 103MPa	Complete transformation of coral to a monophasic CaP	1974	(Roy and Linnehan, 1974)
	<i>Goniopora; Porites</i>	Unspecified	Replication using wax casting	TiO <sub>2</sub> , Al <sub>2</sub> O <sub>3</sub> and HA synthetic replicas of coral structures	1972	(White et al., 1972)
	Cuttlefish	<i>Sepia officinalis</i>	New South Wales, Australia	Hydrothermal Synthesis with superheated steam (CS) - 80°C (32hrs) – 190°C (1.5hrs)	Complete transformation to a monophasic HA	2010
<i>Sepia officinalis</i>		Adriatic Sea	(1) Pyrolysis 350°C (3hrs) (2) HT Synthesis, superheated steam (CS) - 200°C (48 hrs)	Complete transformation to monophasic HA	2009	(Ivanovic et al., 2009)
<i>Sepia esculenta</i>		Unspecified	(1) Acid digest (4%NaClO); (2) Pyrolysis 180°C (16hrs)	Predominantly HA	2008	(Kim et al., 2008)

			HT Synthesis superheated steam (CS) - 200°C (24 hrs)			2008)
Red algae	<i>Corallina officinalis</i>	Donegal, Ireland	(1) Pyrolysis 650°C (12hrs) (2) LP Hydrothermal Synthesis - (OS) - 100°C (24hrs)	Complete transformation to a biphasic CaP with ≈ 95 % HA: βTCP Phases	2008	(Walsh et al., 2008)
	Rhodophyta (species unspecified)	Santa Catarina Island	(1) Acid digest (10%NaClO); (2) HT Hydrothermal Synthesis (CS) superheated steam - 200°C (48hrs)	Complete transformation to a monophasic HA with trace Mg <sup>2+</sup> Na <sup>+</sup> ions	2000	(Felicio-Fernandes and Laranjeira, 2000)
Sea urchins	<i>Paracentrotus lividus</i> ; <i>Heterocentrotus mammillatus</i>	Unspecified	Precipitation- 20°C (2 Mths)	HA	2009	(Marc hegiani et al., 2009)
	<i>Heterocentrotus mammillatus</i> <i>Heterocentrotus trigonarius</i>	Unspecified	HT Hydrothermal Synthesis with superheated steam (CS) - 180°C	Complete transformation of spines to βTCMP	2007	(Veccchio et al., 2007 b)
	Unspecified	Unspecified	Replication using polymer casting	CaCO <sub>3</sub>	2006	(Guillemin et al., 1987)
Snails	<i>Pomacea lineata</i>	Unspecified	Precipitation- Room Temperature – 7, 14, 30 days	Aragonite with HA coating	2010	(dePaola et al., 2010)
Sponges	<i>Spongia agaricina</i>	Caribbean	Replication using HA slurry and sintering at 1300°C (5hrs)	HA	2010	(Cunningham et al., 2010)

Table 3. Examples of mineralizing marine organisms. Those that have already been investigated for bone tissue engineering are highlighted in bold.

Phylum	Class	Common Name	Skeletal Mineral
Heterokontophyta	Bacillariophyta	Diatoms	Silica
Haptophyta	Prymnesiophyceae	Coccolithophorids	CaCO <sub>3</sub>
Radiolaria	Polycystina	Radiolarians	silica
Foraminifera	Granuloreticulosa	Foraminiferans	CaCO <sub>3</sub>
Rhodophyta	Florideophyceae	<b>Red algae</b>	CaCO <sub>3</sub> /calcite
Ectoprocta	Stenolaemata	Bryozoans	CaCO <sub>3</sub>
Porifera	Demospongia	<b>Sponges</b>	CaCO <sub>3</sub> and/or silica
Cnidarians	Anthozoa	<b>Corals</b>	CaCO <sub>3</sub> /aragonite
Mollusca	Gastropoda	<b>Snails</b> , limpets	CaCO <sub>3</sub> /aragonite
	Bivalvia	<b>Clams, mussels</b>	CaCO <sub>3</sub> /aragonite
	Cephalopods	Squid, <b>cuttlefish</b>	CaCO <sub>3</sub> /aragonite
Arthropoda	Crustacea	<b>Lobster, crab</b> , shrimp	CaCO <sub>3</sub> /aragonite
Brachiopoda	e.g. Lingulata	Lampshells	CaCO <sub>3</sub> or CaPO <sub>4</sub>
Echinodermata	Asteroidea	<b>Starfish</b>	CaCO <sub>3</sub> /calcite
	Echinoidea	<b>Sea urchins</b>	CaCO <sub>3</sub> /calcite
	Ophiuroidea	Brittle stars	CaCO <sub>3</sub> /calcite
	Holothuroidea	Sea Cucumbers	CaCO <sub>3</sub> /calcite