

Marine organisms as a source of natural matrix for bone tissue engineering

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Abstract

It is a huge challenge to develop an effective regenerative approach which can be employed to restore diseased or defective part of the bone to its original anatomical and physiological function. Exploration of rich biodiversity in marine ecosystem is one of the most exciting prospects for development of biomaterial for bone tissue engineering. Due to their organic nature, the materials which are fabricated from natural environment are usually safe, biocompatible, biodegradable and osteoconductive which stimulates osteogenesis. Furthermore, marine derived biomaterials are superior to the current available materials which are employed for either *in vitro* or *in vivo* grafts or scaffolds as there are some limitations with the present strategies. Regardless of their immense potential, the natural marine resources are enormously unexploited. This review recounts various biomaterials which can be extracted from marine organism for future medical application.

Introduction

In simple term, biotechnology may be referred as exploitation or use of living organism, biological process or system towards developing commercial products or services. The field of biotechnology is not new to human beings as it has been known since a long period [1]. Cohen and co-workers had first patented technology for developing recombinant DNA by cloning gene and bacterial plasmid and, which marks dramatic changes in the growth of commercial biotechnology [2]. Marine biotechnology deals different scientific and engineering principles and their application for processing biomaterials from marine species like plants and animals [3].

Marine biotechnology, utilizes the rich biodiversity found in the oceans for use in biotechnology. This technology domain has now been known to have tremendous potential by both the perspectives of both molecular biologists and biotechnology industry. Ocean comprises about 70% of the earth's surface and 90–95% of the biosphere by volume of living organisms and thus provides a tremendous range of biological diversity for resources.

Last decade, introduction of new methodologies in biotechnology and better understanding of complex biological molecules help in producing extraordinary new products. There are ample opportunities to explore better marine derivatives by applying these tools [4]. Dramatic advancement in understanding the fundamental features of life in marine ecosystem has provided exciting opportunities to make marine biomaterials. Achievement in marine biotechnology is a fundamental part of the economy in the USA, Japan, China, Korea, and Russia as well as in European Community [5, 6]. Deep ocean exploration for biological and commercial purpose is still insufficient. Nevertheless, experts believe that deep sea holds most diverse ecosystems on earth which have tremendous human benefit potential. There are various uses of marine-derived material, perhaps the most exciting prospect is in the medical domain. More than 15,000 biological products which are still rapidly growing, have been discovered from marine creatures. Among them, 28 natural marine products/ derivatives are being used in clinical applications with many more in its preclinical stage already [7].

Bone is a complex tissue architecture and there are several ways by which the bone may become defective which include vehicle accidents, trauma, tumour, osteoarthritis, osteoporosis, even congenital. Different techniques have been employed to regenerate such defects, like bone grafting using xenograft, autograft and allograft. However, all these techniques have their own merits and demerits, for example, insufficient donor sites and disease transmission [8]. Bone tissues have their natural ability to heal itself [9], but in some critical situations, e.g., inadequate or disrupted vascularization leading to large bone defects, body response to bone injury is insufficient or is

incapable to restore by structure and/or by function. Currently, medical research has aimed to replace the injured tissue with prosthetics or tissue grafts. Some disadvantages include high price, harmful immunological responses and insufficient donor tissues [10-12].

So, there are some challenges in tissue engineering (TE) field, especially to develop suitable models and appropriate technologies for recuperation or replacement of defective human tissues or organs [13]. Popular methods include new tissue development by cell-based therapies (by manipulating the cell biochemistry) [14, 15] and using 3D scaffolds (mainly nonliving origin) for attachment and proliferation of required cells [16].

3D scaffolds are found to be most promising for tissue regeneration (both in terms to its original structure and function) as they provide the required structural support *in vitro* and *in vivo*. [14, 15]. But, factors such as appropriate pore size/ shape/ distribution are crucial for growth and cell migration which lead to better biological performance [17]. Another crucial characteristic of scaffold to be an appropriate implant is its biodegradability (replacement by new tissue), low-to-no immunogenicity and non-cytotoxicity [17]. Various materials (like polymers, ceramics, metals, composites) have been employed to fabricate scaffolds [18, 19]. In spite of their advantages, there are also various limitations involved, especially the high costs of production [19, 20]. To overcome these pitfall, natural materials emerges to be a good substitute for TE applications [21, 22]. Since they are organic in nature, natural materials are often more biocompatible due to better cell surface interaction leading to better cell attachment and growth [23].

In this reference, marine ecology and their rich biodiversity contribute abundant resource for new products and services. Interestingly, in spite of their promising structural and chemical properties, marine organisms still remain largely unexploited for applications related to biotechnology. This hierarchical structural material could be a great source of many novel medical products [21, 22], which eventually may solve bone defect related problems by developing synthetic and natural materials and as a result, considerable attention has been already been paid by researchers and orthopedic doctors at laboratory and clinical level respectively.

1. Natural Vs synthetic materials

The development of ideal biomaterials is of paramount necessity owing to high demand in clinical context to substitute lost or broken body parts and to accomplish uncomplicated healing.

It is vital that biomaterials can positively intermingle with neighboring tissue not only to fill the defect, but also aid the natural regeneration of tissue. To comply with the same, tremendous efforts have been put to develop such biomaterial scaffolds for bone tissue engineering [24]. A variety of

synthetic materials have been developed in the form of scaffolds that mimic extracellular matrix (ECM) properties for regeneration and restoration. The advantages of synthetic materials are based on physico-chemical properties like porosity, surface character, and nature of degradation products for intended application [25]. In contrast, natural materials possess biocompatibility, biodegradability, and intrinsic biological functions suitable for range of tissue engineering applications [26-29]. Natural biological materials show superior biointeractive surface at cell level and thus are more biocompatible than synthetic [30]. Presence of extracellular matrix (ECM) and superb optimized structures, these natural materials are highly acceptable for superior cell adhesion, migration and proliferation [31]. Marine derived products (collagen) are proved to be less immunogenic and show no adversity within human body [32, 33]. Further, molecules responsible for regulation of bone regeneration especially during mineral deposition in fracture site exist in the earliest marine organisms. Two important molecules necessary for bone regeneration like bone morphogenic protein (BMP) and signaling protein, TGF- β are present in the developing skeleton [34] and early marine sponge respectively.

2. Commonly available marine materials for potential use in engineering of biomaterials

2.1. Marine Biopolymers

Marine source materials are consist of abundant natural polymers and complicated polysaccharides too (for example, alginate, chitosan, collagen and fucoidan) and play a vital role in biomedical applications. Example of different marine-derived polymers for bone tissue engineering including their fabrication techniques have been presented in **Table 1**.

2.1.1. *Chitin/ Chitosan*

Chitin is naturally occurring mucopolysaccharides composed of poly (β -(1 \rightarrow 4)-N-acetyl-D-glucosamine). It is found naturally in arthropods' exoskeleton or in fungi and yeast cell walls and crab/ shrimp shells [35]. This natural polysaccharide is also found in diatoms, nematodes, lobsters, krill, and squid [36]. Chitosan is primarily obtained from crustacean shells of marine source [37, 38] after degradation by chitinase enzyme of chitin linkages [39].

Structurally, it is a linear composed of (1 \rightarrow 4)-2-acetamido-2-deoxy- β -D-glucan (N-acetyl D-glucosamine) and (1 \rightarrow 4)-2-amino-2-deoxy- β -D-glucan (D-glucosamine) semicrystalline polysaccharide units and is a deacetylated derivative of chitin [35]. Chitosan and its derivatives are very promising candidates to develop composite scaffold, because it is expected to have a good biodegradable property, with no inflammatory reactions or toxicity [40]. It can also help to promote expressions of osteogenic progenitor cells and hence boost osteogenesis [41]. Commercially it is

produced by deacetylation of chitin. Traditional and well-developed chemical, enzymatic and fermentative methods have been used for isolation of chitin and chitosan from shellfish waste owing their simple process and convenience for large scale commercial production [42].

2.1.2. Alginate

Alginates are broad family of polysaccharides distributed widely in cell of brown seaweeds which are produced industrially. It is widely abundant polymer and the annual production is estimated to approximately 38,000t worldwide [43]. Alginate is manufactured from *Laminaria hyperborea*, *Laminaria digitata*, *Laminaria japonica*, *Ascophyllum nodosum*, and *Macrocystis prrifere* [all brown algae (*Phaeophyceae*)] using alkali solutions (NaOH) treatment. The extract, after filtration using calcium chloride forms gelatinous precipitates. Further processing with diluted hydrochloric acid can transformed alginate salt into alginic acid and ultimately, produces water-soluble sodium alginate with further purification [44]. *Azotobacter* and *Pseudomonas* [45] are the sources for bacterial alginate.

Structurally, alginate is covalently linked, homopolymeric blocks in sequences or in blocks of alternating both β -(1-4)-linked D-mannuronic acid (M) and α -L-guluronic acid (G) residues. Essentially, it is anionic and comprised of such linear unbranched copolymer. [44, 46]. Physical properties and its resultant hydrogels depend on M/G ratio (composition), sequence, length of G-block and molecular weight of alginate [47]. Due to its biodegradability, chelating ability, biocompatibility, non-antigenicity, encapsulation efficiency and ability to mould in different forms (e.g., fibers, sponges, foams, microspheres and gels), alginate is also a promising natural material for bone tissue engineering [44, 46]. Alginate by hydrogen bonding at low pH forms hydrogel. Both chemically unmodified and modified alginate are promising biomaterials for applications like tissue engineering, drug delivery and cell immobilization. Alginate hydrogels helps to improve neo-cartilage as well as bone regeneration [48, 49].

2.1.3. Collagen

In general term, collagen is a main structural protein of the extracellular matrix, organized in a fibrillar arrangement in animal bodies [50, 51]. Main structural and biological part of connective tissues like skin, muscle, bone and cartilage is collagen and is most common protein in mammals and vertebrates. It is significantly used as nutraceuticals, functional food, pharmaceuticals and cosmeceuticals, originated from fish or other sources (invertebrate marine animals like sponges or jellyfish). It is also extensively used for bone-related diseases [52].

From a staggering 75% (by weight) quantity of fish waste (in the form of bones, fins, skins, scales, heads and guts) residues, collagen can be obtained which is also increasing the economic value [53]. Among different types of collagen, the most common is Type I collagen, typically obtained from skin, tendon, bone and muscle (epimysium). Source of Type II collagen is also fish. Again, collagen solubility depends on age of the animals as older animals with more number of crosslinkers are tougher and harder to dissolve than younger animals' source [54]. Based on the sources, different methods are being adopted to get collagen-macromolecules. But bovine source collagen has some drawbacks and carries high risk of transmissible spongiform encephalopathy and that is why marine sources are considered to be a better alternative. This include marine sponge like *Chondrosia reniformis Nardo* [55], rhizostomous jellyfish [56], fish waste like skin, bone, fins [57], jellyfish [58], paper nautilus [59], muscles and skins of marine animals [60], cuttlefish [61], squid skins and *Sebastes mentella* [62].

2.1.4. Fucoidan

Another marine source material found from various species of brown algae and brown seaweed (e.g., mozuku, kombu, limu moui, bladderwrack, wakame, hijiki and sea cucumber) is fucoidan. Edible and dietary products/ species (e.g., *Fucus vesiculosus*, *Cladosiphon okamuranus*, *Laminaria japonica*, and *Undaria pinnatifida*) are other common sources of fucoidan. It is a fucose containing sulfated polysaccharide (may be acetylated and with uronic acids) having primary skeleton of α -1,3-linked sulphated l-fucose and possible repeating sequence of alternating α (1 \rightarrow 3) and α (1 \rightarrow 4) glycosidic bonds [63].

Fucoidans are polymer with sugar backbone containing high proportion of fucose or fuco-oligosaccharides. In general, the first crude extract of fucoidan yields a fraction of 2-10% by weight. Highly branched fucoidans are derived from seaweed. However, another source which have a linear fashion fucoidans are also extracted from echinoderms, particularly, sea cucumbers [64]. It is reported that fucoidan plays an important role in preventing acute and chronic inflammation inhibiting enzyme and complementing system [63]. Fucoidan from dietary supplement is under further development for various biomedical applications [65, 66]. Extensive researches have been done and the published articles related to fucoidans have also been increased threefold between 2000 and 2010 [63].

Table 1: Example of different marine-derived polymers for bone tissue engineering

Material	Source/common name	Genera/species	Fabrication technique
Chitin and Chitosan	Crab	<i>Cancer megister</i> <i>Collinectes sapidus</i>	Chemical hydrolysis method, enzymatic method and fermentation method
	Shrimp	<i>Chinoecetes opilio</i> <i>Pandalus borealis</i>	
	Krill	<i>Crangon crangon</i> <i>Panaeus monodon</i> <i>Euphausia superba</i>	
	Crawfish	<i>Maganyctiphanes norvegicus,</i>	
	lobster	<i>Nyctiphanes australis</i>	
	Crayfish	<i>Procamborus clarkii</i>	
	Plankton	<i>Panulirus homarus</i>	
Alginate	Bryozoans	<i>Astacus astacus</i>	Freeze-drying method, aggregation method and acid precipitation method
	Seaweed (brown algae)	<i>Daphnia</i> <i>Plumatella</i> <i>Laminaria hyperborean</i> <i>Laminaria digitata</i>	
	Bacteria	<i>Laminaria japonica</i> <i>Ascophyllum nodosum</i>	
Collagen	Fish (skin, bone, and fins)	<i>Macrocystis prrifere</i> <i>Azotobacter and</i> <i>Pseudomonas</i> <i>Okamejei kenojei</i>	Chemical hydrolysis method, enzymatic hydrolysis method
	Sponge	<i>Heterodontus japonicas</i> <i>Merluccius merluccius</i>	
Fucoidan	Jellyfish	<i>Oncorhynchus mykiss</i> <i>Priacanthus macracanthus</i>	Precipitation method
	Squid	<i>Chondrosia reniformis</i> <i>Suberites domuncula</i>	
	Seaweed (brown algae)	<i>Rhizostoma pulmo</i> <i>Rhopilema asamushi</i> <i>Nemopilema nomurai</i> <i>Loliolus japonicus</i> <i>Todarode spacificus</i> <i>Hizikia fusiformis</i> <i>Cladosiphon okamuranus</i> <i>Laminaria japonica</i> <i>Undaria pinnatifida</i> <i>Fucus vesiculosus</i>	

2.2. Marine Bioceramics

Marine corals, fish bone, sponges are also a great source of various bioceramic materials having diversified biomedical applications [67] (**Table 2**).

2.2.1. Hydroxyapatite

Hydroxyapatite [HAp; approximate formula $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$], is a naturally occurring mineral, belong to apatite family and has several biological sources to produce them, e.g., fish bone. In fact, emphasis has been given to produce HAp from fish bone owing to concerns on reducing environmental pollution through elimination of waste and results in value-added production [68]. There are several methods available to fabricate HAp from different marine sources which include calcination, hydrolysis (alkaline) and method based on polymers [69-71]. Simplest of them is thermal treatment. Calcination and subsequent alkaline hydrolysis are carried out to isolate HAp from *Thunnus obesus* bone [72]. HAp ceramics was also extracted from other biological sources like cuttlefish bone [70], bovine bone [73], and fish bone [74] which provide inexpensive raw materials from bone and teeth.

HAp is the most important bioceramic material for bone tissue engineering due to its unique bioactivity and stability. Naturally occurring HAp is stable at physiological pH and exhibits bone bonding property, forming strong chemical bonds with surrounding bone which impart a crucial characteristic for rapid bone regeneration after major bone defect. HAp acts as an excellent implant material extensively has been used in biomedical field for implant fabrication of dense and porous biomaterial due to its superior biological properties [75]. HAp, in the form of nanocrystals, has been a promising candidate especially in case of various applications related to bone [76-78]. Due to its similarity of chemical composition as that of bone material, HAp is significantly biocompatible [79].

While synthetic materials have been popular for medical application, natural materials are now identified as a promising candidate for novel biomedical applications [80]. For example, Ozawa and Suzuki prepared HAp from Japanese sea bream by calcining at 1300°C [71]. Some researchers produce HAp from fish scale [81]. Other researchers have used cuttlefish [82] and coral have also been used widely for the production of HAp [83]. Calcium carbonate is the main component of coral, normally in the form of calcite and suitable phosphate precursors are used for converting coral into HAp [84] (**Figure 1**). Several HAp based products are also available commercially like ProOsten and Interpore200 with several biomedical applications [85].

2.2.2. Biosilica

Biogenic silica or Opal or biosilica, comprising of amorphous silica, formed one of the most widespread mineral in many aquatic and terrestrial organisms (like sponges, diatoms, radiolarians and choanoflagellates) [86]. Some sponge species are of prime concern which is a great source of biosilica. There are two main classes of sponges with silica skeleton, viz., demospongiae and hexactinellida. Another class includes calcarea having calcium carbonate skeleton. Biosilica

formation in sponges are enzyme controlled. The axial filament consists primarily of the silicatein, which is a major biosilica forming enzyme and mediate the silicification process around it [87].

Sponges are the only organisms that have the ability to polymerize silica enzymatically and can generate siliceous spicules which are a structural element [88]. Interestingly, siliceous spicules are dissolved by silicasein sponge tissue (it is an enzyme related to carbonic anhydrase) [89, 90]. The biosilica spicules are a part of organic matrix component [91] and it has been manifested that they are nontoxic for mammalian cells and tissues, indicating that they are biocompatible [92]. Remarkably, sponge biosilica is a promising candidate for bio-applications, especially for bone healing and their regeneration strategies in TE [91], because silica ions plays important function to stimulate osteogenesis [93-95]. Bioactive glasses (silica) can form a strong bond; integrate to bone tissue by forming a silica gel layer; triggering osteoprogenitor cells to proliferate and differentiating osteoblasts, resulting formation of organic bone matrix and apatite mineralization subsequently [19, 20].

Table 2: Example of different marine-derived ceramics for bone tissue engineering

Material	Source/ common name	Genera/species	Fabrication technique
Hydroxyapatite	Sponge	<i>Petrosia ficiformis</i> <i>Agelas oroides</i> <i>Chondrosia reniformis</i> <i>Spongia agaricina</i> <i>Spongia zimocca</i> <i>Spongia officinalis</i> Linnaeus	Thermal calcination method, hydrothermal, liquid membrane, precipitation, radio frequency thermal plasma, ultrasonic precipitation, reverse microemulsion, sol-gel, alkaline hydrolysis and polymer-assisted methods
	Corals	<i>Goniopora</i> <i>Porites</i>	
	Snail shells	<i>Cerithium vulgatum</i>	
	Cuttlefish	<i>Sepia officinalis</i> <i>Sepia esculenta</i>	
Biosilica	Sponge	<i>Monorhaphis chuni</i> <i>Tethya aurantium</i> <i>Suberites domuncula</i> <i>Petrosia ficiformis</i>	Thermal calcination method

3. Marine materials for bone tissue engineering

3.1. Marine Biopolymers

3.1.1. Chitin and Chitosan

It's obvious that bone tissue engineering requires a new authenticated biodegradable material with immense potential. In this regard, natural polymer composite materials like chitosan are important biomaterial candidate due to its excellent biodegradability and biocompatibility [96]. Biocomposites based on biodegradable polymers and bioactive ceramics are very appropriate for bone tissue engineering as these new generation biomaterials show bioactive and bioresorbable property alongwith bone tissue regeneration *in vivo* [97]. One of the most promising and challenging biomedical applications of chitin and chitosan are for bone tissue engineering and drug delivery [35, 98] (**Figure 2**).

Chitosan can be reconstructed to several structures like film, fibers, beads and scaffolds [99]. The method used to fabricate biomaterial have high impact on the properties, and several methods (for example, supercritical fluid technology) have been used to construct highly porous bioactive material [100] and the freeze drying method [101]. However, mechanical strength of chitosan is poor. So, it is sometime blended with other polymers and ceramics to enhance mechanical strength. Bioceramics like hydroxyapatite are also often combined with chitosan to make a scaffold for same with better mechanical strength [72]. In bone TE, ability of chitosan to promote bone cell growth and deposition of mineral-rich matrix by osteoblasts cells have been deployed [102]. Nanocomposite scaffolds combining chitosan (CS) and hydroxyapatite (HAp) powder were prepared and were subsequently cross linked [103]. It was observed that the product with a higher ratio of HAp, cross-linking agent, and photoinitiator solution were found to be the most promising materials for bone regeneration. Chitosan can be combined with different class of materials such as alginate, HAp, CP, poly(methylmethacrylate), PLLA, and growth factors for potential application in orthopedics [104].

In 2011, properties of composite scaffolds were studied by combining chitosan (CS) and CPs, obtained by a biomimetic method [105]. The biomaterial was found to be a valuable source for potential application in bone regeneration. A simple technique was employed to fabricate arginine–glycine–aspartic acid (RGD)-conjugated and cross linked chitosan scaffolds and observed that these scaffolds expressed much higher (almost double) calcium deposition by osteoblastic cells than control, thereby greatly enhancing the mechanical property of scaffolds and found to be very suitable for bone tissue engineering applications [106]. Composites of chitosan–alginate gel/mesenchymal stem cells (MSCs)/bone morphogenetic protein-2 (BMP-2) stimulated osteogenesis after this was injected into mouse [107]. Some researchers encapsulated BMP-2 and BMP-7 in poly(lactic acid-co-

GA) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) nanocapsules and incorporated them into fibrous chitosan scaffolds. They showed that growth factor delivery in sequence is a better approach together with mimicking natural process of healing towards their use for tissue engineering [108]. Platelet-derived growth factors integrated in chitosan sponge promote neo-bone formation in rat calvarial defect [109, 110]. Chitin–HAp composite loaded with MSC-induced osteoblasts, promote bone regeneration when inserted into rabbit femur bone defect site [111]. Chitosan in combination with nano HAp 3D biodegradable scaffolds were characterized by much better mechanical, physicochemical, and biological properties compared to chitosan alone. It was reported that the composite is suitable as a scaffold biomaterial for bone regeneration [112].

Chitosan powder can be used in tissue engineering as it significantly enhanced the bone regeneration process in micro drilled bone defects in rat tibias, which can be further investigated as a biomaterial in human bone healing for its biocompatibility and osteoinductivity [113]. Chitosan particles in human BMSC culture media stimulate new bone formation by osteoblastic differentiation and activating angiogenic factors release *in vitro*. On the other hand, chitosan particles alone could not serve the purpose and could not promoted sufficient osteoblast differentiation of BMSCs *in vitro*, thus suggesting some indirect mechanism of chitosan promoting osteogenesis *in vivo* [114]. Another promising tissue-engineered bone substitute is HAp together with chitin. This was noncytotoxic and biodegradable *in vivo* (both in cell cultures and after implantation in intra-muscle of rat model) [111]. The porous chitosan–HAp materials synthesized in an aqueous medium method was found to have good osteoconductivity *in vivo* experiments and proved to be promising materials for healing when used in tibial bone defects in rat [115].

Lyophilized nanocomposite scaffold made of β -chitin hydrogel and bioactive glass nanoparticles (nBGC) had improved porosity, swelling properties, bioactivity and biodegradability compared to control. The scaffolds were found to be noncytotoxic to human osteosarcoma cells (MG63) and primary osteoblasts as well with enhanced attachment and proliferation [116]. Carbonate apatite–chitosan scaffolds (CA–ChSs) were developed into 3D interconnected porous structure by using the lyophilization technique. The scaffolds had no brittleness; can support osteoblast proliferation and differentiation and found to be a good scaffold material for bone tissue engineering [117].

The role of hydroxyapatite/chitosan (nHAp/ CTS) implanted with bone marrow mesenchymal stem cells (BMSCs) was studied for potential application in bone TE both *in vitro* and *in vivo* [118]. Bone regeneration of the scaffolds was promoted by adhesion, proliferation, and activating integrin-BMP/Smad signaling pathway of BMSCs (*in vitro* and *in vivo*). Finally, they concluded that the nHAp/CTS/BMSCs were superior to nCTS/ BMSCs to promote bone regeneration and shows great

potential for bone tissue engineering applications. Chitosan– nano HAp composite scaffold was also successfully developed by a supercritical CO₂-assisted process. The prepared scaffold was investigated using ATR-FTIR, XRD, SEM and EDX analyses. The composite scaffolds shows increase in water uptake, good mechanical strength and no cytotoxicity was observed toward fibroblast cells *in vitro*. Thus, the prepared biomaterial was considered as an optimistic candidate for bone tissue engineering applications [119]. Highly porous nano HAp/ chitosan composite scaffold was fabricated in situ through wet chemical method and characterized in detail. Physico-chemical properties were analyzed by SEM (including porosity), thermo-gravimetric analysis (TGA), XRD, X-ray photoelectron spectroscopy and FTIR. While, biocompatibility was examined through proliferation and morphology of MC 3T3-E1 cells seeded on scaffolds. The scaffolds showed superior biocompatibility than pure chitosan with great potential for bone tissue engineering [120].

3.1.2. Alginate

A major challenge for orthopedic surgeons is the reconstruction of lost bone. Alginate is one of the best-known materials as scaffolding to treat various organ defects. Alginate gels have several application for bone and cartilage regeneration for its properties like, it can be introduced into body with minimal invasion, ability to fill defects of irregular shape and size, easy modification with ligands (e.g., RGD) and controlled release of growth factors (e.g., BMP, TGF- α) [121]. Stem cell transplantation by alginate hydrogels has been studied in bone TE [122]. However, its lack of mechanical strength invite them to be used in combination with hydroxyapatite to enhance the strength thereby to mimic natural function of bone [123].

Main criteria of scaffolds to be used for bone TE include sufficient mechanical strength to support at site of implantation and mechanical integrity for cell growth *in vitro* and *in vivo* [124-126]. To repair defective tissue part, sometime BMP-2 and mesenchymal stem cells (MSC) are often delivered through alginate scaffolds [44, 46]. Chitosan–alginate gel/ MSCs/ BMP-2 composites was investigated for new bone regeneration and it is found to have interesting potential for clinical use as a successful injectable material to generate new bone [107]. Calcium-based composite with Alg-N-succinyl-chitosan is also used for bone tissue regeneration [127].

Sodium alginate/gelatine scaffold is an efficient candidate for bone regeneration [128]. As chitosan/polypyrrole/alginate composite can act as scaffold substrate, they can also be employed for bone TE by electrical stimuli using bioreactor system with a promising substrate for bone healing [129]. Chitosan/alginate hybrid scaffolds improved the structural stability and mechanical strength when implanted in the bone and were shown to stimulate osteogenesis and accelerate vascularization

[130]. Stem cell-scaffold approaches are also in use in which injectable and mechanically robust stem cell (human umbilical cord mesenchymal stem cells or hUCMSCs) were prepared using calcium phosphate cement (CPC) paste with small alginate hydrogel microbeads. The constructs can remain viable and has huge potential for bone TE [131]. CS/Alg/ nSiO₂ scaffolds developed by freeze drying method resulted in controlled biodegradation and enhance apatite formation, which in fact promote bone formation and can be used in bone TE [132]. Fundamental biological events such as vascularization, oxygenation, cell migration, adhesion and proliferation require porous architecture and alginate scaffolds fabricated by aggregation method can serve the purpose [133].

Oxidized alginate microbeads encapsulating periodontal ligaments (PDLSCs) and gingival mesenchymal stem cells (GMSCs) are used to prepare an injectable and biodegradable scaffold [134]. It is demonstrated that the scaffold represent an effective biomaterial for bone TE. A porous scaffold made up of CPC blended with alginate is used to form three-dimensional (3D) matrix, which is used as a novel material for drug delivery and bone TE [135]. MBG (mesoporous bioactive glass) modified to 3D-structure incorporated with alginate scaffolds significantly improved physicochemical, biological, and a drug-delivery property [136]. Composite scaffolds comprising alginate, chitosan, collagen and HAp with an improved porosity were fabricated without chemical cross-linking agent, which can all be used as an interesting scaffold for regenerating bone tissue [137]. Scaffolds based on Alg/O-CMC/CQ-E could be fabricated by lyophilization of combination of medicinal plant *Cissus quadrangularis* extract (CQ-E) with natural biopolymers alginate (Alg) and O carboxymethyl chitosan (O-CMC). This novel “herbal scaffold” is osteoinductive and could serve as an interesting candidate for bone TE therapeutics [138].

Hybrid growth factor delivery system was developed, for example, using injectable alginate hydrogel for delivery of protein and electrospun nanofiber mesh for promoting neo bone regeneration in different bone defects [121],. Skeletal muscle satellite cells induced by PRP (Platelet-rich plasma) encapsulated in alginate gel could be used for bone TE *in vivo* [139]. It is reported that osteoblast differentiation was better in case of unmodified alginate hydrogels than HAp hydrogels. This indicated that alginates are one of the most interesting materials for bone TE applications [140].

RGD-grafted oxidized sodium alginate/N-succinyl chitosan (RGD–OSA/NSC) hydrogel scaffolds were used as implant where low-intensity pulsed ultrasound (LIPUS) was also employed for mechanical stimulation to form better quality new bone and rapid vascularization. Thus the total system might provide very promising approach in bone TE [91]. Modified alginates with RGD or PHSRN (proline– histidine–serine–arginine–asparagine) mixed at different ratios (and different sequences) to form gels closely mimics the chemistry of natural cell adhesive proteins (e.g.,

fibronectin). This can be promising scaffolds for bone TE application [141]. Human embryonic stem cells derived MSCs (hESC-dMSCs) encapsulating in alginate hydrogel microbeads in macroporous CPC construct is having a wide range of orthopedics applications [142].

3.1.3. Collagen

Collagen has its application in various fields. Collagen from marine origin can be used for different strategies depending on desired application and formulation. Collagens from marine fish are having diversified biomedical applications [143]. A scaffold was fabricated combining salmon skin collagen with HAp, based on biomimetic mineralization principle [144]. Beside its mechanical elastic properties, it exhibits several desired characteristic for bone regeneration like good absorption and great pores interconnectivity. These properties allowed adhesion and proliferation of human Mesenchymal Stem Cells (hMSCs) having good base for osteogenic differentiation. Salmon collagen after cross-linking with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was tested for bone TE [145] and for periodontal regeneration applications after dehydrothermal treatment (DHT).

Important sources of collagen include marine sponges and vertebrate (**Figure 3**). Collagen from *C. reniformis* was used to produce hydrogels for bone regeneration with silica as template [146]. It also support hMSCs to adhere, grow and differentiate osteoblast cells. Likewise many researchers worked on collagen (derived from marine sponge) and shown this as good bone tissue engineering scaffolds [23]. Composites based on chitosan, hydroxyapatite and collagen (from *Ircinia fusca*), prepared by freeze-drying shown adherence and proliferation of MG-63 osteoblast-like cells. The results proposed and supported that these composites to be efficient biomaterial for bone tissue repair [33].

Collagen obtained from jellyfish is also an interesting alternative material and typically used as matrix for tissue engineering with low amount of impurities. It is noted that more than 40% of edible jellyfish is collagen on dry weight basis, but in case of an animal 95% of it is water [147]. Porous scaffolds were prepared by freeze-drying collagen (from jellyfish *Stomolophus nomurai meleagris*) with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride/*N*-hydroxysuccinimide (EDC/NHS) cross-linkers. It is reported that the scaffold can be used efficiently in TE applications [58]. Such collagen was also prepared at different ratios cross-linked with EDC-NHS [148]. They are then used as a substitute of bovine or human collagens. Recently, refibrillized collagen (from jellyfish *Rhopilema esculentum*) was used to fabricate porous scaffolds. The biomaterial was reported to have good elasticity which could support hMSCs, chondrogenic markers' expression under chondrogenic stimulation, indicating great use in cartilage tissue engineering along with high

porosity [149]. Other jellyfish species [e.g., *Cyanea nozakii* [150], from large blooms in Chinese seas [151] have also been studied for collagen having future biomedical applications.

Collagen from marine gastropods (e.g., from different body parts of *Ficus variegata*) has also been investigated to develop porous biomaterial after crosslinking with chitosan, hydroxyapatite and glutaraldehyde [152]. Collagen from marine origin is believed to possess low antigenicity [153], but, *in vivo* studies are essential to conclude about compatibility and viability of using them as human implants particularly in tissue engineering and regeneration applications [154, 155].

3.1.4. Fucoidan

Fucoidan helps in bone mineralization by deposition of minerals in the bone matrix by increase in alkaline phosphatase (ALP) enzyme, type-1 collagen expression, osteocalcin and BMP-2 [156]. Out of several biological application of fucoidan, recent studies indicate that fucoidan have a potential in treating osteoarthritis. Studies showed that *Undaria pinnatifida* fucoidan successfully inhibited pain in an animal model of collagen-induced arthritis when administered orally [157]. Osteoarthritis symptoms were reduced by 52% following such oral administration of extracts of fucoidan-rich seaweed [158]. Commercial bone substitute (Lubboc[®]) with fucoidan having low molecular weight (LMWF) was developed to improve bone regeneration [159]. They reported that LMWF helps in supporting human osteoblastic behaviors. Authors concluded that LMWF pretreatment promoted human osteoblast proliferation, expression of Type I collagen which favored ALP activity with enhancement mineralized bone tissue.

In addition, fucoidan has remarkable potential as bone tissue regeneration material as it has better ALP activity and type-1 collagen expression, Runt-related transcription factor 2 (Runx-2), osteopontin and osteocalcin in human adipose-derived stem cells as this has also supplemented osteogenic cell differentiation in human amniotic fluid stem cells [160]. Biocomposite containing polycaprolactone-fucoidan was studied for bone regeneration with excellent cellular proliferation and bone mineralization. It was also observed that such biocomposites with fucoidan can provide better biological stimulator for regenerating bone tissue [161, 162]. The presence of fucoidan in composite scaffold was observed to enhance mineral deposition by 30% [159]. Freeze-drying method was employed for fabricating chitosan–alginate and chitosan–alginate with fucoidan polymer scaffolds. Each of the scaffolds was characterized as bone grafts which were found to have potential for bone TE [163].

3.2. Marine Bioceramics

3.2.1. Hydroxyapatite

In 1926, De Jong first published that HAp is a component of bone [164]. Since 1960's, hydroxyapatite or hydroxylapatite is considered as a naturally occurring biomaterial. Microcrystalline HAp is marketed as bone supplement due to better absorption property and used in various clinical applications. HAp can be fabricated from different marine organisms like coral, fish bone and shell [165, 166]. Commercial bone substitutes are fabricated by hydrothermal hot pressing method from coral sources which involve solidification of synthetic HAp powder [165, 167]. A biomimetic approach was employed by Roy and Linnehan directly converting coral calcium carbonate skeletons into HAp [168]. Commercially available coral-based products (Pro-Osteon™ 200R, Pro-Osteon™ 500R) having HAp as outer layer (2–5 μm thick) and inner aragonite core were also developed [169]. Natural trabecular structure of coral could be retained after hydrothermal conversion of calcium carbonate and was found to be identical with natural bone. The benefit of coralline HAp for bone TE is that they are predominantly safe, biocompatible, and osteoconductive. So, they can be effectively used as bone graft substitute and bone void filler for bone defect regeneration [170].

HAp bioceramics are successfully used in biomedical science like orthopaedics and orthodontics, as a filling material to partially or totally replace parts of bone tissue defect. It creates physicochemical interaction, between bioceramic and bone tissue and promotes formation of new tissues [171]. Several species of marine organisms could be converted into calcium phosphates (CaP) and HAp grafts by different manufacturing process which include hydrothermal synthesis using superheated steam and/ or vapor pressure, microwave etc. [172, 173]. Hydrothermal synthesis vis-à-vis sol-gel is another method which can improve the mechanical properties of bioceramics. Three different species of marine sponges were used as templates to produce HAp structure, viz., *Spongia officinalis* Linnaeus (from Adriatic Sea), *Spongiazimocca* (from Mediterranean Sea) and *Spongia agaricina* (from Caribbean Sea). High solid loaded HAp slurry (~ 80 % wt.) yet low viscosity (126 mPas) was developed for the same purpose. Characterization was done by combined μ-CT, MIP (Mercury intrusion porosimetry) and SEM to determine its application on bone tissue engineering. HAp scaffolds developed from *Spongia agaricina* with 56–61% open porosity (83% of the pores ranging between 100-500 μm and an interconnectivity of 99.92%) were successfully developed using slip casting method [174].

HAp was obtained from a bone of fresh cuttlefishes (*Sepia officinalis*) and was studied for some of their reported desirable biological features like bone tissue regeneration and vascularization. The *in*

in vitro biocompatibility and bioactivity results were interestingly good for suitable clinical bone implants. Its desirable properties like low cost, availability, demands and easy to design in different shape makes the scaffold more attractive for bone tissue-engineering applications [175]. Similarly in another study, Kannan et al. prepared HAp from a bone of cuttlefish (*Sepia officinalis*). Furthermore, this time it was substituted with fluorine at different concentration (46% and 85%), which was fabricated *via* hydrothermal and thermal calcinations method. The resultant F-substituted HAp (FA) was observed to maintain its structural integrity even after the heat treatment. Additionally, the biomaterial forms nano-sized crystallites identical to the size of bone-like apatite as well as providing a promising sign of possible combination with bioresorbable TCP for early bone growth [176]. Oyster shell (*Mytilus galloprovincialis* and *Ostrea edulis*) was also converted to nano-powder of AB-type carbonated HAp using hydrothermal transformation. The features like low cost, easy availability and naturality makes the product more suitable for biological investigation. Besides, the *in vitro* thermal analysis, X-ray and FT-IR further proved their apatite forming ability with a bone regeneration potential [177].

Calcium carbonate skeleton produced by corals can also be converted into calcium phosphate scaffolds by hydrothermal method. For this reason, a study was conducted to understand their intra-articular function as bone graft by Koëter and co-workers [178] in goats by using coral-HAp scaffold for filling the defect which was produced in the femoral trochlea. It was reported that the coral-HAp scaffold was effective as bone filler in the defect site without triggering an inflammatory reaction. The fabrication of porous HAp from corals skeleton was first reported by Roy and Linnehan in 1974 [168]. Henceforth, various marine organisms and their skeletons have been converted to HAp, like cuttlefish [179], marine sponges [180] and nacre seashell [181], for bone grafts substitute while maintaining their normal architectural pattern.

As it is a hard tissue, bone should be able to bear a compressive force and should have sufficient mechanical strength. As a result, ceramics and metals may be more suitable as implants than that of polymers. However, they are not without limitations; since ceramics are brittle, and may be even stiffer than bone itself [182]. These inevitably drive the scientific community to explore some alternative as bone graft substitute by combining two or more materials which can be used in the field of bone tissue engineering to enhance their mechanical properties as well as osteoconductive properties. Acknowledging the significance of combining supplementary elements to the existing scaffolds, Zhang *et al.* [183] incorporated silver to coral-HAp with a view of determining its antibacterial properties and cytocompatibility of the composite scaffold. Based on different characterization, it was observed that the properties of the scaffolds like morphology and cytocompatibility is mainly

determined by the concentration of Ag⁺ in the composite scaffold; in fact, better result was reported with Ag⁺ at a lower concentration. Additionally, the cell morphology, adhesion and proliferation in MC3T3-E1 cells were also dependent on the Ag⁺ concentration. Interestingly, the silver loaded coral-HAp composite shows remarkably outstanding biocidal properties towards both Gram-negative (*Escherichia coli*) and Gram-positive bacteria (*Staphylococcus aureus*). HAp can also be used to supplement other biomaterial to enhance the biological properties. For instance, Roohani-Esfahani *et al.* [184] developed Poly-caprolactone (PCL) and nano (needle shape) or micron HAp coated biphasic calcium phosphate (BCP) for scaffold reinforcement. The coating by micro or nano HAp combined with PCL improved the compressive strength of BCP from 0.1 ± 0.05 MPa to 0.29 ± 0.07 MPa and 2.1 ± 0.17 respectively. In addition, the coating of BCP with HAp and PCL enhance the overall osteogenic properties of the composite scaffold.

Apart from the former advantages, the ceramic scaffolds can also be used in combinations with growth factors for bone TE application. In relation to this context, Nandi and colleague [84] loaded BMP-2 or IGF-1 into a marine derived coral HAp scaffolds and implanted in an artificially created bone defect model of a rabbit. Based on the *in vitro* study, it was reported that the sustain release of IGF-1 and BMP-2 were 77% and 98%, respectively. Moreover, *in vivo* studies like radiology, histology, SEM and fluorochrome labeling demonstrated that the growth factors offers promising effect when implanted *in vivo* as there is significant improvement in bone healing, although IGF-1 showed better outcome. Similarly, attempts have also been made to combine nano-HA/coral blocks with recombinant human VEGF to stimulate vascularization and angiogenesis. When implanted in critical-size defects in beagle dogs, the porous nHA/coral blocks coated with VEGF promoted neo-vascularization and augment new bone formation after 3 and 8 weeks after surgery. As a result, the bioceramic proved to be satisfactory scaffold to improve bone healing and regeneration [185, 186].

A novel composite scaffold was developed by Pallela et al by freeze-drying method using chitosan (Chi), HAp (fish bone *Thunnus obesus*) and marine sponge (*Ircinia fusca*) collagen (MSCol) [33] and characterized by SEM, XRD and optical microscopy. They demonstrated that the scaffold had homogeneous dispersion of HAp and MSCol in chitosan matrix having 60-180 μm and 50-170 μm interconnected porosity for Chi-HAp Chi- HAp-MSCol respectively. MTT assay and Hoechst staining using MG63 suggested higher cell proliferation in composite scaffolds than pure chitosan. So, this Chi-HAp-MSCol composite scaffold is also a promising biomaterial for application in bone TE.

Nano-HAp could also be synthesized or derived from salmon fish bone by alkaline hydrolysis [187]. After thorough characterization by TGA, FTIR, XRD, SEM and TEM, they demonstrated that the

biomaterial was amorphous, having carbonated group, non-toxic and nanostructure size ranging between 6-37 nm. nHAp also interacted with MSCs and increased bone mineralization subsequently. Thus, nHAp from salmon is another promising biomaterial in bone TE [187].

3.2.2. Biosilica

Through *in vitro* and *in vivo* studies, researchers proved that biosilica derived from marine sponges are very biocompatible. Significant calcium phosphate deposition of SaOS-2 cells was noted on biosilica (silicatein) protein-coated surface [188] suggesting its potential bioactivity *in vivo* and stimulation of osteoblast mineralization function.

In addition, it was revealed that due to its beneficial anabolicity on osteoblasts, biosilica prepared with silicatein can be used in bone TE. On the other hand, it had an adverse effect on bone-resorbing cells (osteoclasts) [189, 190]. So, biosilica can be considered as a better approach which can promote mechanism of anabolism in bone. Besides, biosilica stimulated the expression of important cytokine like BMP-2 by inducing progenitors cell differentiation to mature osteoblasts [191]. Osteogenic activity stimulation of SaOS-2 cells was also seen for silicatein/biosilica-modified substrates (obtained from *S. domuncula* sponge). Gene modulation further enhanced proliferation of cell and HAp formation subsequently. The above observation demonstrated that biosilica has an immense potential for treatment and prevention of bone disease.

Of late, Barros et al. used *Petrosia ficiformis* for analyzing the capability of biosilica extracted from sponges to fabricate a novel biomaterial [192]. Sponges were calcined first to obtain bioceramic structures, and were further subjected to 2 (M) KOH or 2 (M) HCl for inducing bioactivity. It was reported that structures thus obtained by calcinations, presented calcium-phosphate like coating and supported cell growth. For bioactive crystal nucleation and cell colonization, acidic treatment was better effective showing versatility of marine sponges in TE strategies. Likewise, scaffolds derived from marine source and composed of β -tricalcium phosphate (β -TCP), biosilica, biodegradable copolymer poly(D,Llactide-co-glycolide; PLGA) was also studied [91]; MTT assay using SaOS-2 cells showed no cytotoxicity. SaOS-2 adherence on silica-containing microspheres was better than only β -TCP microspheres. Furthermore, bone sections of implants containing silica from experimental animals showed superior bone regeneration compared to control implants (with β -TCP only). These evidences concluded that biosilica-based scaffolds are biocompatible and are promising biomaterials for bone repair/regeneration.

4. Marine biomaterial as local drug delivery system

Calcium phosphate derived from mixed HAp and β -TCP are very potent materials to deliver drugs in orthopaedic problems [193]. Antibiotic delivery is of paramount necessity to treat bone infections, the most challenging phenomena in orthopedic surgery [194]. Although bio ceramics are considered as best material for scaffold preparation, some limitations pose hindrance in its vast use to achieve desired porosity as well as shape for bone defect.

Osteomyelitis is most challenging to treat due to poor penetration of systemically administered antibiotic to the infection site as well as bacteria form a biofilm on bone matrix and orthopaedic implants [195]. Recent study shows that marine shell with specific microspherical structure provide appropriate structure for bisphosphonate (pamidronate) and gentamicin delivery due to presence of foraminifera shells [196]. In PLA–calcium phosphate thin film composites, several scientists opined that addition of hydroxyapatite from hydrothermally developed marine materials improve the drug stability, increases encapsulation efficiency, enhance controlled release and bioactivity and continuous supply of Ca^{2+} and PO_4^{3-} ions enhancing osteogenesis and repair subsequently. The composite also have the efficacy against microbial adhesion and can successfully be used during orthopaedic operation to prevent *S. aureus* infection [194].

Chitosan (CS)-based materials are currently being used during orthopedic tissue engineering due to its unique characteristics like negligible foreign body reaction, inherent antibacterial property, ability to be formed into various geometrical shapes. It also enhances osteogenic cell ingrowth and osteoconduction as well as easily delivers pharmaceutical agents, morphogenic factors and gene in a controlled manner due to its gelling properties and cationic nature [197]. CS is also combined with different other delivery materials, polymers, and growth factors due to its inherent antibacterial property thus make it an unique marine biomaterial to be used during orthopaedic surgeries [198-200]. When CS is combined with HAp and plaster of Paris with a purpose to deliver sustain release of vancomycin or fosfomycin to treat orthopaedic infection, it also inhibited *S. aureus* (methicillin-resistant) to grow *in vitro* for 3 months [201].

Though first commercial use of natural skeletons of coral was done in 1975 [202], but, first application started much earlier in 1929. Coral possess uniformly porous interconnected network which improve the drug release rate as well as drug loading when used during drug delivery system. The presence of different types of pores such as macro, micro and nano-pores in exoskeletons allow different diffusion rates of drug in delivery system [203]. Coral exoskeletons are used as precursor material for such development (drug delivery system). *In vivo* study with osteoporotic mice depicts

that drug loaded and chemically modified biomimetic drug delivery with coral significantly increase cortical and cancellous bone density mimicking a promising material to treat osteoporotic patients [204]. Several researchers worked on effect of simvastatin, a drug used in the treatment of cholesterol in drug delivery system. In one study, calcium carbonate precursor materials derived from foraminifera exoskeletons were hydrothermally converted to β -TCP and used as a carrier material for simvastatin. The result depicted that natural exoskeletons from coral can successfully be used in simvastatin delivery for prolong period as well as β -TCP with simvastatin increase the mechanical strength of bone and help in osteogenesis [205].

6. Conclusion

The seas and oceans are a rich source of diverse biomaterials for use of mankind. Exploration of these abundant marine biomaterials into research is of paramount necessity for its effective utilization. Of late, significant attention has been paid by the researchers for its immense use in diverse applications. These biomaterials in bone tissue engineering have paramount necessity as low cost, safe and biocompatible material. The current review highlighted the basic knowledge of several marine organisms and their conversion for application for specific purposes. Most importantly, the converted product necessary for bone tissue engineering are discussed. The unique features of marine organisms as a natural source will pave a new insight of alternate materials for management of bone disorders. It is hoped that the collective information provided in this review will open the floodgates for development of new biomedicine from marine biomaterials, especially in bone tissue engineering.

Conflict of interest

The authors do not have conflict of interest

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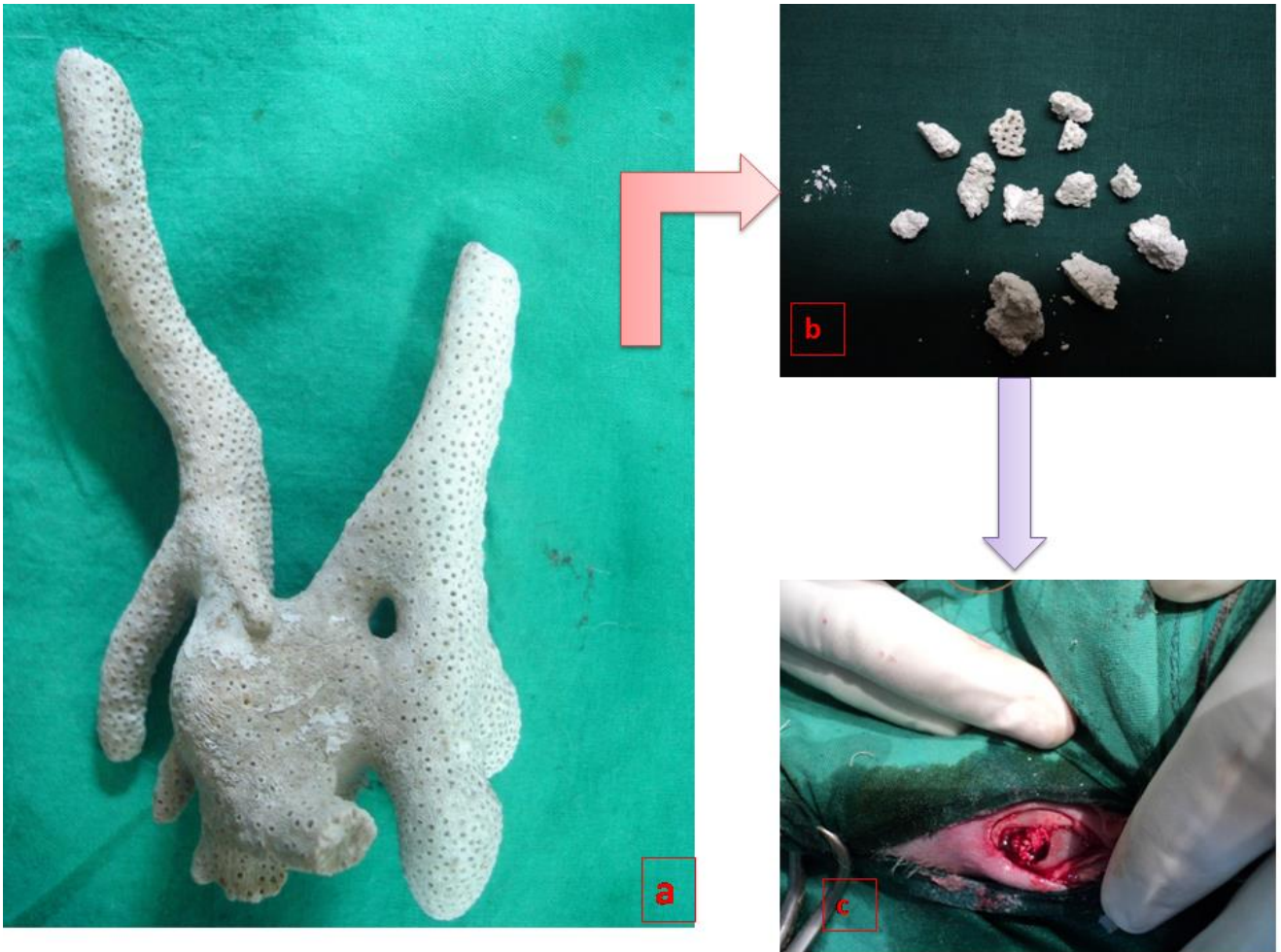


Figure 1: As-received sea coral and hydrothermally converted and sintered (1250 °C) sample (a. sea coral; b. hydrothermally converted coral, c. implanted in bone defect [reproduced with permission])

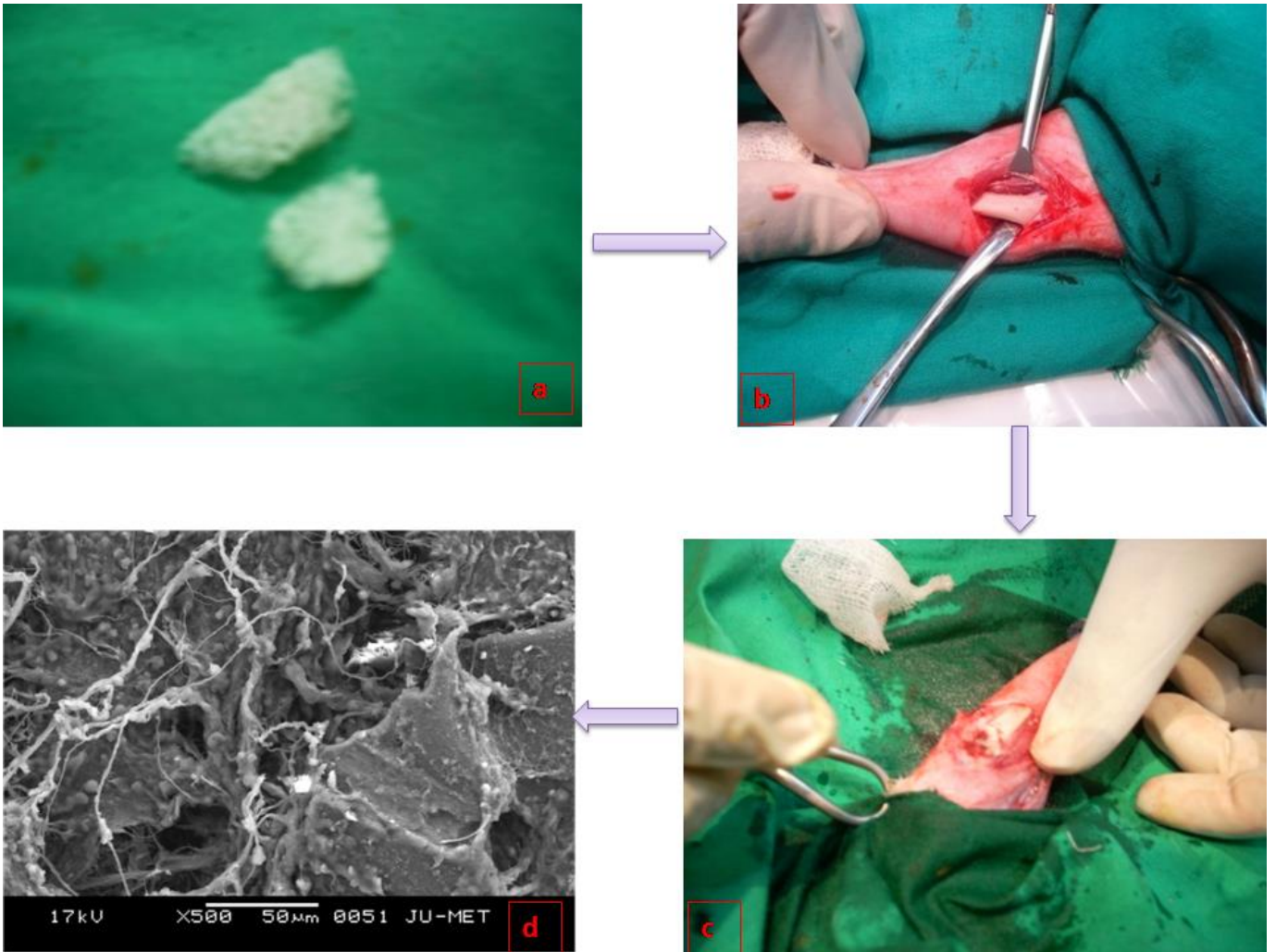


Figure 2: The chitosan implanted bone defect in animal model (a. Converted marine chitosan; b. exposure of bone; c. implantation of chitosan in created defect in animal model, d. SEM picture of chitosan implanted bone) [reproduced with permission]

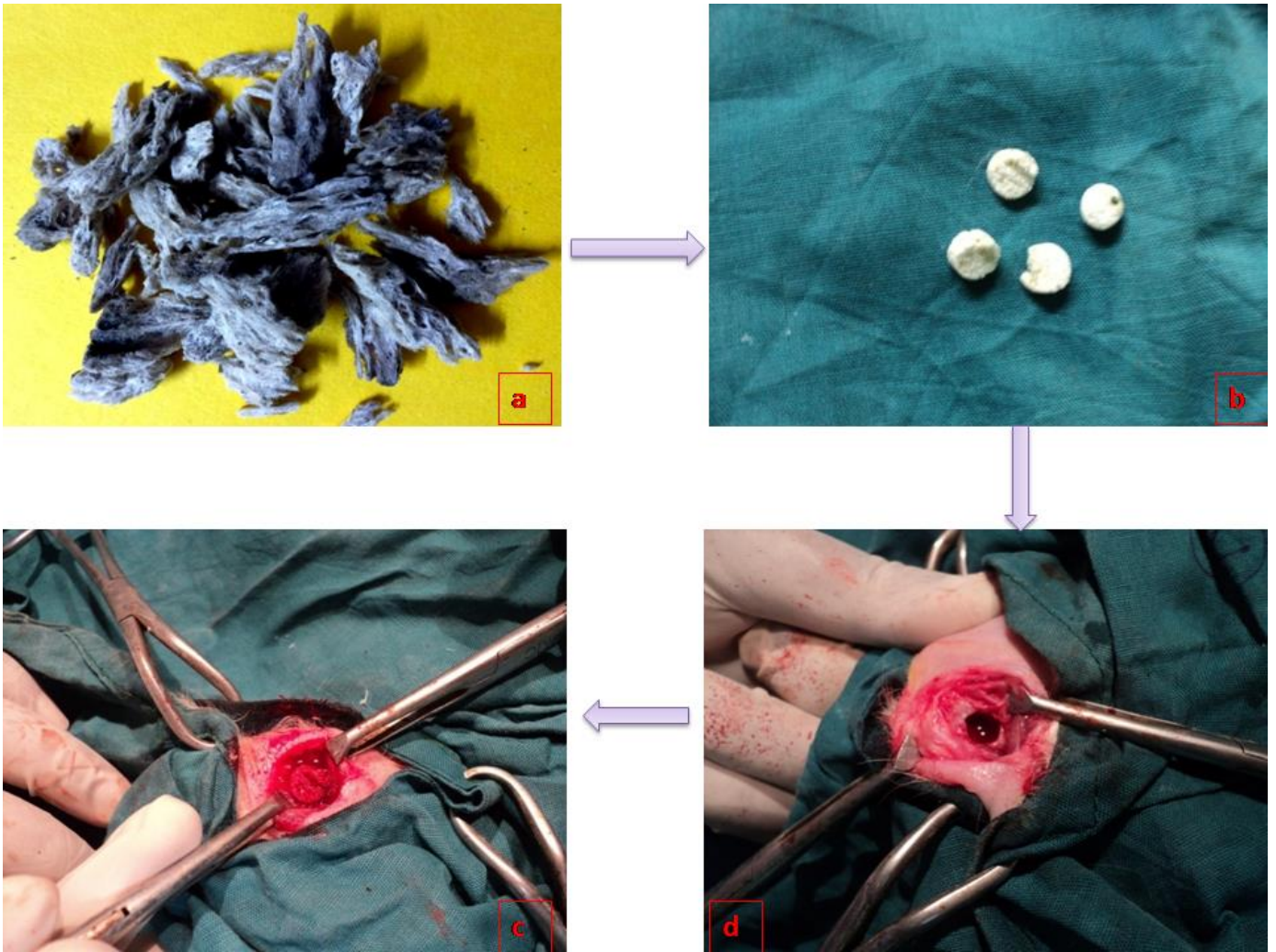


Figure 3: The bioscaffold converted from marine sponge (*Biemna fortis*) to the bone defect site (a. Marine sponge; b. converted marine sponge; c. creation of bone defect in animal model, d. placement of marine sponge scaffold in bone defect) [reproduced with permission]